GENETIC VARIATION WITHIN AND BETWEEN SOME RARE AND COMMON TAXA OF CAPE PROTEACEAE AND THE IMPLICATIONS FOR THEIR CONSERVATION

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SUSAN ANN BROWN

Department of Botany
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The bright yellow blooms of a male L*eucadendron elimense* subspecies *vyeboomense*. This taxon is highly endangered.

The showy red-flushed male flowers of *Leucadendron elimense* subspecies *salteri* offer great potential for exploitation as cut flowers.

ABSTRACT

There are 152 rare, threatened or extinct Cape Proteaceae, many of which are found to occur in small numbers, have few populations, or a geographical range of less than 5 km^2. This suggests that small nature reserves would be an ideal solution to protect such species provided they contain sufficient levels of genetic variation for long term viability. Genetic variation was measured using RAPD analysis in the rare *Leucadendron elimense* with its three closely related subspecies, *L.e. vyeboomense*, *L.e. salteri* and *L.e. elimense*. *L.e. vyeboomense* is restricted to a single endangered population, *L.e. salteri* has seven known populations and is ascribed the status of vulnerable, and the local endemic of the south coast plains, *L.e. elimense*, is considered vulnerable. AMOVA analysis of the RAPD results revealed high levels of genetic variation within populations of all three subspecies at 69.8 to 91.4%. The endangered *L.e. vyeboomense* was genetically most distant from the other two subspecies at a level of 0.40 and 0.39 and also showed the lowest levels of variation within the subspecies at 0.24. Populations of the other two subspecies had levels of variability of about 0.35. Comparable levels were recorded for the ubiquitous *Leucadendron salignum*. *Serruria roxburghii*, an endangered species restricted to two populations on a specialised sandy soil, provided another test of the applicability of the method. Both populations of *S. roxburghii* showed a genetic variability of 0.36 with no significant differences in genetic distances within the two populations. Morphological differences were considered and leaf ratios provided a tool for distinguishing the three subspecies. Total protein electrophoresis and sequencing of the Internal Transcribed Spacer (ITS) region, both showed that *L.e. salteri* was the least similar of the subspecies, and should be considered for possible taxonomic revision to species level. The implications of the thesis for conservation are that RAPD data is directly of use in conservation management decisions. It has shown that despite the small population sizes of the study taxa they have

adequate to high levels of genetic diversity and would be adequately protected in small nature reserves. Small, private reserves should be considered as a potential economical and ecological long term viable option.

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CHAPTER 1

General introduction

Introduction

Genetic variation and rare plants

A major role of conservation is to preserve genetic diversity of species, populations and evolutionary processes in order to prevent genetic deterioration, and hence extinction in the long term (Soulé & Simberloff 1986, Avise 1994). Genetic diversity is generally expected to be low in small isolated populations (Gaston & Kunin 1997, Karron 1997) and endemic species also typically exhibit low levels of genetic diversity (Hamrick & Godt 1989). Because rare species of plants or local endemic species of plants are often found in small isolated populations with a restricted distribution, they might also be expected to exhibit limited genetic variation. Management decisions for such rare taxa ideally necessitate an understanding of their biology and other factors, including genetic variability, that influence their survival. Conservation management decisions for rare taxa, however, often have to be made quickly without adequate ecological or genetic data (Gaston & Kunin 1997). To compensate for this, heavy reliance has been placed on theoretical techniques and empirical generalizations, often taken from island biogeography theory (Doak & Mills 1994). This particularly applies to predictions on genetic variation and inbreeding depression. Such theoretical predictions are often found in practice to be simplistic and untestable (Shaffer & Samson 1985, Soulé 1987). One would therefore expect genetic studies to be a priority in conservation management, especially of rare species. Indeed, Peakall & Sydes (1996) suggest that immediate short term goals in rare plant studies are prevention of extinction, while longer term goals are maintenance of genetic diversity. In order to formulate suitable conservation strategies, a measure of genetic variation and its distribution in rare plant species is therefore essential (Falk 1992). In terms of rare plant species, genetic diversity is best defined as the sum of genetic variation within a population or a species (Peakall & Sydes 1996).

Having identified the need for genetic studies, there are a few cautions. Avise (1994) points out that it is a common assumption that high levels of genetic variability within rare or threatened populations enhance the probability of the population surviving over time. This is because the loss of genetic variation is thought to lead to a potential decline in a species' ability to survive environmental changes and stress, both in the short- and long-term (Ellstrand & Elam 1993,

Milligan *et al.* 1994). Ryan & Siegfried (1994) indicate that in birds there is a link between small population size (with presumed low genetic diversity) and the risk of extinction. An example of causal links is provided by the prairie chicken, *Tympanuchus cupidopinnatus*, which exhibited a decline in Illinois from 2000 individuals in 1962 to 50 in 1994 with an accompanying decline in genetic diversity, measured as mean heterozygosity (Westermeier *et al.* 1998). Many authors, however, caution that no causal link has yet been proven between the level of genetic diversity and a population's long term viability (see, for example, Avise 1994, Milligan *et al.* 1994, Schemske *et al.*1994). Again, using the example of observations of natural populations of birds, there are numerous examples in the literature describing the persistence of small populations (see, for example, Terborgh & Winter 1980, Soulé *et al.* 1988). It is, however, uncertain as to what constitutes a high or low level of genetic diversity, and the maintenance of a constant level of genetic variation is therefore generally considered essential for long-term protection of a taxon (Frankel & Soulé 1981, Simberloff 1988). Any change in the level of genetic variation should be monitored to provide a critical guide for assessing current status and future prospects (Palacios & González-Candelas 1997). Extinction is a normal evolutionary process and even under ideal conditions, long term persistence of both large and small populations cannot be guaranteed (Mangel & Tier 1994). Current population size and patterns of genetic variation are a result of historic events, possibly even genetic bottlenecks (Ellstrand & Elam 1993). Because of this, Ellstrand & Ellam (1993) cast doubt on the practical role of population genetics in plant conservation and the relative importance of genetics versus demographic approaches in practical conservation is also contentious (Schemske *et al.*1994).

Despite the above caveats, genetic research has recently been recommended in recovery plans for several species of rare plants. In New South Wales, Peakall & Sydes (1996) found that 57% of recovery plans make recommendations for genetic studies. A molecular technique which allows for rapid measures of genetic variation could therefore become a critical tool in conservation management decisions. Measures of genetic variation in rare plant species in the context of their conservation have included recent studies on *Helonias* (Liliaceae) (Godt *et al.* 1995), *Liatris* (Asteraceae) (Godt & Hamrick 1995), *Grevillea* (Proteaceae) (Rossetto *et al.* 1995), *Iliamna* (Malvaceae) (Stewart & Porter 1995, Stewart *et al*. 1996), the gymnosperm,

Amentotaxus (Wang *et al.* 1996), *Wyethia* (Asteraceae) (Ayres & Ryan 1997, 1999), *Erodium* (Geraniaceae) (Martín *et al.* 1997), *Limonium* (Plumbaginaceae) (Palacios & González-Candelas 1997), *Haloragodendron* (Haloragaceae) (Sydes & Peakall 1998), *Caesalpinia* (Leguminosae) (Cardoso *et al.* 1998) and *Calamagrostis* (Poaceae) (Esselman *et al.* 1999). These studies have made use of a number of different molecular methods including, amongst others, microsatellites, hybridisation-based fingerprinting, allozymes, isozymes, randomly amplified polymorphic DNA (RAPD) analysis, and restriction fragment length polymorphisms (RFLPs). Since the inception of this project in 1993, recent studies have demonstrated the utility of microsatellites as an accurate measure of genetic variation (See Morell *et al.* 1995, Slatkin 1993, and Michalakis & Excoffier 1996). This method does provide more information than RAPD analysis and overcomes many of the problems of RAPD analysis. The major drawback is that specific primers have to be developed which is both costly and time-consuming (Morell *et al.* 1995). Conservation and small- scale rare plant studies seldom justify such expense. There have been a number of other suitable methods developed, of particular interest is a technique called random amplified microsatellite polymorphisms. This method combines both the sequence tagged site microsatellite and RAPD approaches (Morell *et al.* 1995).

Vegetation and conservation status of South Western Cape

In the southwestern corner of southern Africa lies a vegetation so diverse and rich as to be ascribed the status of Cape Floral Kingdom (Takhtajan 1969). Despite occupying a small area, the level of vascular plant endemism is about 73% of the 8 550 known species (Goldblatt 1978). The Cape Floral Kingdom approximates the same geographical area as the fynbos biome (Moll & Jarman 1984), a vegetation type defined ecologically as evergreen sclerophyllous shrublands, containing predominately small-leafed species. Despite the high taxonomic richness, the vegetation lacks structural diversity (Cowling & Rebelo 1992) and is characterised and often dominated by members of three families; Proteaceae, Ericaceae and Restionaceae (Cowling & Holmes 1992).

Much of the original lowland vegetation of the fynbos region has been replaced by agriculture and the natural vegetation is now mostly restricted to higher elevations unsuitable for farming.

Moll & Bossi (1984) estimated remaining natural land to be only about 66% of its historical extent prior to the arrival of European colonists some 300 years ago. This has resulted in a large number of species, both plant and animal, being brought under threat of extinction. An estimated 60 000 plant species worldwide are considered rare or threatened (Ellstrand & Elam 1993) and of these, 6% lie in southern Africa (based on data from Hilton-Taylor 1996a, 1997). For the southwestern Cape specifically, which marks the area of the Cape Floral Kingdom, 1 546 taxa are listed as rare or threatened (C. Hilton-Taylor pers. comm.).

Rare Cape Proteaceae

The family Proteaceae is represented by some 400 taxa in Africa (Rebelo 1995), of which over 83% are found in the Cape Floral Kingdom (calculated from Gibbs Russell *et al.* 1984, Gibbs Russell 1985) and 78% are endemic (after Bond & Goldblatt 1984). Within the Cape Proteaceae, two taxa are considered to fit the IUCN (International Union for Conservation of Natural Resources) description of extinct, 36 taxa are endangered, 41 vulnerable and 73 naturally rare (calculated from Tansley 1988, Hilton-Taylor 1996a, 1997). These figures become more significant when seen in the light of population parameters common to many Cape Proteaceae. Tansley (1988) showed that rare Cape Proteaceae are characterised by being restricted to few populations (82% are found in five or less populations), by covering a small total area (more than half are restricted to a range of 5 km^2 or less) or by generally being restricted to few plants in total count (more than 90% consist of less than 5000 individuals, many of which are dioecious). These characteristics, occurring on their own or in combination, result in populations being susceptible to sudden extinction by what might otherwise be a relatively minor local disturbance.

Conservation of Small Populations of Rare Cape Proteaceae

The typically restricted distribution of rare Cape Proteaceae means that species could potentially be protected by a system of small nature reserves, as proposed by Tansley (1988). This is not a novel idea and has been suggested in other situations. A system of very small reserves with areas less than 1km^2 has been proposed and partially implemented for the west coast of Newfoundland, Canada (Nantel *et al.* 1998). Similarly, Higgs & Usher (1980) showed that by protecting more small rather than few large limestone pavements in the Yorkshire Dales

National Park, more species would ultimately be protected, and Game & Peterken (1984) found that the IUCN exhortation to concentrate on large reserves would have been misplaced in their study on woodlands in Lincolnshire, England. The best way to estimate the required number of reserves, as suggested by Soulé & Simberloff (1986), is to first identify the target species and then to determine the minimum viable population size while ensuring that the area protected is "not too small". As most populations of rare Cape Proteaceae are already exceptionally small, each whole population is best considered as a minimum size unit.

In broad ecological terms, small reserves protecting these small populations of rare Cape Proteaceae appear to be a solution to their long-term conservation. As already pointed out above, there is much debate about the long-term genetic viability of such small populations. If they have no long term viability (or ecological importance), it would be a needless waste of conservation resources to protect such taxa (Siegfried 1984). The tenacious persistence of the small populations of rare Cape Proteaceae, however, without any kind of protection except, at best, benign neglect, suggests that genetic deterioration is not yet apparent, at least in the phenotypes. This is, however, all speculative because so far no measure has been made of the level of genetic variation within rare Cape Proteaceae. Thus the first important step is to obtain some quantitative measure of the levels of genetic variation within populations of rare Cape Proteaceae and to interpret the usefulness of such information in terms of their conservation. This provided the motivation underlying this thesis. Furthermore, at the time of the initiation of the present study, new genetic techniques were being developed, refined, and applied to problems associated with rare and threatened plants in other countries. The aim of this thesis was to determine if an adequate measure could be made of the levels of genetic variation within rare Cape Proteaceae in comparison to expected levels based on measuring genetic variation in a population from a common widespread taxon. A measure of between-population genetic variation would allow ranking for conservation management and a hierarchical measure of genetic variation would further refine this.

In contrast to other countries, Linder (1996) points out that very little work on genetic variation has been done in the Cape flora. Indeed, few genetic studies have been published on rare South African plant species in the context of their conservation. Two such studies are on

Prunus africana (Rosaceae) (Barker *et al*. 1994) using randomly amplified polymorphic DNA (RAPD) and on the cedar *Widdringtonia cedarbergensis* (Thomas & Bond 1997) using isozyme electrophoresis. The results from these are of limited management value as they showed little differentiation. A study of *Tylosema esculentum*, an exploited leguminous food plant of the Kalahari Desert, has been carried out by Monaghan & Hulloran (1996) using the RAPD technique, to monitor levels of genetic diversity. This plant is not rare but is under considerable threat from possible overexploitation as a highly desirable food source, and a measure of genetic variability was the first step in developing this plant as a cultivated crop.

Methods selected to assess genetic variation

Measures of genetic variability can be made using a variety of genetic markers, each producing information at a different order of complexity. They range, amongst others, from morphological characters, to total proteins, RAPD analysis, and DNA sequencing. The main aim of this thesis is to assess the potential of RAPD's for adequately detecting and measuring genetic variation in populations of rare Cape Proteaceae and assessing the usefulness of such information in terms of conservation management of the populations. The secondary aim of this thesis was to apply RAPD analysis to assess the genetic variability in the different populations of a rare species so as to use this in the preferential selection of certain sites for conservation. This is increasingly important in the light of collapsing existing protective legislation, conservation bodies and funding for conservation in a post-apartheid South Africa. In addition, a brief assessment was made as to information that could be obtained from morphological measurements, total proteins and DNA sequence data that might also be applicable to rare plant management. Since the conception of this study, however, the literature indicated that other researchers were already testing and using RAPD variation successfully for studies of rare plant populations and conservation. Because of the many potential advantages offered by RAPDs, the use of the RAPD technique has remained the primary focus of this project. The methods employed are discussed briefly below.

Morphology

Morphological characters expressed in the phenotype are genetically determined. Only some of the genetic variation is visible in the phenotype of the taxon, yet this is often expressed as

a result of environmental pressures and represents an unknown percentage of the total genetic variability. Morphology can, however, be influenced by environmental conditions, which limits the use of these characters (Newbury & Ford-Lloyd 1993). Nonetheless, morphological characteristics form the basis of most existing taxonomy. Morphology is generally easily observed and quantified, which makes it simple to use. It does suffer from problems of subjectivity in the character analysis (Morell *et al.*1995), although this problem can be reduced, the characters quantified and statistical analysis applied. This method has been used, for example, in a study on the rare *Senecio integrifolius* (Asteraceae) by Widén & Andersson (1993). Morphological observations are of use in conservation management as they allow an assessment of the general health and status of the plant population. The application of morphology to this project is described in Chapter 2.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD analysis is the main focus of this thesis. The RAPD technique was developed by Welsh & McClelland (1990) and its potential use in plants demonstrated by Williams *et al.* (1990). It made use of the polymerase chain reaction (PCR) developed in 1985 by Kary B. Mullis (Saiki *et al.* 1985) and allows a direct observation of genomic DNA using random priming and hence provides a measure of genetic variation.

Huff *et al.* (1993) were among the first to report on the use of RAPD variation within and among natural populations of an outcrossing species of plant. Since then the use of RAPD technique and appropriate data analysis has escalated. RAPD markers offer a number of advantages in the study of rare species, not least of which is that they can detect between-population differences and can sample more of the genome than allozyme markers (Dawson *et al.* 1995), which might underestimate the level of variability and, in some rare plants, might not be able to detect any genetic variation at all (Peakall & Sydes 1996). In species with typically low levels of genetic variation, such as striped bass (*Morone saxatilis*), Bielawski & Pumo (1997) found RAPD technique to be sufficiently sensitive to detect differences despite the low levels of genetic variation recorded. As rare species and small populations are expected to contain low levels of genetic variation, this lends support to the use of RAPD technique for rare species. Increasingly, the RAPD technique offers attractive features for the study of rare

and threatened plant species as seen in recent studies (see, for example, Rossetto *et al.* 1995, Stewart & Porter 1995, Ayres & Ryan 1997, Martín *et al.* 1997, Palacios & González-Candelas 1997, Sydes & Peakall 1998).

Because the PCR reaction amplifies the DNA template, only a small initial quantity of genomic DNA is required (Morell *et al.* 1995). This means that only a small amount of tissue need be removed from the plant, which results in minimal damage (Palacios & González-Candelas 1997). This is of particular importance in Cape Proteaceae, which are susceptible to fungal infections in wounds. In addition, the advent of PCR has made the technique quick, simple, and uses small quantities of template DNA and small reaction volumes, hence small amounts of chemicals. This method, once it is established, produces a measure of genetic variability in a short time span without any prior knowledge of the plant's genetics or sequence data (Martín *et al.* 1997). This feature makes it very attractive for rare plant studies where, by the very nature of being rare, the population biology is often poorly known or recorded. The technique is not particularly demanding (Gillies *et al.* 1997), even for non-molecular biologists (Stewart & Porter 1995) provided basic rules are adhered to.

RAPD markers are able to distinguish genetic differences between populations which makes them useful in conservation studies (Dawson *et al.* 1995), particularly where morphological similarities exist. The amplified DNA provides a large number of potential polymorphic markers (Palacios & González-Candelas 1997), which is particularly useful in studies of rare plant populations where the level of genetic variation is expected to be low and not easily separated (Williams *et al.* 1993). The large numbers of markers can detect a high level of variation within and among individuals despite the loss of resolution due to the dominant nature of RAPD markers (Peakall *et al.* 1995). It consequently provides a valuable genetic tool for small-scale studies that do not justify the cost of developing co-dominant DNA markers (Peakall *et al.* 1995).

Inevitably, there are potential limitations to the method. Most of these are attributable to poor techniques and are easily avoided. Others are attributable to the nature of the DNA fragments visualised by electrophoresis and limits the information that can be extracted from RAPD

variation patterns. Hedrick (1992) points out that no one understands the exact basis of the variation the technique uncovers. The dominant nature of RAPD data (Welsh & McClelland 1990) is potentially a problem in data analysis. The basis for DNA fragment absence is not clearly understood and does not necessarily imply shared ancestry (Gillies *et al.* 1997). This has hampered the use of the RAPD technique in the past (Lynch & Milligan 1994). The RAPD technique produces numerous DNA fragments, which are visualised as bands by gel electrophoresis. The RAPD technique can only detect band presence being the heterozygous state or homozygous co-dominance and band absence being the homozygous recessive. This means that the heterozygous state within populations cannot be estimated accurately (Lynch & Milligan 1994).

Some studies report a low incidence of non-inherited bands (Heun & Helentjaris 1993). These might be PCR artefacts as Morell *et al.* (1995) indicate that most RAPD bands are known to be inherited in a Mendelian fashion. Different band positions may not represent different loci because of the possibility of nested, inverted repeats of primer sequences within a locus (Stewart & Excoffier 1996). Most of these problems are overcome by the large number of bands produced by the RAPD technique (Peakall *et al.* 1995). Conclusions then should be drawn from an adequate number of bands.

Bands visualised by electrophoresis are generally treated as independent characters. In practice, it is possible that many bands might be alleles of the same locus, or at least linked to some degree (Weising *et al.* 1995). This is a problem when seeking genetic relatedness or phylogeny among individuals, which is not an issue in this thesis.

Co-migration of non-homologous bands cannot be excluded (Weising *et al.*, 1995). This is exacerbated on agarose gels as there is limited resolving power and "false matches" are more likely than on polyacrylamide gels. Silver staining will further enhance this resolution. The problem of co-migration in relation to interspecific relatedness is considered at some length by Riesenberg (1996) and by Katzir *et al.* (1996) who verified the integrity and homology of each band by using Southern hybridization. Backeljau *et al.* (1995) warn that some DNA fragments may be amplified to a lesser extent and might not be visible on the gel. Once again the use of

polyacrylamide and silver stain will greatly enhance the visibility of such bands. The above limitations of RAPD's restricts its use in phylogenetic studies at this stage (Backeljau *et al.* 1995) as the required assumptions are not necessarily met. Again this is not directly relevant in this thesis.

In the past, reproducibility of RAPD profiles, between repeat samples, repeat amplification, different laboratories, different workers, different brands of chemicals, and different equipment has been questioned (Ellsworth *et al.* 1993, Linton *et al.* 1994, Micheli *et al.* 1994, Schweder *et al.* 1995). Hadrys *et al.* (1992) and Weeden *et al.* (1993) showed that reproducibility of RAPDs was not a problem if conditions were strictly maintained. This was supported by Penner *et al.* (1993) who showed reproducibility between laboratories. They allowed the laboratories to use their own optimised protocol which was largely dependant on the thermocycler. Across five primers the mean level of reproducibility was 77%. Morell *et al.* (1995) reported that they obtained good reproducibility despite the DNA being extracted by three different methods with multiple PCR reactions and two operators working in two separate laboratories. The application of the RAPD technique to rare Cape Proteaceae is described in Chapters 3 & 4.

Total Protein Electrophoresis

Total proteins provide a crude measure of underlying genetic information and often serves as a starting point for further DNA-based studies. This is a simple and quick technique which allows visualization of patterns of protein variation using gel electrophoresis. It can be used to produce a measure of genetic distance. A study which used this method to differentiate geographically isolated populations was conducted on rare *Tilapia* fish (Nxomani *et al.* 1994). Stress may cause the appearance of stress proteins in a plant, which would be reflected in any protein based profile. This disadvantage of protein based markers is overcome by DNA-based markers which are able to generate information independent of any environmental factors such as stress (Newbury & Ford-Lloyd 1993). The use of total protein analysis is described in Chapter 5.

DNA sequence analysis

Finally, a highly detailed and precise method of measuring genetic variation was investigated, that of DNA sequencing. While this method is very accurate in the information produced, it is rather hit-and-miss as to which part of the genome is selected for study. This method might be expected to verify the taxonomy of the species as a whole and perhaps support the delineation of the subspecies and their relationships. Results of DNA sequencing on selected rare Cape Proteaceae is discussed in Chapter 6.

Selection of sample populations

To assess whether genetic variability, as determined by RAPD analysis, provided a useful tool in terms of implications for conservation of Cape Proteaceae, the study species needed to represent separate but related taxa of different conservation status. This would potentially have allowed for any differences in RAPD results to be attributed to a measure of different levels of genetic variation rather than merely gross taxonomic differences. In addition, a population from a ubiquitous taxon was needed to show whether there was any grouping of genetic variation within closely related taxa or rare versus common taxa. Finally, a test group was needed to apply the selected RAPD technique and see if meaningful results were produced. *Leucadendron elimense* E. Phillips provided three subspecies with different conservation status in six usable study populations. *Leucadendron salignum* P.J. Bergius provided the common widespread species and the rare and endangered *Serruria roxburghii* R. Br. provided the test case with its two remaining populations.

To summarise, the main aim of this thesis was to investigate genetic variation in small populations of rare Cape Proteaceae using the RAPD technique. The specific objectives were:

a) To measure the RAPD variation in the three subspecies of *L. elimense*, which represent different degrees of rarity, and to compare these to variation in a population of a ubiquitous species, *L. salignum*.

b) To test the applicability of the RAPD technique to another genus of Cape Proteaceae, the endangered *S. roxburghii*, and to attempt to identify individuals of this species for preferential translocation.

c) To assess variability in the three subspecies of *L. elimense* using analysis of total protein.

d) To look for sequence variation in the ITS region of the three subspecies of *L. elimense*.

In the final chapter, Chapter 7, I attempt to draw conclusions from the different methods investigated and assesses the implications of genetic data and other data for conservation management of small populations of rare Cape Proteaceae.

CHAPTER 2

Description of study taxa and their morphology, with particular reference to the leaf morphology of *Leucadendron elimense* E. Phillips. (Proteaceae)

Introduction

Morphology is defined as the outward appearance or phenotype of an organism. It is a function of the genotype, but only represents a very small, albeit visible, portion thereof. Although the genotype is the primary determinant of a plant's morphology, other factors can modify it. Environmental factors like soil properties, water, and nutrients, for example, can sometimes exert such influence that a single species may appear unrelated at the extremes of its range if there are no intermediate forms. Such situations occur repeatedly within the Cape Proteaceae, some specific examples being *Leucadendron cinereum*, *Leucadendron linifolium*, *Leucadendron pubescens*, *Leucospermum wittebergense*, *Leucospermum oleifolium*, and *Protea subulifolia*.

Morphology has, until recently, provided the basis for almost all classical and traditional taxonomy. There is, however, an increasing body of literature highlighting discrepancies between the traditional view of taxonomic relationships and evidence from molecular studies. One such example is the classical study on bird relationships by Sibley & Ahlquist (1990), in which they propose a molecular metric that would be applicable to formal avian taxonomy. Although molecular techniques may provide desirable supplementary or complementary taxonomic data, these techniques are expensive and require a degree of expertise to carry out. In contrast, morphological measurements are generally easy to obtain and are readily tested statistically. Morphology is consequently still important for conservation in that the definition of taxa is a necessary basis for identifying units needing conservation. Should taxa be of uncertain status, a waste of resources that might be better directed elsewhere can result. Some taxa, previously thought to be "endangered", have become "not threatened" due to taxonomic revision based on morphological studies. One such example is the Red Wolf (*Canis rufus*), thought to be an endangered species, but which in fact was a hybrid of Grey Wolf (*C. lupus*) and Coyote (*C. latrans*) (Wayne & Jenks 1991). A local plant example is seen in *Xiphotheca fruticosa* (L.) (Fabaceae), previously classified as "naturally rare" and now found to be "not threatened" (Hilton-Taylor 1996b).

The aim of this chapter is to introduce the candidate taxa selected for the study, which sets the scene for the subsequent genetic investigation. The chapter comprises two parts. The first is largely descriptive and provides background information on the taxonomy, general morphology, geography, ecology, demography and present conservation status of the study taxa. The second part provides quantitative measurements of leaf morphology of the three subspecies.

Description of study taxa

Leucadendron elimense

Leucadendron elimense is one of four species belonging to the section Leucadendron, subsection Ventricosa. This subsection is characterised by hairless nuts, inflated on one side and keeled on the other, with a narrowly ridged perimeter (Williams 1972). Unique to this subsection is the cone, which has bracts that are completely free and which appear leaf-like from an elongated receptacle and are arranged in five spiral rows (Williams 1972). All are characterised by wind-dispersed seed. The species in this subsection are all found on plains at the foothills of mountains. All are rare and occur in small populations and hence are considered worthy of conservation.

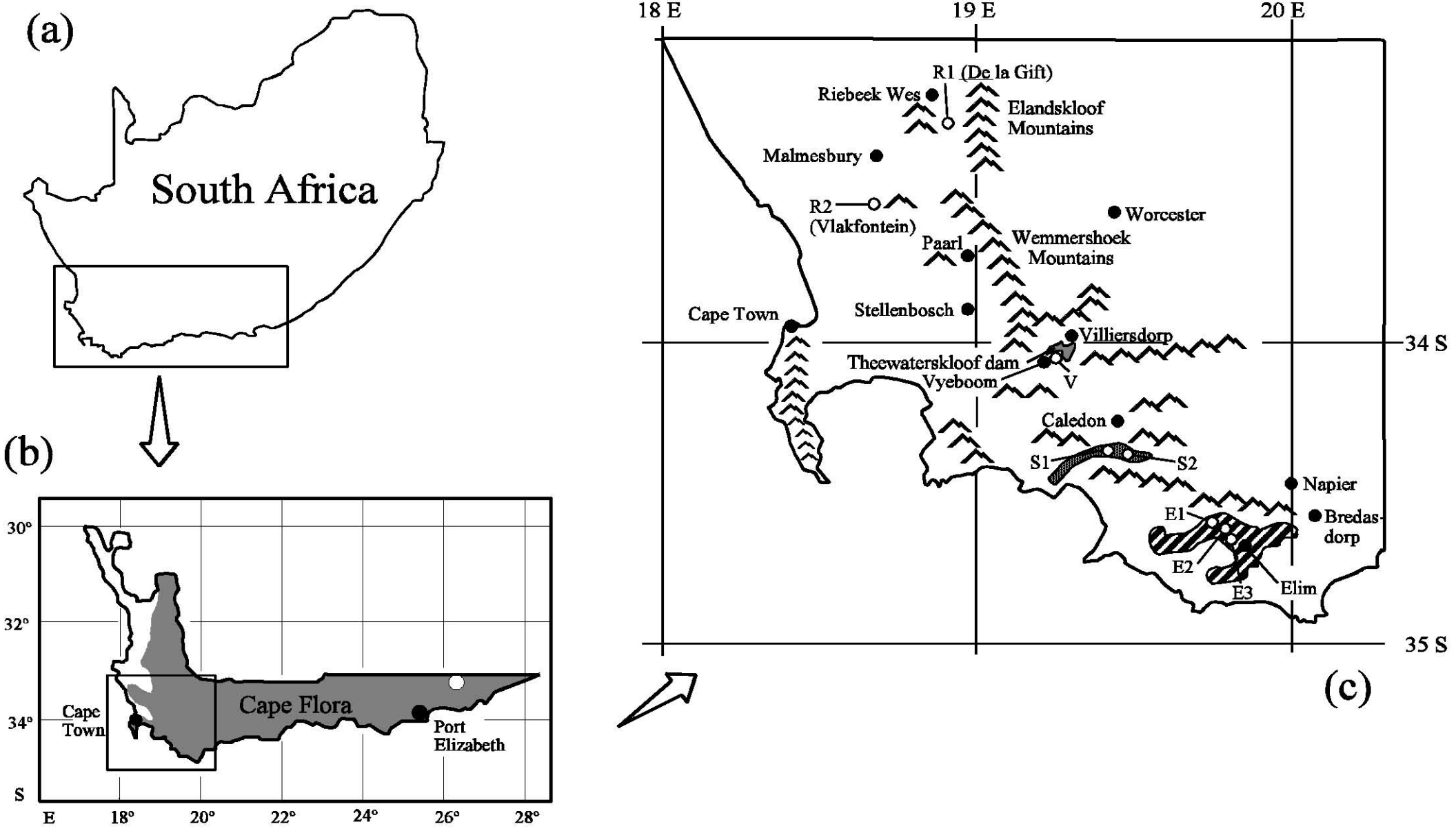
In his authoritative taxonomic revision of the genus, Williams (1972) divided *Leucadendron elimense* into three subspecies, *L.e. elimense* E. Philips, *L.e. salteri* I. Williams and *L.e. vyeboomense* I. Williams. The three subspecies are geographically distinct but otherwise differ only in size, shape and spacing of leaves. *L.e. elimense* is relatively widespread and common throughout its distribution, *L.e. salteri* is more restricted and *L.e. vyeboomense* is highly restricted. The three subspecies consequently represent genetically closely related taxa but present different population dynamics (Tansley 1988) and hence different levels of rarity. If they are genetically similar, one can compare the levels of genetic diversity in a meaningful fashion and any differences found can be assumed to be real. Because of its very small population, one might, for example, expect *L.e. vyeboomense* to show marked reduction in genetic diversity relative to *L.e. elimense*. Furthermore, as with all species of *Leucadendron*, *L. elimense* is dioecious, thereby enforcing obligative outcrossing with no cloning from vegetative reproduction.

The type subspecies *L.e. elimense* is found on the shallow sands of the Elim plains of the south coast of South Africa, from Gansbaai to Bredasdorp (Fig.2.1). Until recently, there were numerous populations totalling at least 10 000 plants. Although it was ascribed the IUCN status "vulnerable" due to grazing pressures (Tansley 1988), it was, at that stage, widespread throughout the Elim region. The subspecies currently numbers approximately 7000 plants in eight known populations, mostly small in number. IUCN status is still "vulnerable". The prime conservation problems for this taxon are agricultural in origin, specifically grazing, burning and ploughing. To a lesser extent, it also suffers through picking as "green filler" for the export flower trade.

Three study populations were selected in 1995. These populations lay in a north-south line along the road to Elim (E1-E3 in Fig. 2.1c). Although at the time of selection the populations were quite distinct, it is possible that in the past they were all part of one large continuous population covering some 10 km^2. Evidence of the dynamic nature of the population structure is seen in fluctuations in populations numbers from one visit to the next. These fluctuations are mostly anthropomorphic rather than natural. Population 1 remained constant between visits in 1994 and 1997. It is heavily choked by *Acacia* spp. and it is possibly that these large bushy shrubs hide *L.e. elimense* plants from grazers. A new population was seen in 1997 on what was recently ploughed land in 1994. This population lies to the north of Population 1 and in 1997 the plants showed three years growth and appeared healthy at over 1 m tall.

L e. salteri occurs in seven previously known small, discreet populations between the Botriver and Shaw's Pass at the foothills of the Babilonstoring in the South western Cape, near Caledon. All these populations are periodically subjected to heavy grazing, brush-cutting and burning. This taxon was ascribed the IUCN status of "endangered" (Tansley 1988) and totals some 1000 plants. The population sampled and designated Population 1 (S1 in Fig 2.1c) was the largest known population and was located on the farm Hartebeesrivier at the foot of Shaw's Pass. The second population (S2 in Fig2.1c) was on the farm Ruigtevlei, 3 km away. This population was not large but appeared to be very old. Prior to the visit in 1994, only three sites were found to still be extant; the two study sites and a third population, which was simply a northern outlier of the Hartebeesrivier population.

Figure 2.1. Locality and distribution of the study populations. In (a) the position of the Cape Floral Kingdom within South Africa is indicated and is enlarged in (b) to show its full extent. The shaded part of the Cape Floral Kingdom shows the widespread distribution of *L. salignum*. The position of the study population is indicated by the open circle. The area showing the study populations of the rare taxa is detailed in (c). Here R1 refers to *S. roxburghii* Population 1on the farm De la Gift, R2 refers to *S. roxburghii* Population 2 on the farm Vlakfontein. For the six study populations of *L. elimense,* V refers to *L.e. vyeboomense,* S1 refers to *L.e. salteri* Population 1 and S2 refers to *L.e. salteri* Population 2. The shaded area over the two *L.e. salteri* populations shows the known extent of this subspecies. E1, E2 and E3 refer to Populations 1, 2, and 3, respectively of subspecies *L.e. elimense*. The striped area over the three populations of *L.e. elimense*, shows the known distribution of this subspecies.


(a)
South Africa
(b)
30°
32°
34°
S
E
18°
20°
22°
24°
26°
28°
Cape Town
Cape Flora
Port Elizabeth
18 E
19 E
20 E
R1 (De la Gift)
Riebeek Wes
Elandskloof Mountains
Malmesbury
R2 (Vlakfontein)
Worcester
Paarl
Wemmershoek Mountains
Cape Town
Stellenbosch
Villiersdorp
34 S
Theewaterskloof dam
Vyeboom
V
Caledon
S1
S2
Napier
E1
Bredas-dorp
E2
E3
Elim
35 S
(c)

An additional four populations are known although each is no more than 50 -500 plants (Pool & Smuts 1993). These are on sites known to have had *L.e. salteri* in the past, suggesting possible cyclic fluctuations in numbers which decline to no visible plants and then increase up to large populations of thousands of plants, presumably from buried seed stores.

L.e. vyeboomense occurs on the banks of the Theewaterskloof Dam, near Villiersdorp and is confined to an area of about 200 hectares (V in Fig. 2.1c). In September 1994, there was one main population of 250 plants but a recount in August 1997 yielded only 50 plants. The IUCN status ascribed is "endangered" but this subspecies will almost certainly soon become "extinct". This taxon is now situated on the edge of a large reservoir of water. Many of the plants closest to the water appear to have fungal infections, possibly as result of too much water too nearby. On the second visit to the area in 1997, all these plants had died. The land occupied by *L.e. vyeboomense* is under dispute as it is wanted by a neighbouring farmer as compensation for land lost to the reservoir. The area occupied by the plants has now largely been fenced and occupies some 7 ha. This is approximately 10% of what was known to exist in 1979. Away from the surviving plants, on the edge of the previous extent of the population in 1979, remain two secondary very small outliers of this population. They are approximately 1 km from the main surviving population and lie on a neighbouring farm in private ownership. The visit to the main population in 1997 showed considerable evidence of physical trampling with many broken plants and also evidence of patchy fire damage.

L. salignum

L. salignum has been placed by Williams (1972) in the section Alatosperma, sub-section Alata, characterised by winged fruit and mature female perianth segments being free and glabrous. This section contains 23 taxa, some of which are common and widespread whereas others are restricted and naturally rare. *L. salignum* is not found in dense stands but is widespread everywhere within the Fynbos biome (Williams 1972). It has the widest distribution of all species of *Leucadendron*, being found from Cape Town east to Grahamstown and north to Nieuwoudtville (Fig.2.1b). This species is characterised by persistent rootstock with many stems making it possible to withstand frequent fire and re-sprout rapidly by coppicing. In addition, the deep root stock appears to favour this plant in dry areas. The conservation status

of this species is "not threatened". Williams (1972) states that, contrary to expectations, there is little morphological variation between populations. He also points out that the populations are not distinct units but exist as a continuum throughout the distribution range. Some populations show unique broad leaves or much redness of the leaves. These characteristics though merge and gradually change back to the more usual form via intermediate forms as one moves through the population.

S. roxburghii

S. roxburghii belongs to the section termed "Curly Spiderheads" (Rebelo 1995) in which the flowers typically are curved in bud, clustered in loose headlets on short stalks. Unlike the dioecious *Leucadendron*, this genus is bisexual with male and female parts in each flower. This presents an advantage for conservation purposes in that it increases the number of possible offspring as every plant potentially can produce seed. *S. roxburghii* previously occurred on recent sands on the west coast plains of the Swartland, which are scattered and unsuitable for cultivation although they are used heavily for grazing. This species is now restricted to two populations (R1 and R2 in Fig 2.1c) near the town of Riebeeck Kasteel. Both of these populations were sampled for this project. Recently an additional very small population has reportedly been found but this has yet to be confirmed. For this study, Population 1 (R1) occurs on the farm De la Gift and Population 2 (R2) occurs on the farm Vlakfontein. Both these populations seem to be characterised by frequent burning. Fires seem to burn erratically and in a patchy pattern, as a result of which some plants survive the fire. Population 1 was subjected to fires in 1993 and 1994, which almost destroyed the population completely.

Because of the restricted size of the populations, restricted number of populations, the presence of grazing animals, and controlled burning by farmers, this species has been ascribed the IUCN status of "endangered". An additional four populations are known although each is no more than 50 -500 plants (Pool & Smuts 1993). These are on sites known to have had *L.e. salteri* in the past, suggesting possible cyclic fluctuations in numbers which decline to no visible plants and then increase up to large populations of thousands of plants, presumably from seed stores.

Methods

Study populations were selected with the help of R. Pool (Cape Nature Conservation). Population parameters such as number of plants, area of population, height, and age of population were measured in the field. Areas were estimated and confirmed using 1:50 000 topo-cadastral maps. Plant numbers were counted in a representative sample area, selected randomly and then estimated for the whole population where numbers were large. For populations smaller than 200 plants, every plant was counted. The age of each population was assessed by counting the number of branching nodes of growth. This assumes new branching to have occurred at annual intervals and has been used by the author on other occasions. It is easily confirmed where the fire regime is well documented.

Information on morphology and ecology was tabulated for all three subspecies of *L. elimense* and for *L. salignum* and *S. roxburghii*. Morphological data for the study taxa were collected from a variety of sources, which included field observations, measurements from herbaria specimens, confirmation from revision by Williams (1972), and from data held by Dr J Rourke (Curator, Compton Herbarium and Proteaceae expert). Only the range of each characteristic was recorded for each taxon. The tabulated data did not relate specifically to the study populations but generally attempted to provide a background on gross similarities and differences between the study taxa.

Results and Discussion

Study Populations

Populations of *L. elimense* studied declined in numbers from 1994 to 1997 (Table 2.1). No measure was made of the extent of the populations in 1997 but most populations had plants present over the same range in the same area as in 1994, although these appeared to be more sparsely distributed in 1997. Most populations of *L. elimense* were 3-5 years old. Although parts of both populations of *S. roxburghii* were subjected to fire (Pool & Smuts 1993), Population 2 nevertheless, increased in number, which is ascribed to seedling recruitment. The age of the plants within each study population ranged from new seedlings in one population of *S. roxburghii* to as old as 30 years in the other population. The varied age structure in the two populations was ascribed to the patchy, incomplete fire patterns.

Table 2.1. Numbers, extent, and age of the selected study populations. Some of this data supplied by Pool & Smuts (1993).

	Number of plants in 1994	Number of plants in 1997	Extent of population in 1994 (ha)	Height of population in 1994	Estimated age of population in 1994
L.e. vyeboomense	200	50	2	0.5 m	3 years
L.e. salteri Population 1	5000	500	50	2 m	3 years
L.e. salteri Population 2	100	100	0.1	2-3 m	12 years
L.e. elimense Population 1	50	50	0.01	1.5 m	5 years
L.e. elimense Population 2	1000	<10 seedlings	600	1-1.5 m	5 years
L.e. elimense Population 3	200	50 & 50 seedlings	1	0.5-1.5 m	5 years
S. roxburghii Population 1	3000	3000	60	up to 0.5 m	New seedlings to 3 years
S. roxburghii Population 2	4000	6000	100	0.2 -1.5 m	1-30 years

Past records from Department of Nature Conservation (R Pool pers. com.) show fluctuation in population sizes for rare Cape Proteaceae, from small to large to extinct to small again. For example, some populations of *L.e. salteri* have been known to be present, to disappear and then reappear again, all within a 20 year period. The same is known for *S. roxburghii*, which was for many years thought to be extinct. A similar situation has been observed for *Serruria decumbens* (Thunb.) R.Br., which had not been located for over 50 years on the Cape Peninsula, and was then rediscovered by this author during field work in 1985. These fluctuations in numbers give credit to the theory of chaotic dynamics and rarity and the possibility that such chaos allows for competitive advantages of some sort (Rosenzwieg & Lomolino 1997, Gaston & Kunin 1997). Thus while such fluctuations are a serious problem for conservation management in the field, they appear to be normal and must thus be taken into account, particularly when ascribing IUCN status.

Comparative ecology

L. elimense and *S. roxburghii* are both ascribed a threatened status (Tansley 1988) as is shown in Table 2.2. There are no records to suggest that any of these taxa have ever been prolific so they would certainly have been considered naturally rare at best. Each of these restricted taxa has very specific edaphic requirements (Table 2.2). *L.e. vyeboomense* occurs on granitic sands, *L.e. salteri* on shales while *L.e. elimense* occurs on shales, or shallow sand over shale, and most specific of all is *S. roxburghii* which occurs on isolated patches of recent sand surrounded by shale. This apparent soil requirement means that there usually is a tight association with a particular vegetation. All the study taxa are characterised by being dominant, fairly densely distributed and highly visible in the area covered by the population (Table 2.2). This might be a disadvantage and is somewhat unusual for rare taxa which are often cryptic, infrequent, and well scattered (M^{c}Kinney 1997), which may be a means of escaping notice and thus aiding survival. The altitudinal range (Table 2.2.) shows some overlap between the subspecies of *L. elimense* and does not appear to be a critical factor. In other rare Cape Proteaceae, such as *Paranomus centaureoides* Levyns and *Orothamnus zeyheri* Pappe ex Hook.f. altitude plays a much more important role.

Table 2.2. Conservation status and ecological characteristics of the study taxa.

Characteristic	*L.e. vyeboomense*	*L.e. salteri*	*L.e. elimense*	*L. salignum*	*S. roxburghii*
Conservation Status	endangered	endangered	vulnerable	not threatened	endangered
Soil	granitic sand	shale	shale/sand	granitic sand, shale, sandstone	recent sand
Community Position	dominant	dominant	dominant	dominant/sub-dominant	dominant
Vegetation Association	renosterveld	renosterveld	Elim sands, renosterveld	renosterveld, mountain fynbos	lowland sand
Altitude (above mean sea level)	300-400	50-350	50-150	0-2000	100-200

The vegetation community associated with the study taxa is renosterveld or lowland sandveld. These are similar open shrublands of the Fynbos biome with the lowland sandveld of the *S. roxburghii* populations containing many renosterveld elements.

Comparative morphology

Table 2.3 allows for an quick overview of some of the morphological characteristics of the study taxa. If the three subspecies of *L. elimense* are compared (Table 2.3), it is seen that eight out of 19 categories (42%) selected show differences. Of these differences, the most visible ones are leaf characteristics, which are analysed further below for the six study populations of *L. elimense* . A comparison of *S. roxburghii* with the three subspecies of *L. elimense* showed 15 of the 19 characters (79%) differing. *L. salignum* differed from the *L. elimense* subspecies in 10 of the 19 characters (53%). These differences largely mirror the suggested taxonomic distances between the subspecies within the same species (42% difference), the same genus but different species (53% difference) and the different genera (79% difference). Overall, Table 2.3 allows for a summarised comparison of the morphological characters of the study taxa.

If the three subspecies of *L. elimense* are compared, *L.e. salteri* is most different. It is tallest, largest leaved and has red colouring on the floral bracts. The month earlier flowering time for *L.e. vyeboomense* is certainly attributable to the inland location where winters are colder and longer. *L.e. vyeboomense* has an expected life span which is half that of the other two subspecies. It also differs in leaf shape, being more lanceolate as opposed to elliptic.

The selected taxa do not show much morphological plasticity. In *L.e. elimense*, morphological variations do occur at the northern and eastern extremes of its range, particularly with regard to reduced leaf size and shape (Williams 1972). Rebelo (pers. comm.) also reports a large-leafed population of *L.e. elimense* at Baardskeerdersbos, to the west of the distribution range. Unfortunately, this has not been confirmed because the population appears to have been ploughed in 1995. There is, however, a possibility that these plants may still survive as seeds. A possible reason for the large leaf size is that the area in which they grew is used for agricultural crops and has, through time, been well fertilized.

Table 2.3. Morphological characteristics measured in the study taxa.

Characteristic	*L.e. vyeboomense*	*L.e. salteri*	*L.e. elimense*	*L.salignum*	*S.roxburghii*
Maximum height (m)	1.5	3.0	2.0	2.0	2.0
Mean mature height (m)	1.0	1.5	1.5	0.5	1.0
Fire survival	seed	seed	seed	rootstock & seed	seed
Years to flowering	3	3	3	4	3
Age to maximum seed	8	8	8	8	12 (in wild)
Lifespan (years)	10	20	20	10 -30 (varies)	20 (5 in cultivation)
Sexual orientation	dioecious	dioecious	dioecious	dioecious	monoecious
Flower colour	yellow	yellow, red bracts	yellow	yellow to red	pink
Expected pollinators	insects	insects	insects	insects	insects
Inflorescence position	terminal	terminal	terminal	terminal	terminal spike
Fruit	winged seed	winged seed	winged seed	winged seed	small hairy seed
Flowers	Sept. to Oct.	August to September	August to September	April to November	September to November
Seed dispersal	wind	wind	wind	wind	mymerochorus

Table 2.3 continued on next page ....

.... Table 2.3 continued

Characteristic	*L.e.vyeboomense*	*L.e.salteri*	*L.e.elimense*	*L.salignum*	*S.roxburghii*
Leaf length (mm)	39 - 36	55 - 78	13 - 57	20 - 60	10 - 13
Leaf width (mm)	8 - 10	15 - 25	5.5 - 21	3 - 5.5	2 - 3
Leaf ratio/area	0.25	0.30	0.40	0.10	0.20
Leaf shape	lanceolate, acute	elliptic, obtuse	elliptic, obtuse	oblanceolate-linear, acute	flagelliform
Leaf covering	glabrous	glabrous, sub-glabrous	glabrous, sub-glabrous	glabrous	densely villose, glabrous when mature
Leaf colour	green	green	green	yellow, green, red	silver blueish green, red tipped

A measure of the leaf ratios as opposed to gross size might also show a similarity with other populations of *L.e. elimense.*

Phenotypic plasticity can have taxonomic consequences which are of conservation importance. A good example of this is seen in *Protea subulifolia,* which ranges in habitat from sandstone soils at high altitudes (1 600 metres above sea level) with a rainfall of 900 mm to heavy clay shales on the plains to sandy coastal soils at low altitudes (60 metres above sea level) with a rainfall of 200mm. A change from dark brownish needle leaves 85 mm long on tall stems of almost 0.9 m to pale pinkish ericoid leaves 5 mm long on 0.3 m long stems is seen along this habitat gradient. Each of the many populations of *P. subulifolia* are only partially distinct in morphological characters yet when members of different populations with their accompanying different morphology are grown under uniform environmental conditions these differences persist, suggesting that these differences are genetically stable (Rourke 1980). Karron (1997) indicates that there are several studies that show that phenotypic differences among populations growing in distinct habitats have a genetic basis. An example is the ecotypic study on *Plantago* by Van Dijk (1989). Morphological plasticity can be problematic when using morphology to characterise populations, and hence taxa. A study of the phenotypic plasticity and the underlying genetic variation within the Cape Proteaceae could provide some information about taxonomic and environmental relationships.

Leaf morphology of the three subspecies of *L. elimense*

Methods

Measurements of leaf size

The single most distinguishing character for separating the three subspecies of *L. elimense* is the differences in the leaves, particularly their size (Table 2.3). In order to assess how size differs in this parameter, leaf lengths and breadths were measured six centimetres from the growing tip in both male and female plants. This was an arbitrary measurement which did not necessarily come from the identically positioned leaf on each stem measured. Assuming that conditions were similar within the populations, similar rates of growth in the stems would be expected across the population, resulting in more-or-less the same leaf position being measured. A minimum of 15 plants of each sex were sampled from each population. Leaf

measurements for the study populations were reduced to a ratio to minimise the effect of gross size. Comparison between plants of the same sex within and between populations of the same subspecies, between different sexes and between sub-species were made using t-test (where only two populations) or one way ANOVA (where three or more populations). Where the assumptions for normality and equal variance were not met, the non-parametric Mann-Whitney rank sum test or Kruskal-Wallis one way ANOVA on ranks was used instead.

Results and Discussion

Leaves of the three subspecies are clearly different in size (Fig. 2.2), with those of *L.e. salteri* being the largest, those of *L.e.elimense* the smallest and those of *L.e. vyeboomense* being intermediate.

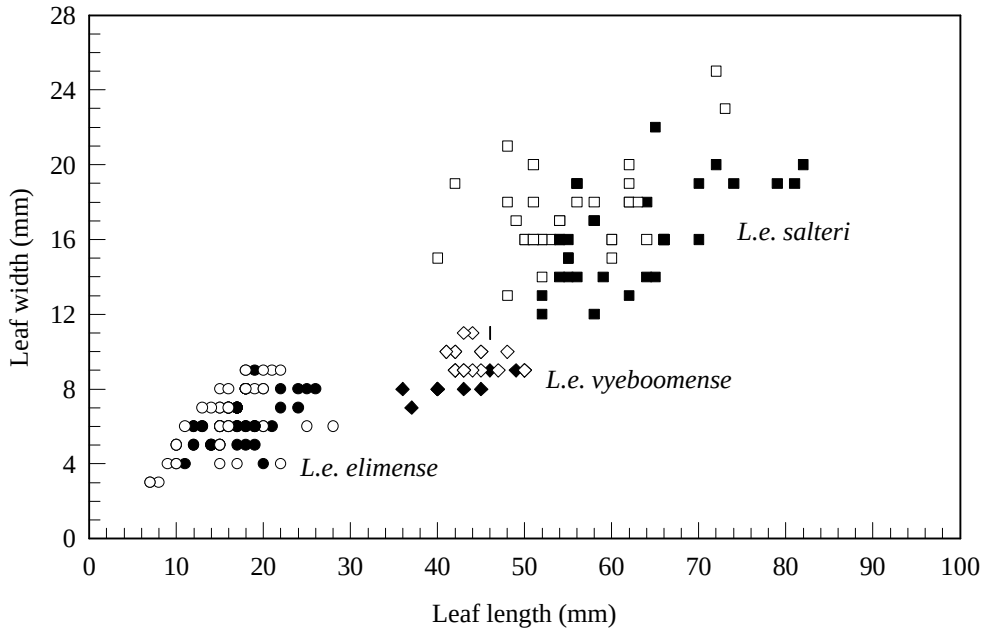


Figure 2.2. Leaf size of the three subspecies of *L. elimense.* Closed symbols are male plants and open symbols are female plants.

The mean leaf measurements for each study population of *L. elimense* are shown in Table 2.4. For consistency, these measurements were restricted to leaves sampled 6 cm below the growing tip and hence do not reflect the maximum or minimum measurements possible for

leaves in the populations. The largest leaves (mean length 67.87 mm, width 17.33 mm) measured were found in male plants in Population 2, of *L.e. salteri*. The smallest leaves (mean length 14.25 mm, width 5.94 mm) measured were found in female plants in Population 3, of *L.e. elimense*. A comparison of the leaf ratios for the three subspecies shows the largest ratios in *L.e. vyeboomense* which were 5.01 for males and 4.58 for females, and the smallest ratios of 2.78 for males and 2.57 for females in *L.e. elimense* (Table 2.4). This cline in leaf ratios is reflected in the geographical distribution of the three subspecies (See Fig.2.1c).

A comparison in the leaf ratios between the populations of the three subspecies indicated no significant difference between males (*L.e. elimense* $F = 1.05$, $P = 0.36$; *L.e. salteri* $t = 0.77$, $P = 0.45$;) or females (*L.e. elimense* $H = 3.32$, $P = 0.19$; *L.e. salteri* $U = 249$, $P = 0.57$) so plants of the same sex were pooled within subspecies. Leaf sizes of males and females of all three subspecies were, however, significantly different (*L.e. elimense* $U = 2640$, $P = 0.004$; *L.e. salteri* $t = 5.65$, $P < 0.0001$; *L.e. vyeboomense* $t = 2.49$, $P = 0.02$).

A comparison of the leaf ratios between the three subspecies of *L. elimense* (carried out on the male and female plants separately because of the significant differences in gender in all three subspecies) showed that the differences between all three subspecies were significant (males $F = 108.9$ $P < 0.0001$; females $H = 51.8$ $P < 0.0001$). Since there is little evidence for environmental influences on morphology in the species, this is strong support for the taxonomic subdivision of *L. elimense* into the three subspecies, as ascribed by Williams (1972). This has little direct relevance for conservation except in terms of the taxonomic grouping distinguishing three taxa rather than one, requiring conservation management and input and that conservation of all three subspecies as separate integral units is essential.

Table 2.4. Measurements (Mean ± S.D.) of leaves within the study populations. Leaves were taken from about 6 cm from the tip of the branch. Leaves from male plants (**%**) are separated from female plants (**&**) as there was statistically significant sexual dimorphism.

Subspecies and population	mean **%** leaf length (mm)	mean **%** leaf width (mm)	**%** leaf ratio	mean **&** leaf length (mm)	mean **&** leaf width (mm)	**&** leaf ratio
L.e. vyeboomense	42.87 ± 4.36	8.60 ± 0.83	5.01 ± 0.49	44.63 ± 2.39	9.81 ± 0.83	4.58 ± 0.47
L.e. salteri Population 1	57.13 ± 4.88	15.20 ± 2.15	3.82 ± 0.54	55.94 ± 9.04	17.56 ± 3.25	3.23 ± 0.45
L.e. salteri Population 2	67.87 ± 8.57	17.33 ± 2.72	3.97 ± 0.52	54.56 ± 5.94	17.25 ± 1.39	3.18 ± 0.44
L.e. elimense Population 1	15.47 ± 2.85	5.93 ± 1.39	2.65 ± 0.36	17.44 ± 5.37	6.19 ± 2.14	2.98 ± 1.06
L.e. elimense Population 2	18.53 ± 4.71	6.71 ± 1.11	2.76 ± 0.53	16.44 ± 1.15	7.19 ± 0.84	2.30 ± 0.19
L.e. elimense Population 3	16.80 ± 2.24	5.87 ± 0.83	2.94 ± 0.73	14.25 ± 4.95	5.94 ± 0.73	2.42 ± 0.57
L.e. salteri whole subspecies	62.50 ± 8.76	16.27 ± 2.64	3.89 ± 0.52	55.25 ± 7.56	17.41 ± 2.46	3.20 ± 0.44
L.e. elimense whole subspecies	17.00 ± 3.65	6.19 ± 1.17	2.78 ± 0.56	16.04 ± 4.39	6.44 ± 1.71	2.57 ± 0.75

Conclusions

L. elimense, with its three subspecies *salteri*, *vyeboomense*, and the type subspecies *elimense*, differ in conservation status to the common and widespread *L. salignum* and the highly endangered *S. roxburghii*. These rare Cape Proteaceae are characterised by dynamic fluctuations in both population size and numbers. Such fluctuations make it difficult to assess the true conservation status at any one time and must be considered in the drawing up of a management plan. The ecological and morphological characteristics are seen to show relatively little variation between the subspecies of *L. elimense*, and more variation between *L. elimense* and *S. roxburghii*, a between-genus comparison. This result supports the existing taxonomic ranking of the study taxa in broad terms (but see Chapters 5 & 6) but has no direct conservation value.

The single most distinguishing feature seen to separate the three subspecies of *L. elimense* is the leaf morphology, which is shown here to be significantly different between male and female plants within subspecies and between subspecies. This supports the taxonomic separation of the subspecies based on a morphological species concept, but has little direct value for conservation decisions in terms of management of populations or of the selection of populations within a subspecies.

CHAPTER 3

Assessment of genetic diversity in the three subspecies of the rare and endangered *Leucadendron elimense* E. Phillips (Proteaceae) using RAPD analysis, and the implications for their conservation

Introduction

The family Proteaceae is represented by some 400 taxa (330 species) within the Cape Floral Kingdom (see Chapter 1). Of these, 152 are considered rare or threatened (after Hilton-Taylor 1996a, 1997), an increase of some 23% since my assessment in 1988 (Tansley 1988). Many of the rare or threatened species are characterised by having restricted ranges, having few populations, and/or few individuals (Tansley 1988) yet until recently these taxa maintained a constant presence without any apparent signs of stress or extinction. Genetic diversity may, however, be reduced in such populations relative to populations of common taxa (Gaston & Kunin 1997, Karron 1997).

An example of a species exhibiting the above characteristics is the cone bush, *Leucadendron elimense,* which was selected for this study because it has three subspecies which are not only genetically closely related, but all are rare to varying degrees with different population dynamics (See Table 2.1, 2.2, and 2.3). Because of its very small population size, one might, for example, expect *L.e. vyeboomense* to show marked reduction in genetic diversity relative to *L.e. elimense*. Furthermore, as with all species of *Leucadendron*, *L. elimense* is dioecious, thereby enforcing obligative outcrossing with no cloning from vegetative reproduction.

To date no measure has been made of the genetic diversity within any rare Cape Proteaceae, yet this should be an important component in determining conservation strategies for the Cape Flora as a whole (see for example Rebelo 1992, Rebelo & Tansley 1993, Linder 1996). To this end, the aim of this study was to measure levels of genetic variability within and between populations and subspecies of *L. elimense*. These differences can, hopefully, be used to select preferentially certain populations and focus conservation efforts positively and productively. In addition, a ubiquitous species was selected to test whether genetic variability in rare Proteaceae is reduced compared to a common, widespread species.

Materials and methods

Plant material

For *L.e. vyeboomense,* the single remaining population was sampled, whereas for *L.e. salteri* two major populations of the subspecies were sampled and three populations of *L.e. elimense*.

The location of these populations is shown in Fig. 2.1c. Populations were counted and aged according to the number of internodes, the plants producing a single node per growth year. Leaves were collected from growing tips of 20 individual plants of known sex in each population and immediately stored at -20°C. In addition, leaf material was collected and processed from a population of *Leucadendron salignum*, a widespread common species (Fig. 2.1b) to provide a comparison.

Extraction of Genomic DNA

Three methods for extracting genomic DNA were screened. Two of these methods were found to be unsuitable. The first method was already extensively in use by the laboratory. It was modified from a protocol from Sambrook *et al.* (1989). Leaf tissue was ground in a pestle and mortar, then lysed for 10 min in a chilled buffer (0.32 M sucrose, 10mM Tris HCl, pH 7.6, 5 mM $MgCl_2$, 1% Triton X-100 (Kirby 1990). The sample was centrifuged at 13 000 rpm for 5 min. The resultant pellet was resuspended in a solution of 10 mM Tris HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA (Kirby 1990) for 10 min. This was centrifuged at 13 000 rpm for 5 min. after which the lysis buffer was removed and replaced with 250Fl new pellet lysis buffer containing 1mg/ ml proteinase-K and incubated at 65°C for 2 hours. This was followed by a phenol/chloroform extraction and a second chloroform extraction. The supernatant was added to 1 ml ice-cold ethanol to precipitate the DNA overnight. The sample was then centrifuged at 13 000 rpm and vacuum dried. The pellet was resuspended in 50 Fl TE buffer(10mM Tris HCl,pH 7.5, 1mM EDTA). This method yielded very small amounts of DNA.

The second method tried was that of Chalmers *et al.* (1992).This method used a single leaf punch with the lid of a sterile Eppendorf tube. The leaf material was ground with liquid nitrogen and 10 mg of Polyclar AT (BDH). 400 Fll of extraction buffer (200mM Tris HCL, pH &.), 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 10mM mercaptoethanol) were added. The sample was mixed, then centrifuged at 13 000 rpm for 1 min. The supernatant was extracted with phenol/chloroform, then chloroform, and the resultant aqueous fraction was added to 300Fl ice-cold isopropanol for about 5 min. Centrifugation at 13 000 rpm followed for 5 min. The DNA pellet was vacuum dried and then rehydrated with 50Fl TE buffer 10mM Tris HCl, pH 7.5, 1mM EDTA). With the *Leucadendron elimense* leaf material, the DNA pellet was dark brown,

suggesting contaminants still present. The complete failure of the amplification tended to confirm this. Unfortunately, this method was not suitable for the extraction of genomic DNA from *Leucadendron elimense* leaves. This method also produced small DNA yields. DNA yields were measured spectrophotometrically.

The most successful method, which was selected for use, was one modified from that of Doyle & Doyle (1990). Using this method, DNA has been successfully extracted from a variety of higher plant species including, amongst others, palms, sedges (known to contain high levels of silicas), orchids, walnuts, oaks, beeches, legumes, lobelias, brassicas and portulaccas. This method has even been used successfully on herbarium material (Doyle & Dickson 1987), although when attempted in this study was of limited success. The method produced a consistent yield of genomic DNA from samples of all subspecies, which amplified using both RAPD primers and sequencing primers. DNA produced was stable for at least 30 months at 4°C. Doyle & Doyle (1990) started with 1 g of tissue, which yielded DNA at a concentration of 1mg/g fresh weight. Only 100ng of template DNA was required in the present study for amplification, so much less tissue was initially required.

The whole leaf was washed and sterilized with ethanol and water. Two leaf discs were punched out using the inner seal on a 1.5 ml safe-lock Eppendorf microfuge tube lid. The samples had a combined mass of approximately 290 mg. These were ground to a fine powder in liquid nitrogen in the microfuge tube using custom-made glass or perspex grinding rods.

CTAB isolation buffer [3% (w/v) CTAB (BDH), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol (Sigma), 20 mM EDTA, 100 mM Tris-HCL (Sigma), (pH 8.0)] was preheated to 65°C in a water bath. To the leaf powder, 200µl hot CTAB isolation buffer was added with 1% (w/v) Polyclar AT (BDH) (polyvinylpyrollidine insoluble). This was about 0.002 g Polyclar which has been shown to be successful at binding polyphenolic compounds (Jobes *et al.* 1995). After mixing, the tubes were incubated at 65°C for 60 minutes with gentle periodic shaking.

The sample was extracted using 200µl chloroform-isoamyl alcohol (24:1 - v:v) and after centrifugation at 13 000 rpm for five minutes, 150µl of the aqueous phase was transferred to

a clean 1.5 ml microfuge tube containing 300Fl ice-cold isopropanol. At this stage the DNA could be seen. After leaving overnight at 4°C, the DNA was centrifuged at 13 000 rpm for five min. Most of the isopropanol was then poured off and replaced with 200Fl wash buffer (76% ethanol, 10mM ammonium acetate). This was left overnight. During this wash, the chlorophyll disappeared and samples were centrifuged at 13 000 rpm for five minutes. The wash buffer was poured off and the samples dried in a vacuum centrifuge and subsequently rehydrated with 49 Fl TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA).

RNase A (Sigma) was added to a final concentration of 10 Fg/ml (giving a final sample volume of 50Fl) and incubated at 37°C for 60 min. The samples were diluted with 100Fl TE buffer. To this, 672Fl ice-cold ethanol was added along with 75Fl 7.5 M ammonium acetate (pH 7.0) which delivered a final concentration of 2.5 M. The tubes were swirled gently to mix. After four hours at 4°C, samples were centrifuged at 13 000 rpm for 7 minutes. The supernatant was discarded and the samples dried using a vacuum centrifuge. Often the DNA pellet appeared gelatinous at this stage although this did not cause any apparent problems for amplification. The pellet was resuspended in 50 Fl TE buffer. Half of each sample was stored at 4°C and the other half was stored at -20°C. The samples stored at 4°C seemed to be perfectly stable and were subsequently used for sequencing 30 months later without any problems.

The quality of the DNA was checked on a 0.8% agarose gel stained with ethidium bromide. This showed the DNA to be of high molecular weight in a bright band not far below the well. The ultraviolet spectrophotometer was used to gauge the amount and quality of DNA present in the template. Using wavelengths of 260 and 280 nm, readings for ten samples showed a range of 165ng/Fl to 480ng/Fl(mean 250 ng/Fl). Fairly high levels of proteins or other compounds were indicated by low 260 to 280 nm ratios (Kirby 1990). Quantification by absorbance at 260nm generally gives unreliable results (Glasel 1995, Manchester 1996), presumably due to residual CTAB (Doyle & Doyle 1990). This effect could not be verified by a CTAB dilution series. Nevertheless the possible CTAB effect could not be discounted. Relatively small variations in DNA concentrations between samples were observed and because the size of the original leaf punch was strictly controlled, the DNA template concentrations were not individually adjusted.

Polymerase chain reaction (PCR)

Amplification of the DNA by PCR was done using a Hybaid Omnigene Thermocycler. PCR conditions were optimised to produce consistent, reproducible fragment profiles (see below). Some studies duplicate every PCR reaction to ensure reproducibility (Sydes & Peakall 1998, Palacios & González-Candelas 1997, Stewart & Porter 1995, Paran *et al.* 1997). In the present study it was not economically viable to duplicate every reaction. Consequently only five samples from each population were extracted in duplicate and amplified. Good reproducibility was found. In addition, the same samples were amplified at different times and run on different gels. These also showed good reproducible RAPD banding patterns. At the top of some lanes, smears made it difficult to read banding patterns. This region lay outside the recommended range of band sizes generally measured in other similar studies (See for example Kappe *et al.* 1995, Stewart & Excoffier, 1996, Gillies *et al*, 1997) and was possibly caused by compounds, such as tannins not removed in the extraction method (G Scott pers com.). Consequently it was not considered a problem.

The level of reproducibility achieved is shown in Fig. 3.1 where pairs of duplicate samples are visualised on a polyacrylamide gel by electrophoresis. Although reproducibility was tested with all primers and all populations, only the results from plants 2, 3, 4, and 6 in *L.e. elimense*, Population 1 are presented in the thesis (Fig. 3.1). Here genomic DNA was extracted from two different leaves of the each of the samples on different days and amplified separately. The pairs of duplicates were then run on the same gel to allow comparison. Careful appraisal of these four pairs of replicate samples shows that duplicate samples, although extracted form different leaves on different days and amplified at different times, have identical banding patterns. It will, however, be noticed that the intensity of the bands differs between replicates. This might be a loading discrepancy in that the total sample amplified was not loaded onto the gel. Throughout this study, banding intensity was not considered.

A set of 12 custom-made random primers (Ransom Hill), designated Rhodes 1-12, and 20 primers of kit AI (Operon), designated OPAI 1-20, were screened and five primers were subsequently selected for reproducible and distinct banding patterns. Primer sequences are given in Table 3.1. Each reaction had a total volume of 50Fl overlaid with 30 Fl mineral oil

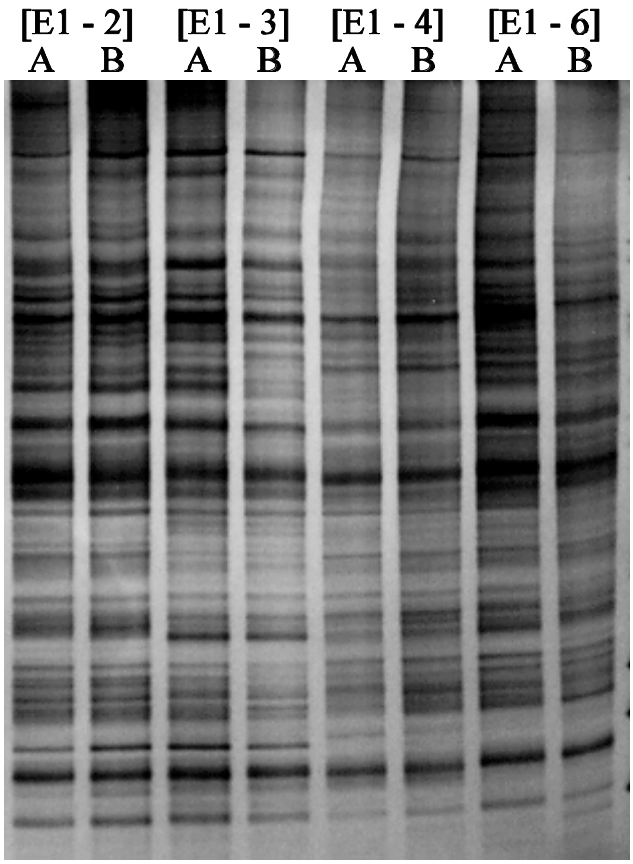


Figure 3.1: Pairs of duplicate samples from four different plants, 2, 3, 4, and 6 from *L.e. elimense* Population1 (E1), are shown in neighbouring lanes, A and B, to verify the level of reproducibility achieved. Samples labelled A were extracted from different leaves of the plant and were amplified prior to and separately from the extraction and amplification of the duplicate samples labelled B. The duplicate samples from each plant are shown here on a single gel. Slight differences in the loading of the lanes caused different densities of bands. Bands were therefore scored simply as present or absent.

(Sigma) and was carried out in 0.3 ml thin-walled omnistrips. The PCR mix contained reaction buffer (Advanced Biotechnologies Ltd) delivering 50 mM KCL, 10 mM Tris-HCL (pH 8.8), 2 mM MgCl, and 0.1% Triton X100. Primer concentration was 200nM. dATP, dCTP, dGTP and dTTP (Promega) were added at a concentration of 0.1mM each. One unit of Thermoprime^{plus} DNA Polymerase (Advanced Biotechnologies Ltd.) and 33.5 Fl H_2O completed the PCR mix. 2 Fl DNA in TE buffer was added to each 50 Fl reaction thereby delivering approximately 500ng DNA (as determined spectrophotometrically).

PCR conditions began with an initial denaturing at 94ºC for 2.5 min, followed by thirty cycles of denaturing at 94ºC (30 s), annealing at 40ºC (30 s) and extension at 72ºC (60 s). A final extension of 4 minutes at 72ºC completed the thermocycling. Negative controls with water replacing the template DNA were always included. Magnesium chloride is known to influence PCR success. This was screened at concentrations of 1 - 5 mM. Fig. 3.2 shows the optimization of magnesium chloride concentrations in the PCR reaction. The sample shown in the figure is sample 17 from Population 3, *L.e. elimense*. The titration was repeated with samples from the other subspecies and other primers. The optimal concentration was 2 mM. This was also screened in combination with variable amounts of template DNA. Different amounts of DNA simply resulted in different densities of certain bands.

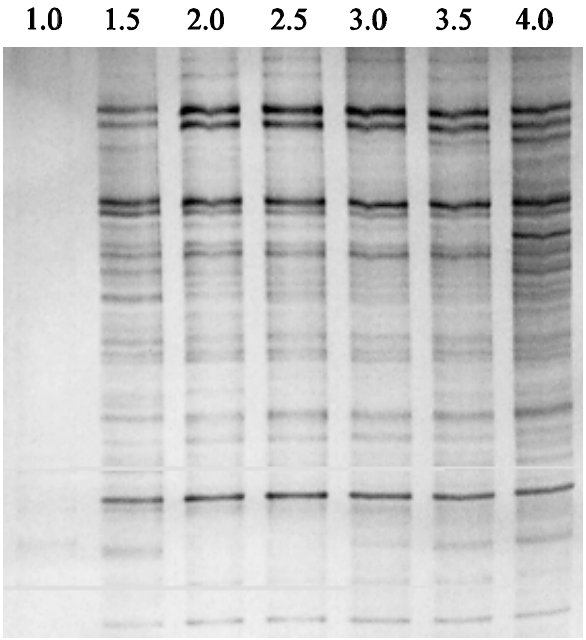


Figure 3.2: Using primer Rhodes 12, sample 17, Population 3, *L.e. elimense*, is shown amplified using concentrations of 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4-5 mM MgCl. Higher concentrations resulted in complete absence of bands.

DNA concentrations were tested in the 50Fl PCR reaction at 2Fl (approximately 500ng/Fl reaction), 1Fl (approximately 250ng/ reaction) and at 0.4Fl(approximately 100ng/reaction). There were no visible differences between the use of 1Fl or 2Fl, except that there were more PCR failures with the 1Fl reactions. As this was attributed to pipette inconsistencies at this range, it was decided that the larger amount of 2Fl would be used.

Gel Electrophoresis

Amplification products were resolved electrophoretically on 10% denaturing polyacrylamide gels (20 x 20 cm) run at 180 V for about 5 hours in TBE (pH 8.00) and visualized by staining with silver nitrate. The use of silver-stained polyacrylamide gels provided greater resolution than agarose gels, particularly in the range of 25 -1000 base pairs (Morell *et al.* 1995). The range and size of reproducible fragments scored for each primer is shown in Table 3.1 and lies within this range. The increased sensitivity of the silver staining was able to detect bands containing 0.15 pmoles of DNA/200bp band (Bassam *et al.* 1991). Molecular size markers were provided by digesting pBR322 with the restriction enzyme, Hinf I (Boehringer Mannheim), and the double digestion of ëDNA with the restriction enzyme Hind III and EcoR1 (Boehringer Mannheim).

Gels were photographed and the bands were scored manually. Manual scoring has been successfully used in other studies (Morell *et al.* 1995). Scoring was done using good quality photographs overlayed with clear film onto which the banding patterns were transferred. These were verified with the original gels. It has been reported by Penner *et al.* (1993) that very large and very small fragments of DNA do not necessarily amplify consistently, so in this study, only the central region of fragments were scored. An attempt to use the automated Gel Compar computer software (Applied Maths 1995) led to the creation of artifactual bands. Although tedious and potentially subjective, the manual scoring of the bands nevertheless proved to be the best option for the present study. A second person scored some of the gels independently to confirm accuracy and to minimise subjectivity.

Because it was not possible to obtain an accurate measure of the amount of DNA in each sample before amplification, there was some difference in amount of amplification product.

Fragments are repeated in the genome to varying degrees which results in a varying intensity of bands (Bielawski *et al.* 1995, Kambhampati *et al.* 1992). Therefore no account was taken of band intensity and bands were simply scored as present or absent. All polymorphic and monomorphic bands were included without any bias in scoring polymorphic versus monomorphic bands, as suggested by Clark & Lanigan (1993).

Data Analysis

RAPD analysis has been successfully completed on a threatened species of Proteaceae in Australia, *Grevillea scapigera*, by Rossetto *et al.* (1995). In order to allow broad comparison to this study, the data analysis was carried out in a similar manner for *L. elimense*. Quantitative measures of fragment patterns can be a measure of genetic distance. Genetic distance is a quantitative estimate of the genetic divergence between two sequences and is the converse of genetic similarity (Avise 1994).

Presence/absence data were analysed to obtain an estimate of similarity for each pair of individuals in each population. The similarity coefficient S is given by $S = M/N_t$ (Apostol *et al.* 1993), where M is the number of matches of shared bands and shared absence of scored bands between plants within the population and N_t is the total number of bands identified for a particular primer or group of primers for the species. Variability was calculated as $V = 1 - S$ (Apostol *et al.* 1993). Essentially this is a measure of genetic distance or dissimilarity. Mean population dissimilarity was estimated by averaging the dissimilarities of all pairs of individuals in the sample. This method was used by Kappe *et al.* (1995). In addition to the within-population mean distances calculated across the range of primers used, average genetic distances were calculated between populations, both within and between subspecies units to allow further comparisons. This average genetic distance was calculated using the coefficient of Apostol *et al.* (1993) with the pooled primer data (using primers Rhodes 3, 7, 12 and OPAI-13). In addition, for the pooled primer data, the coefficient of Dice (1945) and Nei & Li (1979) was used to generate tables of genetic distance. This shows a similarity F where $F = 2m_{xy} / 2m_{xy} + m_x + m_y$ and m_{xy} is the number of shared presence of bands between x and y, and m_x and m_y are the number of bands in x and y, respectively. Dissimilarity is thus $1 - F$.

Pease & Fowler (1997) suggest the use of adaptations of analysis of variance (ANOVA) as a more systematic approach to conservation studies. Such a statistic is provided by analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992), which allows for a study of hierarchical genetic structure of populations using molecular information. AMOVA was used to estimate the variance components among individuals within populations, between populations within each subspecies, and between subspecies. AMOVA is mathematically identical to ANOVA (Peakall *et al.* 1995) and the theoretical considerations have been discussed previously (Excoffier *et al.*1992). It is of particular use in an outcrossing species such as *L. elimense* where there is within-taxon variation (Morell *et al.* 1995) which may not be distinguishable by eye, and here AMOVA is a powerful statistical tool. Euclidean distances are required for the distance matrix used in AMOVA. The coefficient of dissimilarity (Excoffier *et al.* 1992) is $1 - n[1-(m_{xy}/n)]$ where n is the number of individuals sampled in the population, m_{xy} is the number of shared presence between two individuals of bands scored.

The unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal 1973), was used to generate dendrograms. The four coefficients tested were those of Nei & Li (1979), Apostol *et al.* (1993), Excoffier *et al.* (1992), and Jaccard (1901 cited in Armstrong *et al.* 1994). Details of the first three coefficients are given above and the coefficient of Jaccard is $J = m_{xy} /(N_t - m_{oo})$, where m_{xy} are shared presence of bands between x and y, N_t is total number of band positions, and m_{oo} is shared absence of bands between x and y. An estimate of variability is calculated as 1-*J*. Essentially this is a measure of genetic distance. A co-phenetic correlation coefficient (r_{cs}) was obtained as a measure of goodness of fit between the original dissimilarity matrix and the co-phenetic values from the UPGMA dendrogram (Sneath & Sokal 1973). The UPGMA method tends to maximise r_{cs} (Sneath & Sokal 1973). To summarise, a consensus tree of the four coefficients was produced using the strict consensus method (Sokal & Rohlf 1982).

Three dimensional visualization of the clustering was obtained using multivariate analysis. Such an ordination tests the robustness of the groups formed by the UPGMA tree. The ordination analysis was done using Principle Co-ordinate Analysis (Gower 1966) to generate three dimensional scatter plots. Both clustering and ordination were done using programmes within

NTSYS-pc (Version 1.80) of Rohlf (1993). Measures of genetic distance required for these programmes and AMOVA were generated using RAPDistance (Armstrong *et al.* 1994).

Results

DNA Fragment description

Fig. 3.3 shows a typical example of a RAPD profile on a polyacrylamide gel. The high background presence evident in the gels could not be reduced. It is, however, not unexpected as Proteaceae are characterised by various tannins, phenolic and polysaccharide compounds. The negative controls, from which DNA was omitted, always were free of DNA fragments, indicating that no contamination occurred at the PCR stage.

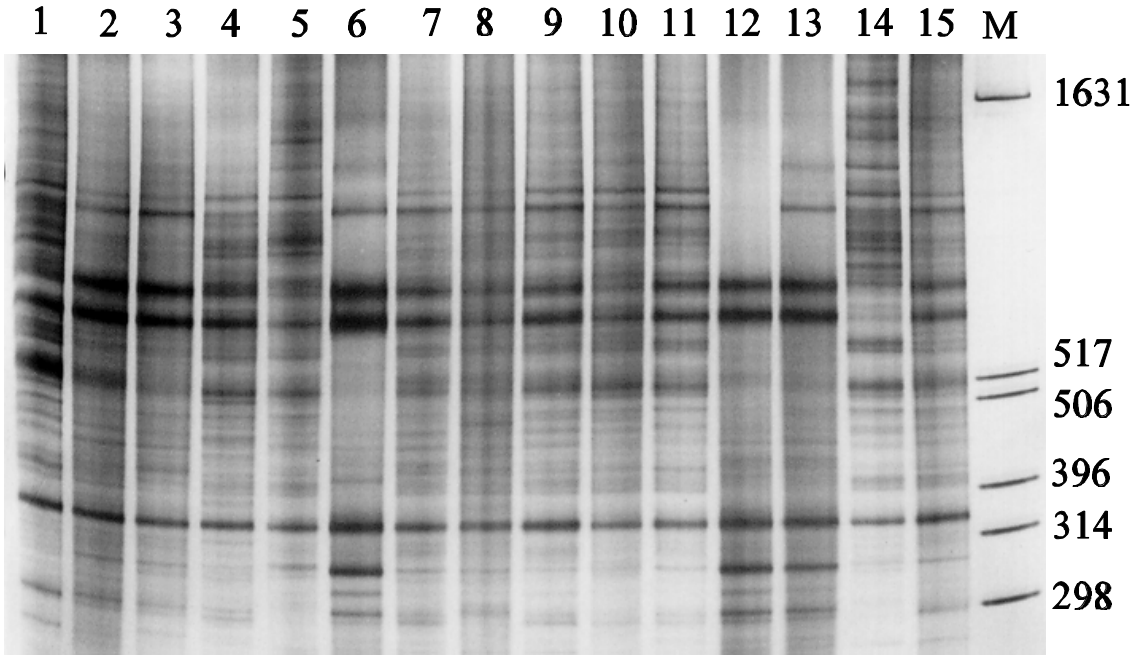


Fig. 3.3. A typical example of a polyacrylamide gel stained with silver nitrate. This specific example is Population 3 of *L.e. elimense* amplified with primer OPAI- 13. The scale on the right shows the molecular weight marker in base pairs. The numbers across the top of the gel are names ascribed to each sample within the population.

The fragment pattern data are summarised in Table 3.1. The data for each of the five primers were analysed separately to accommodate as large a sample size as possible. Here a total of 98 (96 polymorphic) scorable bands were used. In addition, four of the primers (Rhodes 3, 7, 12,

and OPAI-13) were pooled and analysed together, producing a total of 83 scorable bands (of which 82 were polymorphic). The sample size here was reduced to 57 individuals as not all samples amplified with all four primers. Nevertheless, the combined analysis did serve to average the different results from the different primers and approximated the mean calculated from the five primers fairly closely (see Table 3.2 below).

Table 3.1. Sequences of the 10 mer RAPD primers used in this study. The size range of fragments scored came from the central region of each gel lane where bands were reliable, reproducible fragments for each primer. The total number of possible bands per primer within the selected size range is given, followed, in parentheses, by the number of monomorphic bands. The average number of bands generated per plant over the fragment size range scored is shown along with the complete range of fragment numbers generated per primer per plant. The pooled primers included Rhodes 3,7,12 and OPAI-13.

Primer Name	Primer sequence 5' to 3'	Size range of bands (base pairs)	Number of bands scored	Average no. bands/plant (range)	Total no. bands present (as %)
Rhodes 3	CGG CCC CTG T	350-900	15 (0)	11.78 (3-15)	966 (78.54)
Rhodes 7	TCA CGA TGC A	400-830	13 (0)	8.77 (3-12)	605 (67.45)
Rhodes 12	ATT GCG TCC A	350-950	29 (1)	12.29 (2-26)	1106 (42.38%)
OPAI-05	GTC GTA GCG G	500-830	15 (1)	9.39 (6-13)	601 (62.60%)
OPAI-13	ACG CTG CGA C	350-950	26 (0)	12.86 (3-20)	1093 (49.46%)
Pooled primers		350-950	83 (1)	48.98 (28-62)	2792 (59.02%)

For the purposes of this study, a particular RAPD banding pattern or a genetically distinct individual was termed a genotype (See Sydes & Peakall 1998). When the RAPD banding pattern data from the different primers were analysed separately, duplication of genetically distinct individuals or genotypes occurred, particularly within populations. Primer Rhodes 7, for example, showed an exceptionally low genetic diversity of 0.07 for *L.e. vyeboomense* (13 samples; Table 3.2). Only four genotypes were distinguished within this population by primer Rhodes 7. Similarly, primer Rhodes 7 showed a mean genetic diversity of 0.22 for 13 samples of *L.e. elimense* Population 1 where nine genotypes were distinguished.

Primer Rhodes 3 showed a mean genetic diversity of 0.13 for *L.e. elimense* Population 2, which had 15 samples but only seven genotypes. In the pooled analysis there were 57 genotypes and no duplication of genotypes occurred. This is not unexpected because the chance of finding two individuals with the same banding pattern is the mean similarity raised to the power of the mean number of bands (Wolff & Peters-Van Rijn 1993). For the whole species, using the pooled primers this gives a probability of 6.8×10^{-10} of two individuals having the same genotype.

Fragment distribution

The total presence of bands ranged from 42% to over 78% (Table 3.1). This indicated that bands scored were generally common to about half to three-quarters of the samples and were not restricted to a few individuals. This meant that there were few rare bands or unique bands. The distribution pattern of the scored RAPD band frequency for the five primers is shown in Fig. 3.4a for all samples of all three subspecies. Of the total of 98 bands only two were invariant or monomorphic, occurring in every individual sampled. One third of the bands occurred in more than 70% of the samples, while only 5% occurred in less than 20% of the samples. There was a marked peak around 30-40% of the samples having a maximum of 17% of the bands present. No bands were exclusive to a single individual and no bands were found to be exclusive to a specific sex of plant in any of the subspecies.

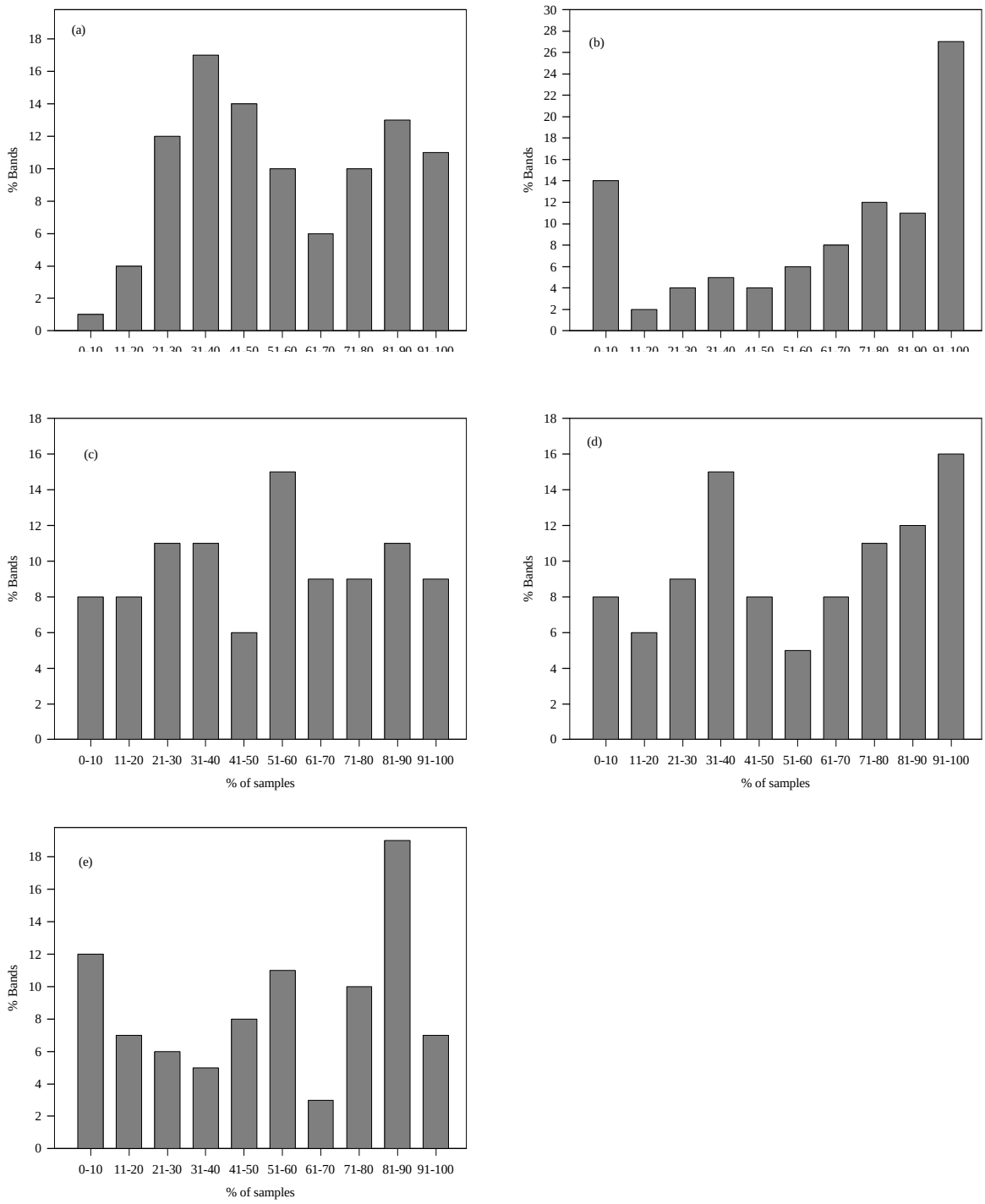


Fig. 3.4. Frequency distribution of individual RAPD bands for all five primers in *L. elimense*. The number of plants showing each band as a percentage of the number of plants sampled for that particular primer is plotted against the number of bands. Total number of bands is 98. The frequency distribution has been plotted for (a) All the *L. elimense* populations. (b) *L.e. vyeboomense* population (c) the two *L.e. salteri populations* (d) the three *L.e. elimense* populations (e) the *L. salignum* population.

The distribution pattern in Fig. 3.4a is bimodal. In Fig. 3.4b, c, and d the distribution of the band frequencies were plotted for the three subspecies separately. This produced generally similar patterns of distribution of bands in *L.e. salteri* (Fig. 3.4c) and *L.e. elimense* (Fig. 3.4d). *L.e. vyeboomense*, showed a peak at each end of the histogram (Fig. 3.4b). This was similar to the pattern obtained for *L. salignum* (Fig. 3.4e).

Genetic Variability

Estimates of mean genetic variability or, more precisely, measurements of genetic distance, calculated for individual primers, the pooled primers, for individual populations, for the three subspecies, and for the species as a whole, showed a range from 0.07 to 0.50 for the individual primers and a range of 0.24 to 0.35 for the mean of the five primers and also for the pooled primers (Table 3.2). Using the coefficient of Nei & Li (1979), produced genetic variability of 0.19 to 0.37. Both coefficients for the pooled primers showed that *L.e. vyeboomense* had the lowest genetic distance (mean 0.24), followed by *L.e. elimense* (mean 0.31) and *L.e. salteri* as a subspecies had the highest level of genetic distance at 0.35. From Table 3.2, it is seen that primer Rhodes 7 showed the greatest range of genetic distance (0.07 - 0.39) whereas primer OPAI-13 showed the smallest range (0.19 - 0.37).

An analysis of Table 3.3 allows a population versus population assessment of genetic distance. Here the single population of *L.e. vyeboomense*, shows the greatest genetic distance from the other populations, it separates from *L.e. salteri* Population 2 at a level of 0.44, followed by a distance of 0.41 versus *L.e. elimense* Population 2.

Table 3.4 allows for a comparison between subspecies and within subspecies. Again *L.e. vyeboomense* shows the greatest distance at 0.39 versus *L.e. salteri* and 0.40 versus *L.e. elimense*. A comparison of the within subspecies genetic distance, shows *L.e. vyeboomense* having by far the lowest level at 0.24. *L.e. elimense* versus *L.e. vyeboomense* showed a distance of 0.40 but only 0.36 versus *L.e. salteri*. This genetic distance pattern reflects the greater geographical separation between *L.e. elimense* and *L.e. vyeboomense* than between *L.e. elimense* and *L.e. salteri*.

Table 3.2. Mean genetic variability (*V*) for the six populations of *L. elimense* as calculated for each of five primers used and for the pooled primers (excluding OPAI-05). The genetic distance between each pair of individuals within the population, subspecies, and all samples was calculated and then averaged. Genetic variability was calculated as $V = 1 - S$. n is the number of individuals sampled, followed, in brackets, by the number of genotypes. The last column shows genetic variability calculated from $1 - F$.

Plant populations	*V* Rhodes 3	*V* Rhodes 7	*V* Rhodes 12	*V* OPAI-05	*V* OPAI-13	*V* Mean of all primers	*V* Pooled primers	1 - *F* Pooled primers
L. e. vyeboomense, only population	0.35 n=13 (12)	0.07 n=13 (4)	0.29 n=15 (15)	0.28 n=14 (12)	0.22 n=15 (13)	0.24	0.24 n=12 (12)	0.19 n=12 (12)
L. e. salteri Population 1	0.28 n=14 (13)	0.39 n=12 (12)	0.39 n=15 (15)	0.25 n=5 (5)	0.21 n=12 (12)	0.30	0.29 n=8 (8)	0.25 n=8 (8)
L. e. *salteri* Population 2	0.35 n=11(11)	0.35 n=9 (9)	0.27 n=15 (11)	0.50 n=3 (3)	0.26 n=13 (12)	0.35	0.34 n=3 (3)	0.37 n=3 (3)
L. e. elimense Population 1	0.19 n=15 (12)	0.22 n=13 (9)	0.33 n=15 (15)	0.29 n=13 (12)	0.28 n=15 (15)	0.26	0.27 n=13 (13)	0.23 n=13 (13)
L. e. elimense Population 2	0.13 n=15 (7)	0.20 n=11 (8)	0.36 n=15 (15)	0.29 n=15 (15)	0.37 n=15 (15)	0.27	0.29 n=11(11)	0.22 n=11(11)
L. e. elimense Population 3	0.23 n=14 (13)	0.31 n=11(11)	0.26 n=15 (15)	0.34 n=14 (14)	0.19 n=15 (15)	0.27	0.24 n=10 (10)	0.24 n=10 (10)
L. elimense whole species	0.29 n=82 (74)	0.34 n=69 (53)	0.43 n=90 (86)	0.35 n=64 (61)	0.32 n=85 (82)	0.35	0.35 n=57 (57)	0.30 n=57 (57)

Table 3.2 continued on next page ....

.... Table 3.2 continued

Plant populations	*V* Rhodes 3	*V* Rhodes 7	*V* Rhodes 12	*V* OPAI-05	*V* OPAI-13	*V* Mean of all primers	*V* Pooled primers	1 - *F* Pooled primers
L.e. salteri Whole subspecies	0.38 n=25 (24)	0.39 n=21 (21)	0.37 n=30 (26)	0.34 n=8 (8)	0.27 n=25 (24)	0.35	0.35 n=57 (57)	0.30 n=11 (11)
L. e. elimense whole subspecies	0.19 n=44 (39)	0.27 n=35 (28)	0.39 n=45 (45)	0.35 n=42 (41)	0.33 n=45 (45)	0.31	0.32 n=34 (34)	0.28 n=34 (34)
L. salignum	0.39 n=7 (7)	0.35 n=7 (7)	0.31 n=10 (10)	0.23 n=8 (7)	0.23 n=8 (8)	0.30		

Table 3.3. Average genetic distance between the populations using the dissimilarity coefficient of Apostol *et al.* (1993). The genetic distance was calculated for each of the pairs of individuals from the two populations and then averaged. This is done using the pooled data from primer Rhodes 3, 7 12 and OPAI-13. (n) refers to the number of individuals used in each comparison.

	L.e. vyeboomense	*L.e. salteri* Pop. 1	*L.e. salteri* Pop. 2	*L.e. elimense* Pop. 1	*L.e. elimense* Pop. 2
L.e. salteri Population 1	0.38 (n=20)				
L.e. salteri Population 2	0.44 (n=15)	0.35 (n=11)			
L.e. elimense Population 1	0.38 (n=25)	0.34 (n=21)	0.37 (n=16)		
L.e. elimense Population 2	0.41 (n=23)	0.36 (n=19)	0.41 (n=14)	0.35 (n=24)	
L.e. elimense Population 3	0.40 (n=22)	0.34 (n=18)	0.35 (n=13)	0.33 (n=23)	0.34 (n=21)

Table 3.4. Average genetic distance between the three subspecies using the dissimilarity coefficient of Apostol *et al.* (1993). The genetic distance was calculated for each of the pairs of individuals from the two populations and then averaged. This is done using the pooled data from primer Rhodes 3, 7 12 and OPAI-13. (n) refers to the number of individuals used in each comparison. The self comparisons are average within population measures of genetic distance.

	L.e. vyeboomense	*L.e. salteri*	*L.e. elimense*
L.e. vyeboomense	0.24 (n=12)		
L.e. salteri	0.39 (n=23)	0.33 (n=11)	
L.e. elimense	0.40 (n=46)	0.36 (n=45)	0.32 (n=34)

Genetic variability in the population of the widespread, common *L. salignum* ranged from 0.23 to 0.39 with an average of 0.30. This appears to be a wide range, 25 percent above and below the mean, but is comparable with the range observed in *L. elimense* (Table 3.2).

The number of individuals sampled does not appear to influence the level of variability. For primer OPAI-13, *L.e. elimense*, Population 2 showed a genetic variability of 0.37 from a sample of 15 individuals, yet the whole subspecies *L.e. elimense* (3 populations) showed a genetic variability of 0.33 for 45 individuals (Table 3.2). For primer OPAI-05, *L.e. salteri* Population 2 showed a mean genetic variability of 0.50 from three individuals, yet a mean genetic variability of all primers was 0.35 for the population as a whole (Table 3.2). This difference suggests that three samples is too few.

The results of the AMOVA analysis for both the individual and pooled primer data are given in Table 3.5. These data include the nested analysis showing the hierarchical structure, the population data showing the effect of removing the taxonomic subspecies division, and the subspecies data showing the removal of the population subdivisions.

When the results were analysed primer by primer, the within-population variation showed a range of 67.41 - 90.06% which went up slightly to 69.79 - 91.45% with the removal of the subspecies structure. The among-subspecies variability ranged from an extreme low of -6.66% to a high of 12.80% although these were not considered statistically significant for any of the individual primers. The removal of the among-population structure, showed the within-subspecies variability to range from 76.97-96.02%.

Similar patterns were generally reflected by all the primers as can been seen graphically, by the pie charts in Fig. 3.5. Here the high percentage variance within-populations is obvious. The variation between subspecies is not significant for any of the primers, except OPAI-05 from the nested analysis. There is some differences in the partitioning of variance between-subspecies and between-populations from one primer to the next (see Fig. 3.5). Primer Rhodes 3 shows a negative value of -6.66% for the among-subspecies variation suggesting that there is no between-subspecies variation visible with this primer.

Table 3.5. Analysis of molecular variance (AMOVA) for the six populations of *Leucadendron elimense*, using 98 RAPD markers. The data is presented for the individual primers and the pooled group of four primers. Statistics include degrees of freedom (*df*), sum of squared deviations (SSDs), mean squared deviations (MSD), variance component estimates (*Var*), the percentage of total variance (% Total), and the probability (*P*) of obtaining a more extreme component estimate by chance alone. 1000 permutations were used for each analysis.

Source of variation	*df*	SSD	MSD	*Var*	%Total	*P*-value
Pooled Primers						
(A) Nested analysis						
Among subspecies	2	131.74	65.87	1.49	9.46	0.009
Populations/subspecies	3	118.55	39.52	3.17	20.09	<0.001
Individuals/subspecies	51	566.69	11.11	11.11	70.45	<0.001
(B) Population analysis						
Among populations	5	250.29	50.06	4.20	27.44	<0.001
Within populations	51	566.69	11.11	11.11	72.56	<0.001
(C) Subspecies analysis						
Among subspecies	2	131.74	65.87	3.32	20.72	<0.001
Within subspecies	54	685.24	12.69	12.69	79.28	<0.001
Primer OPAI-13						
(A) Nested analysis						
Among subspecies	2	40.22	20.11	0.31	6.93	0.108
Populations/subspecies	3	36.57	12.19	0.61	13.63	<0.001
Individuals/populations	79	280.90	3.56	3.56	79.44	<0.001
(B) Population analysis						
Among populations	5	76.79	15.36	0.83	19.01	<0.001
within populations	79	280.90	3.56	3.56	80.99	<0.001
(C) Subspecies analysis						
Among subspecies	2	40.22	20.11	0.635	14.08	<0.001
Within subspecies	82	317.48	3.87	3.87	85.92	<0.001

Table 3.5 continued on next page

Table 3.5 continued...

Source of variation	*df*	SSD	MSD	*V*	%Total	*P*-value
Primer OPAI-05						
(A) Nested analysis						
Among subspecies	2	11.61	5.81	0.11	4.15	<0.001
Populations/subspecies	3	12.33	4.11	0.16	5.80	0.006
Individuals/subspecies	58	141.91	2.45	2.45	90.06	<0.001
(B) Population analysis						
Among populations	5	23.94	4.79	0.23	8.55	<0.001
Within populations	58	141.91	2.45	2.45	91.45	<0.001
(C) Subspecies analysis						
Among subspecies	2	11.61	5.81	0.20	7.41	<0.001
Within subspecies	61	154.25	2.53	2.53	92.59	<0.001
Primer Rhodes 3						
(A) Nested analysis						
Among subspecies	2	9.30	4.65	-0.16	-6.66	0.788
Populations/subspecies	3	26.43	8.81	0.49	20.40	<0.001
Individuals/populations	76	156.35	2.06	2.06	86.26	<0.001
(B) Population analysis						
Among populations	5	35.73	7.15	0.37	15.35	<0.001
within populations	76	156.35	2.06	2.06	84.65	<0.001
(C) Subspecies analysis						
Among subspecies	2	9.30	4.65	0.09	3.98	0.034
Within subspecies	79	182.78	2.31	2.31	96.02	0.034

Table 3.5 continued on next page

Table 3.5 continued ...

Source of variation	*df*	SSD	MSD	*V*	%Total	*P*-value
Primer Rhodes 7						
(A) Nested analysis						
Among subspecies	2	17.39	8.69	0.12	5.92	0.236
Populations/subspecies	3	17.44	5.81	0.38	18.23	<0.001
Individuals/subspecies	63	99.34	1.58	1.58	75.85	<0.001
(B) Population analysis						
Among populations	5	34.83	6.97	0.47	22.96	<0.001
Within populations	63	99.35	1.58	1.58	77.04	<0.001
(C) Subspecies analysis						
Among subspecies	2	17.39	8.69	0.33	15.58	<0.001
Within subspecies	66	116.79	1.77	1.77	84.42	<0.001
Primer Rhodes 12						
(A) Nested analysis						
Among subspecies	2	96.52	48.26	0.86	12.80	0.147
Populations/subspecies	3	73.63	24.54	1.33	19.79	<0.001
Individuals/populations	84	381.46	4.54	4.54	67.41	<0.001
(B) Population analysis						
Among populations	5	170.16	34.03	1.97	30.21	<0.001
within populations	84	381.47	4.54	4.54	69.79	<0.001
(C) Subspecies analysis						
Among subspecies	2	96.52	48.26	1.56	23.03	<0.001
Within subspecies	87	455.10	5.23	5.23	76.97	<0.001

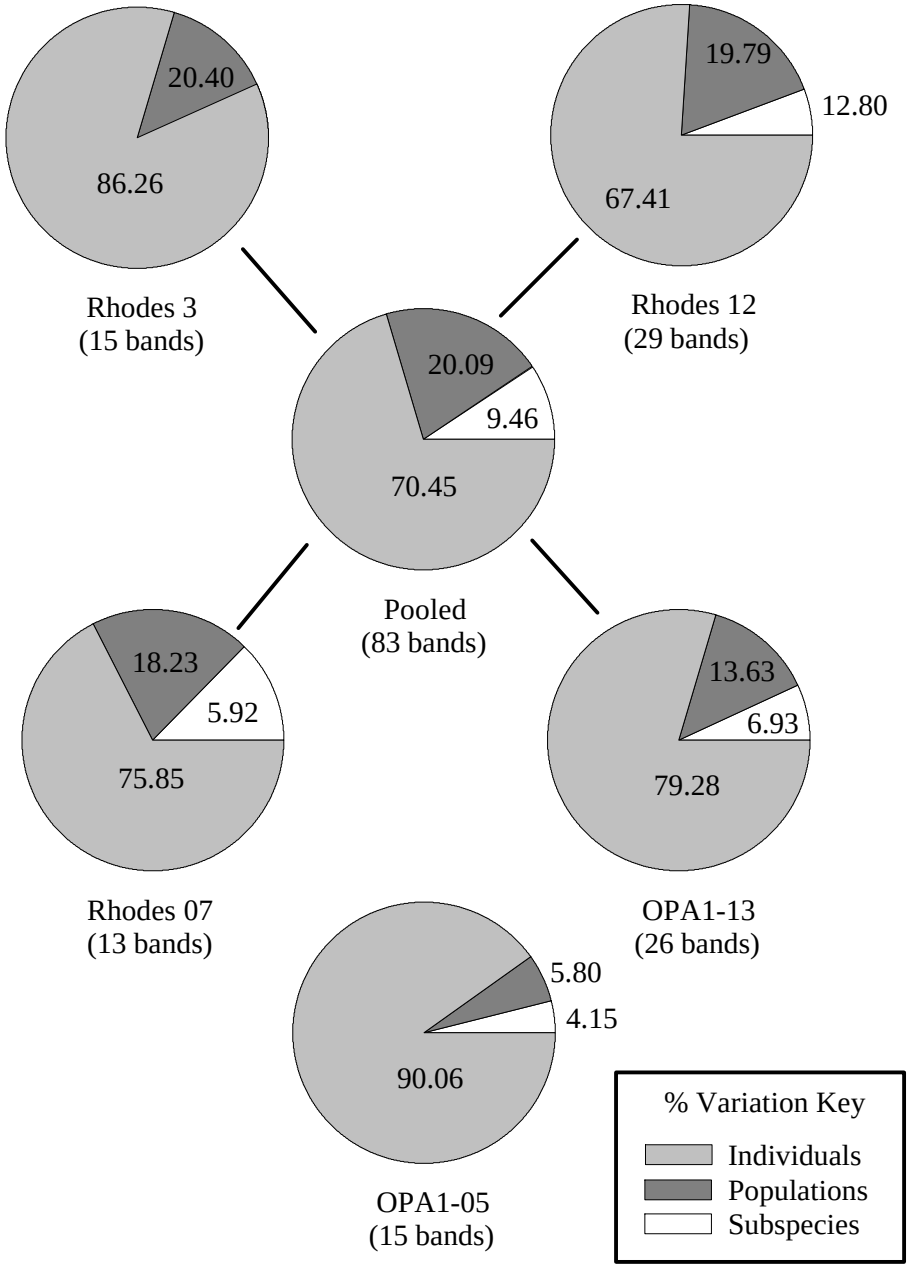


Fig. 3.5. Proportions of total variance attributable to subspecies differences, to population differences within a subspecies, and to variation among individuals within a population, from the nested AMOVA analyses of individual RAPD primers represented as pie charts. The numbers indicate the numbers of fragments scored for the primer in question. The central figure represents the results of pooling four of the primers. The variation between subspecies was only significant for OPAI-05.

Clustering of Populations

Although four different coefficients were used to generate the UPGMA trees in Fig. 3.6, the resultant trees show general overall conformity. For all four dendrograms in Fig. 3.6, the co-phenetic correlation coefficient r_{cs} was 0.7, making all dendrograms acceptable. This might, however, be a function of the data rather than the coefficient selected. The clearest pattern of clustering was in Fig. 3.6a, which was generated from the Euclidean distance equation of Excoffier *et al.* (1992) used to generate the AMOVA. Here *L.e. vyeboomense* branches away from the other populations at a level of 32.4 (39%) (I in Fig.3.6a), with within-population distance measurements ranging from 0.9 - 27.1 (1-33%). *L.e. salteri* and *L.e. elimense* are separated from each other at levels of between 25 -29.5 (30-36%). There are two individuals from *L.e. salteri* Population 2, which are separated from the rest of the samples at a level of 34 (41%) (J in Fig.3.6a). In Fig. 3.6b, using the coefficient of Nei & Li (1979), *L.e. vyeboomense* is most clearly isolated from the other samples at a level of 0.32 and within-population distances range from 0.07 to 0.29. The *L.e. salteri* and *L.e. elimense* populations separate at levels of between 0.25 and 0.30. There are outlying samples from *L.e. salteri* Population 2, which separate at a level of 0.45 and 0.36 from all the other samples, as well as one from *L.e. salteri* Population 1, separating at 0.38. This sample is well contained in the main population (Fig. 3.6a).

In Fig. 3.6c, using the coefficient of Apostol *et al.* (1993), *L.e. vyeboomense* separates at a level of 0.39, and the *L.e. salteri* and *L.e. elimense* populations separate at levels of 0.30 to 0.36, these levels being equivalent to those seen in Fig. 3.6a. Although four different coefficients were used to generate the UPGMA trees in Fig. 3.6, they show general overall conformity. In Fig. 3.6d, using the coefficient of Jacquard (in Armstrong *et al.* 1994), *L.e. vyeboomense* was separated at a dissimilarity level of 0.41. This is higher than the level observed for the other three coefficients. This dendrogram produced many outlyers, no doubt a function of the parameters included in the coefficient. In addition, the outlying samples are separating at extremely high levels, *L.e. salteri*, Population 2, sample 09 separates at 0.62, the highest level of separation in any of the four dendrograms. The consensus tree (Fig. 3.6e), compiled from the other four trees allowed for a measure of agreement of the four trees.

Fig. 3.6. UPGMA analysis of *L. elimense* plant relationships from the pooled primer data, using the Euclidean distances generated from (a) the dissimilarity coefficient of Excoffier *et al*. (1992), (b) the dissimilarity coefficient of Nei & Li (1979), (c) the dissimilarity coefficient of Apostol *et al*. (1993), and (d) the dissimilarity coefficient of Jacquard (1901 cited in Armstrong *et al*. 1994). v = *L.e. vyeboomense* (only population); s1 = *L.e. salteri* (Population 1); s2 = *L.e. salteri* (Population 2); e1 = *L.e. elimense* (Population 1); e2 = *L.e. elimense* (Population 2), e3 = *L.e. elimense* (Population 3). For (a), the scale is 0 to n number of bands. Here n is 83 for the pooled primer data. For (b), (c), (d), the scale is 0 to 1. A strict consensus tree of the four coefficients shown in (a) to (d) is presented in (e).

Fig. 3.6 (a)

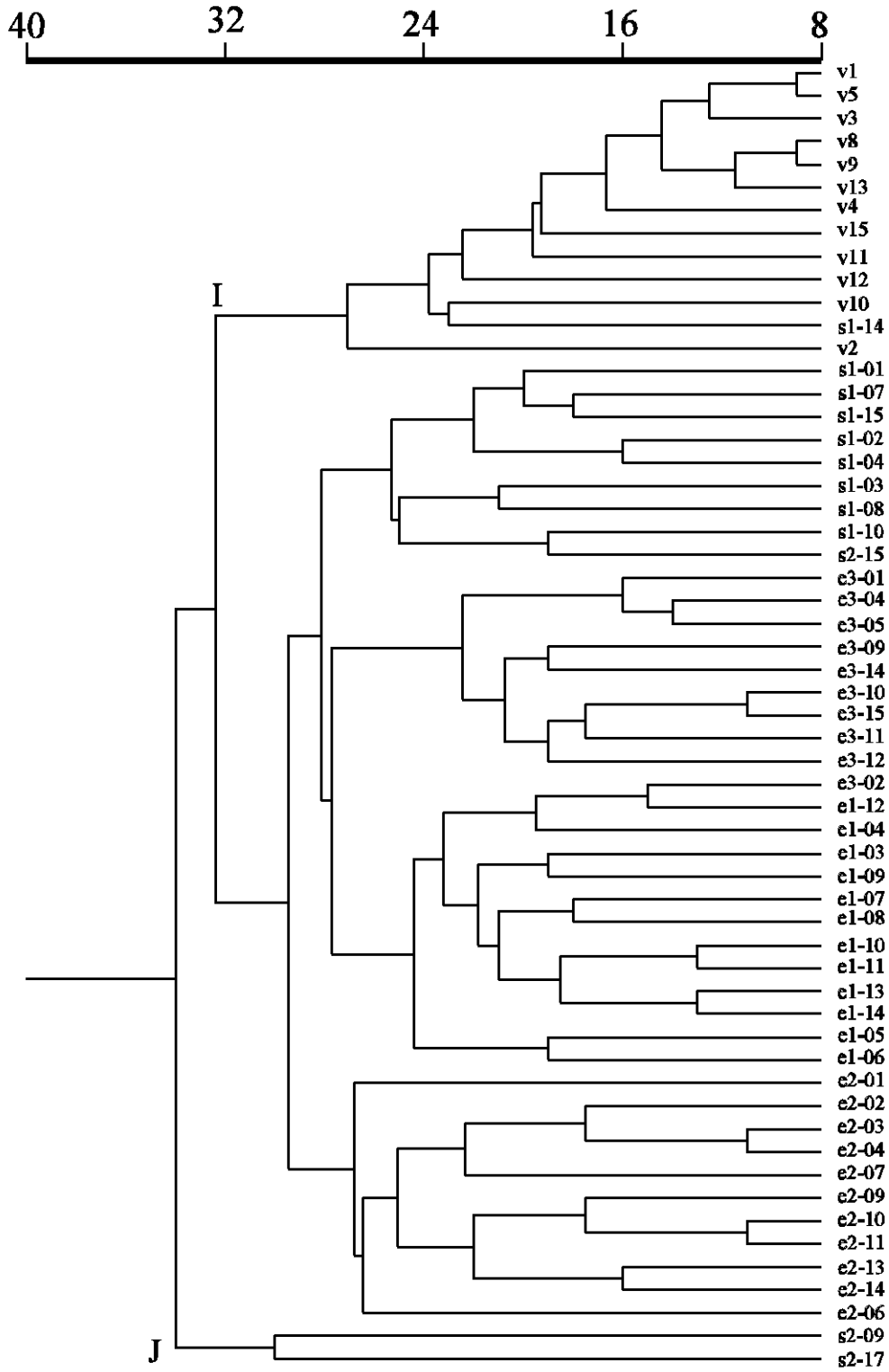
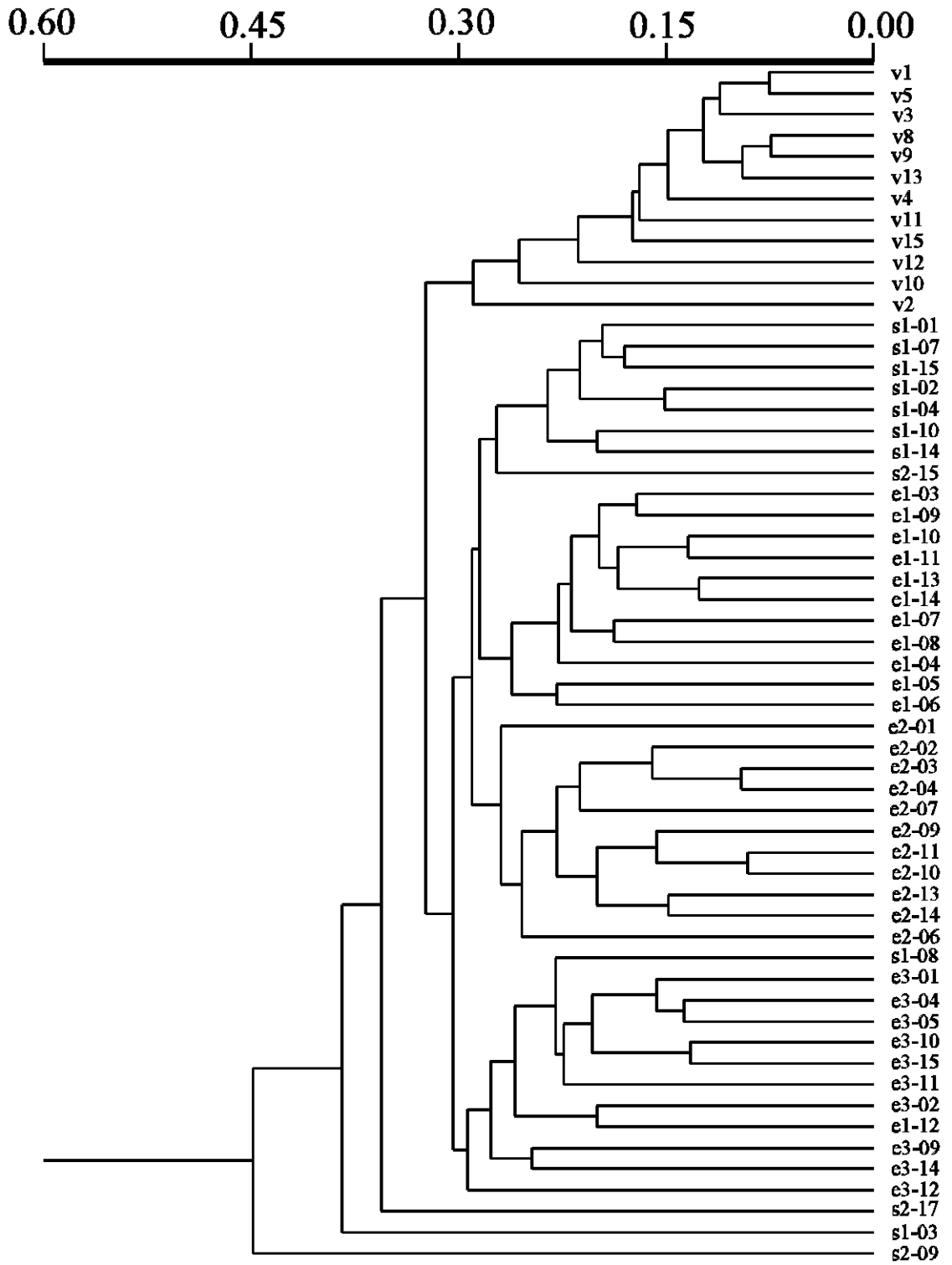

40
32
24
16
8
I
J
v1
v5
v3
v8
v9
v13
v4
v15
v11
v12
v10
s1-14
v2
s1-01
s1-07
s1-15
s1-02
s1-04
s1-03
s1-08
s1-10
s2-15
e3-01
e3-04
e3-05
e3-09
e3-14
e3-10
e3-15
e3-11
e3-12
e3-02
e1-12
e1-04
e1-03
e1-09
e1-07
e1-08
e1-10
e1-11
e1-13
e1-14
e1-05
e1-06
e2-01
e2-02
e2-03
e2-04
e2-07
e2-09
e2-10
e2-11
e2-13
e2-14
e2-06
s2-09
s2-17

Fig. 3.6 (b)


0.60
0.45
0.30
0.15
0.00
v1
v5
v3
v8
v9
v13
v4
v11
v15
v12
v10
v2
s1-01
s1-07
s1-15
s1-02
s1-04
s1-10
s1-14
s2-15
e1-03
e1-09
e1-10
e1-11
e1-13
e1-14
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e2-02
e2-03
e2-04
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e2-09
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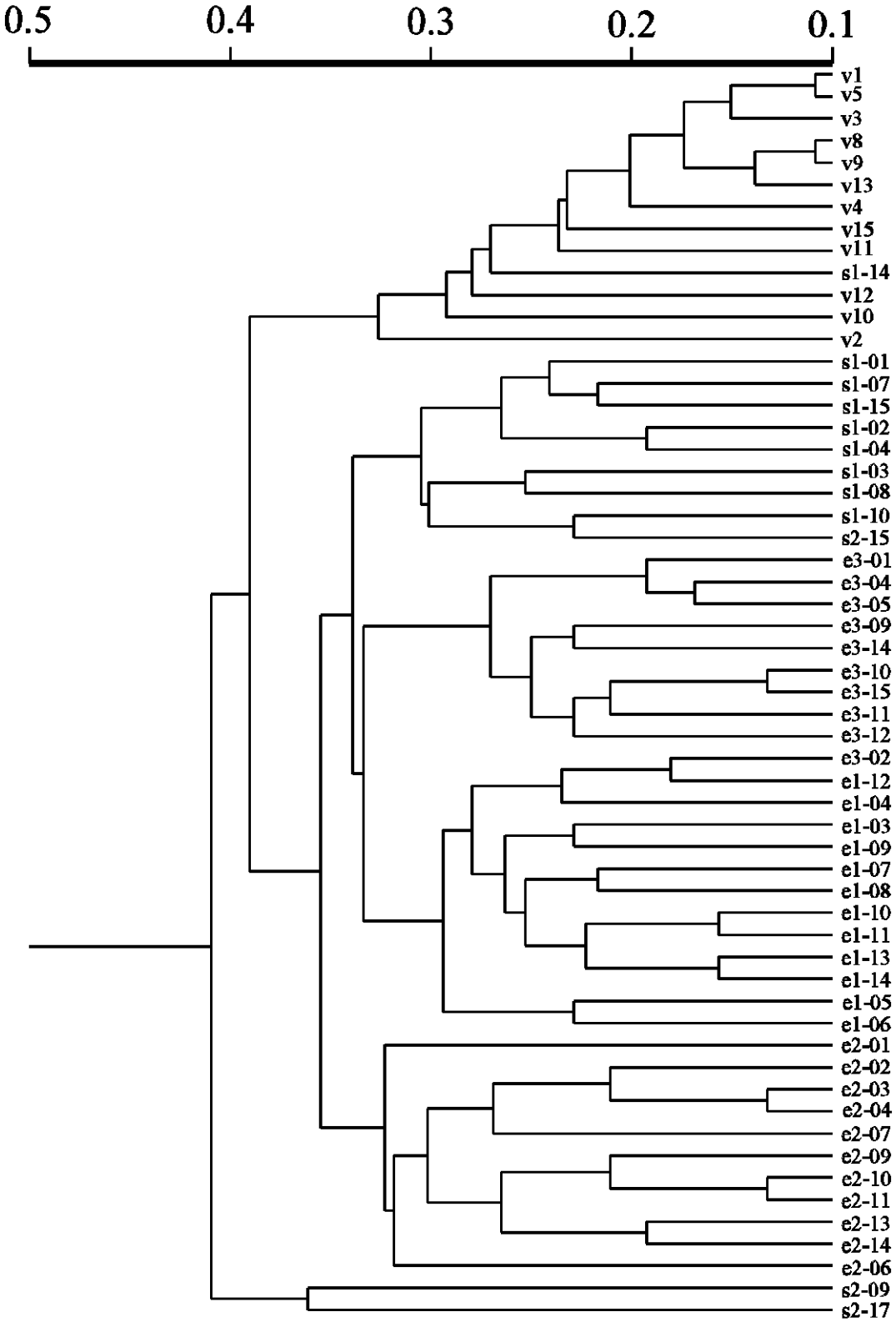
e2-10
e2-13
e2-14
e2-06
s1-08
e3-01
e3-04
e3-05
e3-10
e3-15
e3-11
e3-02
e1-12
e3-09
e3-14
e3-12
s2-17
s1-03
s2-09

Fig. 3.6 (c)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0.4
0.3
0.2
0.1
v1
v5
v3
v8
v9
v13
v4
v15
v11
s1-14
v12
v10
v2
s1-01
s1-07
s1-15
s1-02
s1-04
s1-03
s1-08
s1-10
s2-15
e3-01
e3-04
e3-05
e3-09
e3-14
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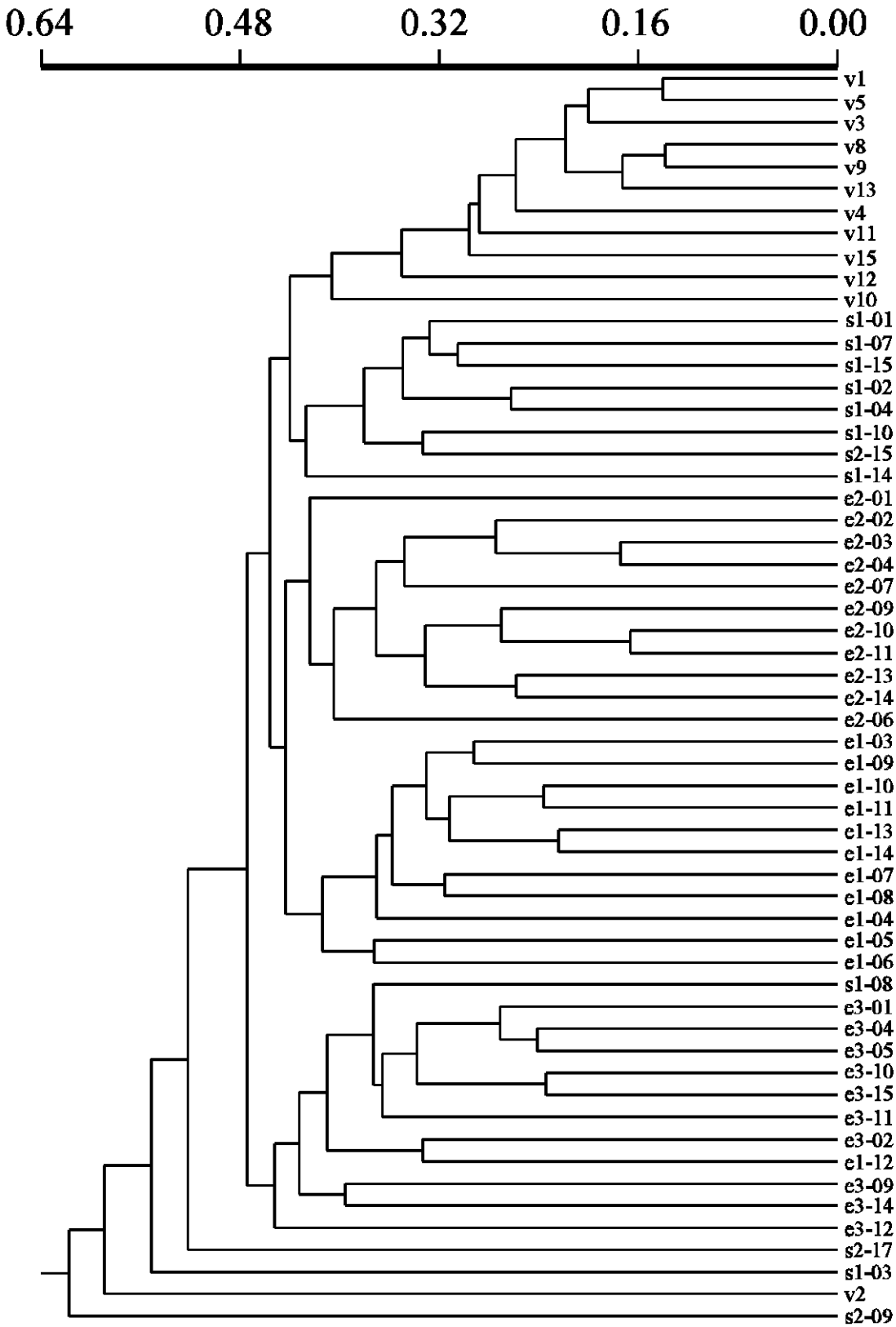
e1-06
e2-01
e2-02
e2-03
e2-04
e2-07
e2-09
e2-10
e2-11
e2-13
e2-14
e2-06
s2-09
s2-17

Fig. 3.6 (d)


0.64
0.48
0.32
0.16
0.00
v1
v5
v3
v8
v9
v13
v4
v11
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v10
s1-01
s1-07
s1-15
s1-02
s1-04
s1-10
s2-15
s1-14
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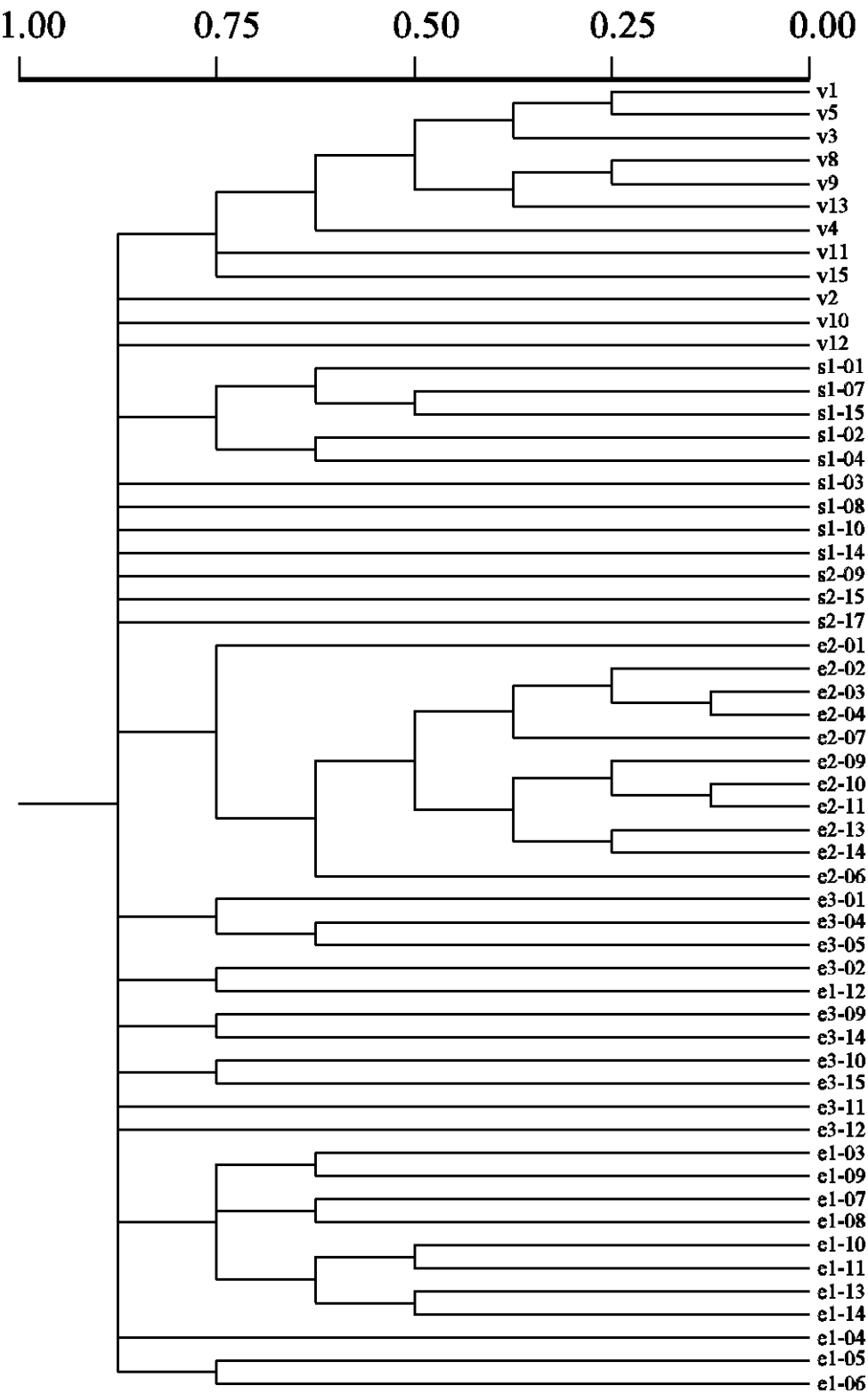
s1-08
e3-01
e3-04
e3-05
e3-10
e3-15
e3-11
e3-02
e1-12
e3-09
e3-14
e3-12
s2-17
s1-03
v2
s2-09

Fig. 3.6 (e)


1.00
0.75
0.50
0.25
0.00
v1
v5
v3
v8
v9
v13
v4
v11
v15
v2
v10
v12
s1-01
s1-07
s1-15
s1-02
s1-04
s1-03
s1-08
s1-10
s1-14
s2-09
s2-15
s2-17
e2-01
e2-02
e2-03
e2-04
e2-07
e2-09
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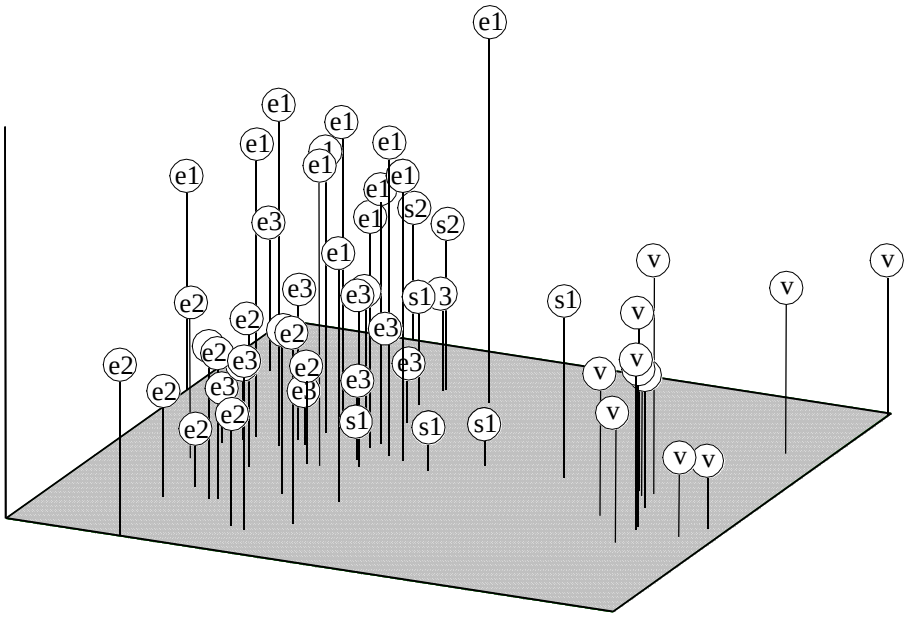
e1-14
e1-04
e1-05
e1-06

(a)



(b)

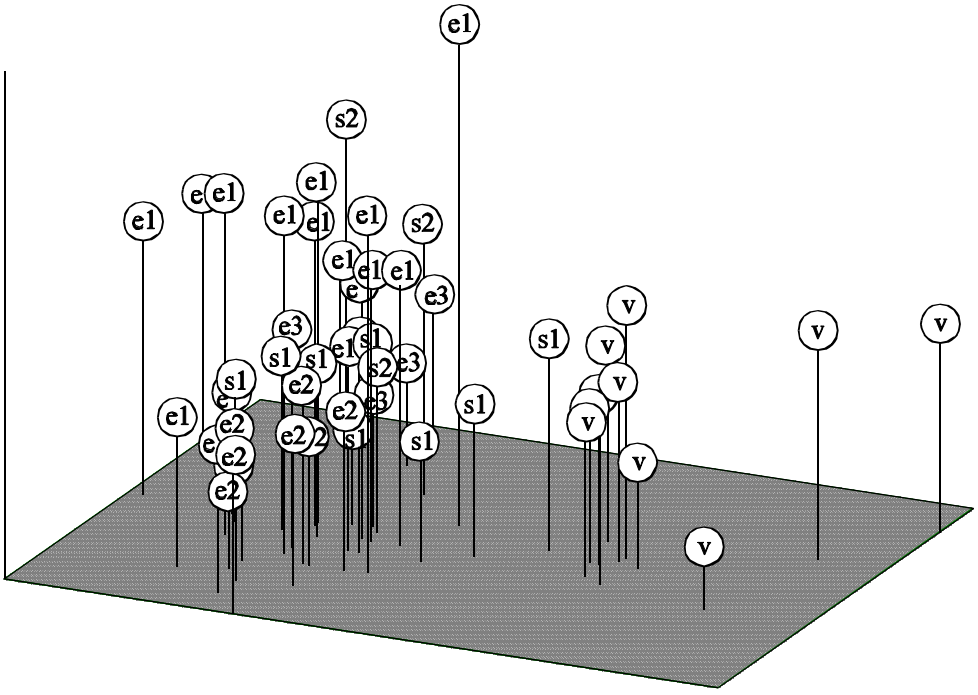


Fig. 3.7. Principle Co-ordinate Analysis of dissimilarity tables presented as a three-dimensional model based on the Euclidean distances using the dissimilarity coefficient of Excoffier *et al.*(1992) (a), and the dissimilarity coefficient of Nei & Li (1979) (b). v = *L.e. vyeboomense*, s1 = *L.e. salteri*, Population 1, s2 = *L.e. salteri*, Population 2, e1 = *L.e. elimense*, Population 1, e2 = *L.e. elimense*, Population 2, e3 = *L.e. elimense*, Population 3.

The tree showed bifurcation as well as multiple branching with 33 subsets . This summarises the clustering analysis and indicated that the *L.e. vyeboomense* population was largely well separated from the other two subspecies, which is reflected in its geographical separation from them. In addition, Population 2 of *L.e. elimense* forms the most distinctive subset. The three populations of *L.e. elimense* were geographically close without any physical barriers and this was also reflected in the cluster analysis. The clear separation of *L.e. vyeboomense* was also evident in the Principle Co-ordinate Analysis (Fig. 3.7).

Discussion

Primer selection

Because primers are selected randomly, their choice is theoretically endless. Strictly speaking, however, the choice of primers is not random as all have a cytosine-guanine content of at least 50% with no internal palindrome (Williams *et al.* 1990). The present study indicates that screening of suitable primers is important because primers may emphasise genetic variability at different taxonomic levels, as can be seen from the AMOVA in Table 3.4 and graphically in Fig. 3.5. Primer Rhodes 3, for example, did not distinguish among-subspecies variation at all. Screening of a range of primers will allow for selection of primers that consistently produce polymorphic bands. Some primers, in particular Rhodes 3 and 7, did not distinguish individuals within populations. This low variation is not seen in the other primers for the same populations. It might therefore be argued that this is a function of the particular primer and is not necessarily a true reflection of the number of genotypes within the populations. On the other hand, it could also be argued that this restricted number of genotypes is not reflected in all populations by primer Rhodes 7 or even Rhodes 3. Further support for this is that these primers did not show low variation in the other species studied. It must be remembered that by the very nature of being random, individual RAPD primers detect high or low levels of genetic diversity in a random and unexplained pattern.

When all four primers were pooled, there was no evidence of duplicate banding patterns and all 57 samples were unique. Although some primers show greater differences than others, all

show the same general pattern as is shown for the AMOVA results in Fig. 3.5 and the genetic variation in Table 3.2.

Silver staining on polyacrylamide gels enhances the detection of within population differences as a large percentage of the genome can be scanned to produce many scorable bands (Morell *et al.* 1995). This makes it particularly useful in studies of rare species if a low genetic variability or clonality is anticipated. This study used five primers and produced 96 polymorphic scorable bands. In comparative studies of Nesbitt *et al.* (1995) 10 primers resulted in 149 polymorphic bands in *Eucalyptus globulus* (Myrtaceae) and Huff *et al.* (1993), used seven primers to score 98 polymorphic bands in the buffalograss, *Buchloë dactyloides*. Both these studies used polyacrylamide gels. Less bands per primer were scorable when agarose gels were used in studies such as those of Ayres & Ryan (1997) on *Wyethia reticulata* (Asteraceae), Stewart & Porter (1995) on *Iliamna* (Malvaceae), Dawson *et al* (1995) on *Gliricidia sepium* (Leguminosae) and Fabbri *et al.*(1995) on olive (*Olea europaea*) cultivars where between 21 - 47 bands from 2 - 17 primers were scored.

Effect of using different coefficients of variability

When the coefficient of Nei & Li (1979) (1-*F*) was applied to the pooled primer data, it became apparent that it produced a slightly lower estimate of variability than the coefficient of Apostol *et al*. (1993) although the range was similar (see Table 3.2). The difference between the coefficients is that only *V* takes into account the shared absence of fragments scored whereas (1- *F)* emphasises shared presence of fragments scored. This means that if there is more shared presence than shared absence of fragments, the genetic variability calculated from the coefficient of Nei & Li (1979) will be larger than if the coefficient of Apostol. *et al.* (1993) was used and *visa versa*. If the number of shared presence of fragments equals that of the shared absence, then the two equations will produce identical results. Thus, as the genetic variability for *L. elimense* using (1-*F*) was less than for *V*, there was a greater shared presence of fragments than shared absence. This is confirmed in Fig. 3.4a where most bands show a high percentage presence. By computing these two coefficients, it can be determined immediately if there are many rare fragments, a potentially important consideration for rare plant species. The distribution of bands within *L.e. vyeboomense* (from Fig. 3.4b) indicated that rare bands

were common in *L.e. vyeboomense*. In addition, by the high presence of bands which were common to most of the samples, rare absence of some bands is also a feature of *L.e. vyeboomense*. This would suggest unique bands present in the population of *L.e. vyeboomense* which are not distributed likewise in the other two subspecies (Figs. 3.2c and 3.2d). The presence of rare bands can indicate the presence of a rare gene (Graham *et al*. 1997). This would suggest that a particular site was important for genetic conservation.

Fragment Polymorphism

The total presence of bands in *L. elimense* was high, so that at least half the bands were present in most individuals with 42 to over 78% total presence, depending on the primer selected (Table 3.1). In comparison, total number of bands present in all samples of *Lactoris fernandeziana* (Lactoriaceae) was approximately 75% (Brauner *et al.* 1992). In the present study, although only a few of the scored bands were unique to only a limited number of individuals, high levels of polymorphism were observed, with 96 out of 98 bands scored being polymorphic. As the two monomorphic bands were present in all individuals sampled in all three subspecies of *L. elimense,* these may be considered as marker bands. In fact these two bands were present in all samples of Proteaceae investigated in this study. Their presence in other families must, however, be investigated before it can be determined if these are only always present in Proteaceae.

L. elimense is an obligative outcrossing species with separate male and female plants. This might favourably influence the level of polymorphic bands observed. No bands were exclusive to either male or female plants. In real terms this was a small sample size and sex linked bands might well be observed in a much larger sample. In addition the pooling of all the male samples on the one hand and the female samples on the other, would improve the chance of identifying sex linked bands. A large number of primers should be screened, specifically looking for sex specific bands. This has been done successfully in *Silene latifolia* (Caryophyllaceae) by Mulcahy *et al.* (1992). It is increasingly used in bird studies, particularly in species which do not exhibit sexual dimorphism (Griffiths *et al.* 1998, Lessells & Mateman

1998). In plants, such as *L. elimense*, where there is gross morphological sexual dimorphism, sex linked RAPD bands are of secondary interest.

In *Haragodendron lucasii* (Haloragaceae), Sydes & Peakall (1998) observed extensive clonality resulting in low levels of polymorphic banding across eight primers. In one population of 700 plants, only three genets were delineated. If this plant population was assessed by field observations, the true genetic variability and extent of clonality would not be visible, hence the true conservation status would be masked. In such plants exhibiting clonality or extensive vegetative reproduction, RAPD techniques are an essential element of study and consideration for long term viability, protection and possible translocation. The rare *Iliamna* species studied by Stewart & Porter (1995) also showed clonality and they found the RAPD technique a useful tool in identifying the number of genotypes and the selection of material for conservation. In *Yushania* (Bambusoideae, Poaceae), RAPD techniques identified 31 clones in 53 samples (Hsiao & Riesenberg 1994). The breeding system in *L. elimense* precludes the possibility of clonality here. No similar study on clonality in candidate taxa has, however, been attempted in the Cape Flora.

Unique individuals

In all four dendrograms from Fig 3.4, there are individuals which are shown to be different and distant from other members of the population, as has been pointed out in the results. Careful observation of the original leaf tissue and photographs of the gel electrophoresis does not reveal any further information. There is good amplification and no indication of infection on the leaves. As far as these results can be interpreted, it would appear that these are real differences. These individuals potentially could hold unique genetic information, represent different age cohorts and could contribute significantly to the overall level of genetic variability and hence fitness and long term success of the population.

Estimates of genetic Variability

Similarity among banding patterns within populations can be directly correlated with genetic diversity (Gray 1995). The mean level of genetic variability (*V*) (Apostol *et al.* 1993) for the whole species *L. elimense* from pooled primer data was 0.35 (Table 3.2). The level of

variability of 0.32 was estimated in a similar study on an endangered Australian member of the Proteaceae, *G. scapigera* (Rossetto *et al.*, 1995), and is considered high for a rare plant species. This suggests that there were high levels of genetic variability within all six populations sampled from the three subspecies of *L. elimense*. The study on *G. scapigera* used the same method of data analysis although the primers were different. This limits the validity of a direct comparison with this study although the selection of high variability primers producing many polymorphic bands increases confidence levels. It is more meaningful to compare the results from within this study to those obtained for *L. salignum*, a ubiquitous species which has the widest distribution of all *Leucadendron* taxa (Williams 1972). Mean genetic variability of *L. salignum* was 0.30 (Table 3.2), which is within the range obtained for *L. elimense* but lower than the mean genetic variability for *L. elimense* as a whole. It was, however, higher than the figure obtained for *L.e. vyeboomense* as estimated from four of the five primers. There were different levels of genetic variability with the different primers, and where high levels were observed in *L. salignum* (0.39), in primer Rhodes 3, the genetic variability was only 0.29 in *L. elimense* (whole species), as shown in Table 3.2.

Differences in estimates of genetic variability between primers or pooled primer data might be a function of the particular primers selected (see below), although broad patterns of genetic variability were generally similar with most of the primers (as in Table 3.2). This was also found to be the case in a study on tilapia fish (Bardakci & Skibinski 1994). When estimated from the pooled data, the genetic variability of *L.e. elimense*, the relatively common subspecies, was 33% more than that of the endangered *L.e. vyeboomense*. This trend was mirrored in all but one of the individual primers, primer Rhodes 3 (calculated from Table 3.2). 33% represents a marked difference and was visible as a separation of the two subspecies in the dendrograms (Fig. 3.6) and the Principle Co-ordinate Analysis (Fig. 3.7). It was not, however, possible to determine if this low genetic variability has always been the case, or if there has been a decrease in the level of variability within the endangered *L.e. vyeboomense*. *L.e. vyeboomense* is restricted to a single population of which few plants remain and it is highly endangered. It is possible that one or more of these factors has influenced its genetic diversity. While the level of genetic variability within *L.e. vyeboomense* was low relative to other populations of *L. elimense* in this study, at 0.24 it probably is not particularly low for a rare

taxon. The genetic variability for *L.e. salteri* was approximately the same as within *L.e. elimense* or higher. This was particularly true for Population 2 of *L.e. salteri*.

Careful analysis of Table 3.2 shows that the levels of genetic variability for the three populations of *L.e. elimense* were not consistent. If the pooled primer data were assessed, the range of genetic variability within the three populations was from 0.24 - 0.29. The highest measures of genetic distance within the Population 2 of *L.e. elimense* indicate that this population contained many genetically dissimilar individuals. Therefore the genetic variation within this population could be considered high, making this population a good candidate for conservation should it become necessary to select one population over another.

Possible Effect of Frequent Fires on Genetic Variability

The Cape Flora is a fire-prone vegetation (van Wilgen *et al.* 1992). *L. elimense* is killed by fire and is perpetuated by obligative re-seeding. Such re-seeding species are sensitive to fire frequency (Bradstock *et al.*1994, Bradstock *et al.* 1996). Should the fire interval be too short, the plants might be killed before reaching sexual maturity resulting in an inadequate seed store for the next generation, low numbers, and lack of genetic variation. The ideal fire interval in the Cape flora in terms of vegetation management has long been a contentious issue. The literature cites intervals of anything between four and 40 years, with about 15 years being generally suggested (see Bond 1980, Bond 1984, van Wilgen 1981, van Wilgen 1987, Manders & Cunliffe 1987, Kruger 1984). The estimated age to first flowering in the three subspecies of *L. elimense* is about three years, the age to sexual maturity when flowering abundantly is about six years, and the age to senescence when flowers are sparse and seed set poor, is almost twenty years. When the populations in this study were sampled in 1994, the *L.e. vyeboomense* population was about three years old, as was *L.e. salteri* Population 1. All populations of *L.e. elimense* were about 5 years old. Only Population 2 of *L.e. salteri* was over 11 years old. Sampling in 1997 showed evidence of fire at all sites except *L.e. salteri* Population 2. Plant numbers had declined from 200 to 50 in *L.e. vyeboomense* and from 5000 to 500 in *L.e. salteri* Population 1. Numbers were stable at 100 for *L.e. salteri* Population 2. *L.e. elimense* Population 3 had declined from 200 plants to 50 adults plants and 50 new seedlings. *L.e. elimense* Populations 1 still contained 50 plants but was severely choked by alien *Acacia*. *L.e.*

elimense Population 2 could not be found except for a few young seedlings. This lack of recruitment suggests a lack of seed reserve within the soil and the sexual maturity at six to eight years suggests a minimum fire interval of about ten years. These results tend to support Krauss (1997) who worked on another member of the family Proteaceae, *Persoonia mollis*, and suggested that high frequency of fire played a major role in affecting levels and distribution of genetic variation through local extinction and subsequent recolonisation.

Possible Effects of Population size on Genetic Variability

Theory predicts that small populations will be expected to be genetically depauperate as a result of the founder effect, genetic drift and inbreeding (Baskauf *et al.* 1994). Genetic drift results in a change in allele frequency from one generation to the next and will be expressed in small populations as a decrease in fitness and genetic variation (Ellstrand & Elam 1993). This was monitored in the Prairie Chicken, *Tympanuchus cupido-pinnatus*, where several alleles from a large population were found to be absent in a small population (Westermeier *et al.* 1998). In a study of restricted and widespead species of *Astragalus* (Fabaceae), Karron *et al.* (1988) found lower levels of genetic polymorphisms in the restricted species relative to the widespread species. They did not, however, consider the levels in the restricted species to be low relative to other rare plants. The two restricted species numbered approximately 1 500 and 3 000 individuals each, which was considerably less than the numbers for the widespread species. In comparison, in 1994, when the leaf material was collected for the three subspecies of *L. elimense*, *L.e. vyeboomense* numbered 200 individuals whereas *L.e. salteri* numbered about 5000, and *L.e. elimense* numbered about 10 000 individuals (see Chapter 2).

Small population size presents a large edge relative to the total area of the population, exposing relatively more individuals to potential environmental stresses than in larger populations. One might expect this to lower the level of genetic variability (Godt *et al.* 1995). Certainly some studies have shown that genetic variation decreases with a decrease in population size. In *Echinacea* (Asteraceae), allozyme studies showed that rarer species were often genetically depauperate (Baskauf *et al.* 1994). Frankham (1997) used allozyme studies and showed that in general, for island populations, genetic variability was lower than for related mainland species and that rare plant species are essentially island species. Low levels

of genetic variation are recorded for rare western pond turtles (Gray 1995) and in the rare *Limonium dufourii* in the family Plumbaginaceae (Palacios & González-Candelas 1997). Using RAPD analysis, low levels of variation were found a wide range of other organisms, such as the fragmented, disjunct Newfoundland populations of red pines (*Pinus resinosa*) (Mosseler *et al.* 1992), in the small, scattered populations of *Phoca vitulina* (harbour seals) (Kappe *et al.* 1995) and in wheat aphids (*Diuraphis noxia*) (Puterka *et al.* 1993). In contrast, evidence from *Helonias bullata* (Liliaceae) shows that the ends of disjunct ranges exhibited particularly high levels of genetic variation (Godt *et al.* 1995) and Widén & Andersson (1993) observed higher levels of genetic variation in small populations of *Senecio integrifolius* (Asteraceae) than in larger fragmented populations. It must be emphasised, however, that small population size is in itself a hazard because small populations are easily wiped out by events that might have a relatively small impact on larger populations. An example of this is the rare Proteaceae, *Sorocephalus tenuifolius*, which was thought to be extinct in 1985 after the site of the only known population was ploughed to plant an apple orchard (pers. obs.). Fortunately more recently, other small remnant populations have come to light (Hilton-Taylor pers. com.).

Many rare Cape Proteaceae are found naturally in small populations (Tansley 1988), suggesting potentially low levels of genetic variation. No apparent correlation or pattern was, however, seen between levels of genetic variation and observed population size in *L. elimense* at the time of sampling (see Table 3.2), nor was there any correlation between the genetic variability and the number of individuals sampled in a population. Indeed, in *L. elimense*, high levels of variation were observed in small populations with all primers, particularly in Population 2 of *L.e. salteri* (Table 3.2). Although fluctuations in population size occur in *L. elimense*, at no time can they be regarded as large, with numbers fluctuating from low to no extant plants. The genetic reserves held in seed banks cannot be discounted and possibly should be considered as a component of population size. The present study nonetheless, does serve to show that genetic variability, at least in Proteaceae, is unrelated to extant population size and provides further justification for the use of small nature reserves to conserve rare Proteaceae, as suggested by Tansley (1988).

Analysis of Molecular Variance

Although not originally designed for use with RAPD data, AMOVA has proved useful in other similar studies (Huff *et al.* 1993, Rossetto *et al.* 1995, Peakall *et al*. 1995, Martín *et al.* 1997, Palacios & González-Candelas 1997, Cardoso *et al.*1998). AMOVA can be used to produce a hierarchical analysis of molecular variance directly from the matrix of squared-distances between all pairs of genotypes (Excoffier *et al.* 1992). This is particularly useful in *L. elimense* where a hierarchical system already exists with individuals within populations, populations within subspecies and three subspecies within the species. The ability to test statistical significance of such hierarchical data is not provided by any other types of analyses (Morell *et al.* 1995).

In families which tend to be predominantly outcrossing species, such as the Proteaceae, genetic analysis suggests that differentiation between populations is expected to be less strong than in taxa which tend to be self-fertilizing (Linhart & Grant 1996). It is also follows that obligative outcrossing species will produce most of the genetic variation within the population level (Cardoso *et al.* 1998, Apostol *et al.* 1996). This is indeed the case for *L. elimense*, where the AMOVA analysis showed that most of the genetic variation was seen to be held within each population (Table 3.5). This result was reflected by all the primers making the results unequivocal, despite the small sample size within some of the populations for some of the primers. For all primers, the within-population variation accounted for about 75% of the total variation seen (range 69 to >90% from Table 3.5). In contrast, the variation among subspecies was very low (average of 5% across the five primers). The removal of the subspecies structure resulted in a among-population variation averaging 18% and a within-population variation of 82% for the five primers. The removal of the population structure resulted in an among-subspecies variation of 13% and a within-subspecies variation of 87%.

In *G. scapigera*, the AMOVA produced results essentially identical to those produced for *L. elimense* using primer Rhodes 3, with a between-'location' group variation of -5.83% (*L. elimense* -6.66%), among-populations/groups variation of 18.19% (*L. elimense* 20.40%) and a within-population variation of 87.64% (*L. elimense* 86.26%) for the nested analysis (Rossetto *et al.* 1995). This similarity might just be coincidental or it might signify that the

pattern exhibited by both *L. elimense* and *G. scapigera* are typical of the patterns of diversity exhibited by Proteaceae, most specifically rare Proteaceae.

In *Banksia cuneata* (also a member of the Proteaceae), allozyme studies showed that most of the genetic variability also lay within populations, some between populations within groups but very little variation lay among groups (Coates & Sokowski 1992). Similar patterns were observed in *Erodium paularense*, a member of Geraniaceae (Martín *et al.* 1997) where the variability within population was 80-85%. In the Spanish cedar, *C. odorata* too, most of the variation lay within populations (Gillies *et al.* 1997). Nesbitt *et al.* (1995) observed most genetic variation within populations of *E. globulus*. Here the variation was 73,8 and 94,9 % while the variation between subspecies was only 3.5 and 9.5 %.

This pattern is not always the case and the following examples show low within- population variation relative to between-population variation. For *B. dactyloides*, AMOVA revealed large regional differences (58.4%) and smaller (but significant) population differences within regions (9.7%) and fairly large individual differences within populations (31.9%) (Huff *et al.* 1993). In western pond turtles (an obligative outcrossing species) more genetic variability was observed between populations and between regions than within populations where the level was considered low (Gray 1995). In wild barley, *Hordeum spontaneum*, genetic variability within populations was low at only 43 % (Dawson *et al.* 1993) and in the leguminous tree, *G. sepium*, low within-population genetic variation was observed (Chalmers *et al.* 1992).

In another Proteaceae species, *Banksia spinulosa*, patterns of mating were observed to be interrelated to population genetic structure (Carthew 1993). In *B. spinosa*, pollen and seed dispersal is often limited, which results in subpopulation genetic structure. Genetic differentiation among plant populations over a small scale is well documented (Linhart & Grant 1996). This is a strong possibility for *L. elimense* which has large nut-like seeds, making it difficult to disperse far from the parent plant. Pollen is distributed by birds and insects in *L. elimense* and their territoriality may restrict pollen movement, often within a section of the plant population. In addition, the successful colonisation by offspring close to parents will result in a high probability of interbreeding between siblings and between parents and siblings.

Taxonomic implications

Genetic distance provides a measure of taxonomic isolation in some organisms, however this is not true for all organisms (Harrison 1991). Caution must be applied to applying RAPD results to cladistic phylogenetic analysis because of the potential for unrelated co-migrating products or multiple related products (See Smith *et al.* 1994). Although not the primary aim of this project, using RAPD analysis and AMOVA, the results suggest that the subspecies are merely well differentiated populations and perhaps should not have subspecies status at all. From the measure of genetic variation between subspecies (Table 3.4), the distictiveness of *L.e. vyeboomense* away from the other two subspecies is suggested. This could be interpreted as a taxonomic distinction. In the dendrograms, the clusters contained individuals from largely within single populations. The Principle Co-ordinate Analysis (Fig. 3.7) again showed a clear grouping of the *L.e. vyeboomense* away from the other two subspecies but populations of *L.e. elimense* and *L.e. salteri* were less clearly separated, even in three dimensions. Other studies which separated groups successfully using Principle Co-ordinate Analysis, were the systematic studies on the Juniper tree, *Juniperus* (Adams & Demeke 1993), cabbage and cauliflower cultivars (*Brassica* taxa) (Demeke *et al.* 1992, Hu & Quiros 1991), and deer (Cervidae) (Comincini *et al.* 1996). *Salicornia* species (Chenopodiaceae) were separated (Luque *et al.* 1995), cultivars in *Panicum* millet were detected by RAPDs (M'Ribu & Hilu 1994). Closely related taxa in the beach clam *Donax* (Adamkewiez & Harasewych 1994) were distinguishable using RAPD technique.

Implications for conservation

A most valuable implication for conservation is that RAPD technique allows for a cheap and quick quantitative measure of genetic variability of the taxon under investigation from relatively few individuals of a species with minimal impact on the population. If groupings are required, UPGMA or Principle Co-ordinate Analysis are able to show clustering of genetically similar individuals. AMOVA presents a structured hierarchical pattern of variation and appropriate quantitative statistical analysis. This allows plant collections to be planned to maximise the genetic variation (Dawson *et al.* 1995) by selection of appropriate individuals or populations.

In terms of *L. elimense,* all six populations showed similar, high levels of genetic variation, with most of the variation occurring between populations within subspecies. This would suggest that any individuals could be collected for relocation with equal success. The high genetic variability is likely to be an advantage to the plants adapting to successful relocation.

Even though genetic studies are useful to rare plant management they must be seen as secondary to protection, management and monitoring of the rare population. It must be remembered that rare plant studies all aim to make recommendations which will help prevent the extinction of the taxon. Peakall & Sydes (1996) go further and suggest that immediate short term goals are prevention of extinction, while longer term goals are maintenance of genetic diversity. A caution to apply to the use of genetic studies in rare plant conservation is that current population sizes and patterns of genetic variation are a result of historic events (Ellstrand & Elam 1993). RAPD markers do not allow for a measure of rate of mutation. Genetic variation within a population is a function of the mutation rate, effective population size, dispersal ability and migration rates (Harrison 1991). It is possible that anthropogenic factors have altered these in the last four hundred years, since the arrival of European settlers, which might have resulted in population fragmentation, reduction and stress. As these would be "recent" in evolutionary terms, they may not be reflected in the genome yet. It cannot be predicted with any certainty that such changes will have an effect on the genome. While undoubtedly most rare Cape Proteaceae have suffered from agriculture, it is seen from herbaria records of the early collectors that these rare Cape Proteaceae always were scarce, occurring at best in small numbers at few sites. Typically rare plant populations are small and many authors warn of the fixation of deleterious alleles within such small population (Lynch *et al.* 1995) as these are always associated with increased inbreeding relative to large populations (Milligan *et al.* 1994). This factor cannot be measured.

Although the RAPD technique allows for the genetic variability to be quantified, it is not possible to determine a critical level of variation necessary for the successful perpetuation and hence conservation of the taxon or population. This critical level is maintained by the population numbers being above what is often termed the minimum effective population size (Avise 1994). Being below this level, would result in a loss of genetic variability which would

decrease the potential for future evolutionary responses to environmental changes and lower the fitness through loss of heterozygosity (Milligan *et al.* 1994).

Conclusions

RAPD analysis, when used in combination with AMOVA, is a powerful tool in assessing genetic variability within populations as well as the hierarchical partitioning of genetic variation. Different coefficients of similarity/dissimilarity can alter the apparent level of variation. *L. elimense*, when compared to *G. scapigera*, shows a high variability level of 0.35 for the species as a whole. This variation is evenly distributed throughout the individuals within populations and no evidence of clonality is seen. Hierarchically for *L. elimense*, most of the genetic variation lies within populations within subspecies. No individuals showed unique marker bands, with most bands being present in most individuals sampled. Levels and patterns of genetic variation were similar between all three subspecies, despite their different population dynamics, structure, and numbers. All of these features suggests that the three subspecies of *L. elimense* are genetically sound, or that fragmentation, environmental pressures and reduction in numbers have been too recent to be reflected in the genome in any form of bottleneck. This makes *L. elimense* a suitable candidate for conservation management, protection or even relocation. It is also a candidate for protection by small nature reserves.

CHAPTER 4

RAPD profiling in conservation management: an application to estimate levels of genetic variability in individuals and populations in the endangered *Serruria roxburghii* R.Br. (Proteaceae)

Introduction

Serruria roxburghii is known historically from the area around Paarl Mountain, Paardeberg and Riebeek-Kasteel in the South Western Cape (Rourke & Ward-Hilhorst 1997). It is found on sandy patches which are scattered amongst the shale-derived soils. As this is a geological situation of long standing, it is likely that *S. roxburghii* has never been particularly common, and has always occurred as small scattered populations which were naturally rare. Herbaria records ceased after 1850 and this species was thought to be extinct until its rediscovery in 1976 by staff of the Bolus Herbarium, University of Cape Town (Pool & Smuts 1993). The increase in grazing and burning pressures along with the arrival of alien invasive plants has resulted in this species no longer being naturally rare and it currently is ascribed the IUCN status of endangered (Tansley 1988, Hilton-Taylor 1996a). Concern for the survival of the species has been expressed as there are currently only two small established populations of this plant known, one of 3 000 plants in 60 ha and the other of 6 000 plants in 100 ha, plus a recently rediscovered relict patch of a few plants (R. Pool pers comm.). Land owners are keen to protect and enhance the survival of these two populations as far as economically possible. It consequently became critical to obtain as much information as possible, both ecological and genetic, to protect these last two remaining populations. An indication of the levels of genetic variation within and between these two populations of *S. roxburghii* is essential to focus the limited conservation resources to maximise efficiency and effectiveness. This study was consequently undertaken to provide some indication as to which population contained the greater genetic variation and thus potentially the better chance of successful long term survival, should a choice have to be made between the populations. The surviving relict patch, although unlikely to be viable, might be valuable in adding to the gene pool.

The aim of this chapter was to measure genetic variation in *Serruria roxburghii* using the RAPD technique optimised as described in Chapter 3 for *Leucadendron elimense*. Essentially this would test the applicability of the method across more than one genus of Cape Proteaceae without modification.

Material and Methods

Collection of plant material

The number of plants found in Population 1, on the farm De La Gift, near Riebeek-Kasteel, was small as it had suffered from a fire shortly prior to collection of leaf material. Leaf material was collected from all plants found and labelled R1-1 to R1-15. A group of six young seedlings was included. These were labelled R1-20 to R1-26. Leaf material was also collected from the second population (Population 2) on the farm Vlakfontein a few kilometres away and labelled R2-1 to R2-11. This population had also recently been burnt, and the young growth after the fire was heavily grazed which resulted in only 11 samples being collected. The localities of these populations is shown in Fig. 2.1 in Chapter 2.

DNA analysis

In order to make the results directly comparable to the results for *L. elimense*, all methodology and data analysis was the same as already described in Chapter 3 for *L. elimense*. A minor difference had to take into account the fact that the leaves of *S. roxburghii* are terete, conical in cross section, and finely dissected. Therefore the leaf had to be bunched before two punches could be taken using the inner lid of an Eppendorf microtube so that approximately 290 mg, the same amount of tissue as used for *L. elimense*, was obtained. Methods of DNA extraction, PCR, visualisation and analysis have been described in detail in Chapter 3 and are not repeated here.

Results

A typical example of a polyacrylamide gel for *S. roxburghii* is shown in Fig. 4.1 and similar distinct banding patterns to the gels for *L. elimense* were obtained. If the lanes in Fig. 4.1 are compared to the dendrogram in Fig. 4.3b, it is seen that sample 12 was absent as it was too faint across most of the lane to score accurately.

Fragment description and distribution

The fragment pattern data for *S. roxburghii* is summarised in Table 4.1, where primers used are described and fragment sizes selected for the particular primers. The same size range of fragments were chosen as were used in *L. elimense* to allow comparison. The average number

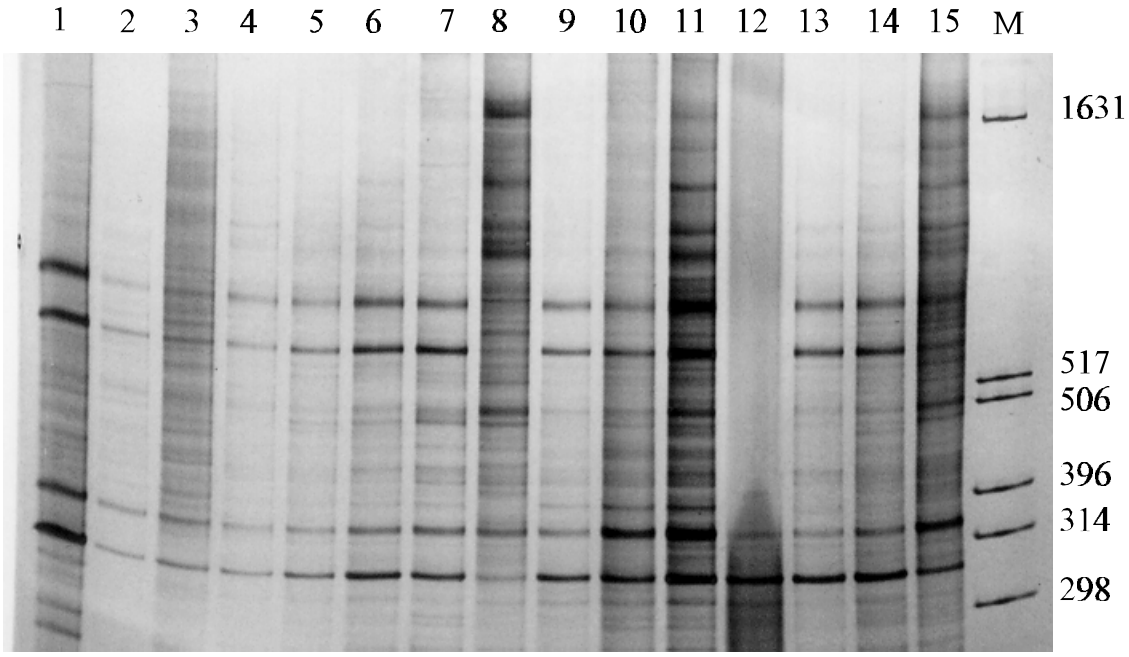


Fig. 4.1 An example of a banding pattern obtained for *S. roxburghii* on polyacrylamide gel stained with silver. This includes samples1 to15 from Population 1 amplified with primer OPAI-13.

of bands per plant, expressed as a percentage across the five individual primers was 63.21%, indicating that bands were generally more often present than absent. Of the total of 98 bands scored for the five primers, 92 were polymorphic. The number of duplicate banding patterns (RAPD genotypes) in each primer was few (Table 4.2). The chance of finding two individuals with the same banding pattern is the mean similarity raised to the power of the mean number of bands (Wolff & Peters-Van Rijn 1993). For the pooled primer data this is 8.45×10^{-13}, which is even smaller than for *L.elimense*.

The distribution pattern of the fragments is shown in Fig.4.2. There was a sharp peak of band frequency with 20% of the bands occurring in 41-50% of the samples (Fig.4.2a). Only 7% of the bands occurred in 0-20% of the samples, indicating few rare or unique bands. No bands were exclusive to single individuals. Rare absences were also not a feature as only 27% of the bands occurred in more than 80% of the samples. A comparison of the two populations is

Table 4.1. Sequence of 10 mer primers selected for use in this study. The size range of fragments scored came from the central region of each gel lane where bands were reliable, reproducible fragments for each primer. The total number of possible bands per primer within the selected size range is given, followed, in brackets, by the number of monomorphic bands. The average number of bands generated per plant over the fragment size range scored is shown along with the complete range of fragment numbers generated per primer per plant.

Primer Name	Primer sequence 5'to 3'	Size range of bands (base pairs)	Number of bands scored	Average no. bands/plant (range)	Total no. bands present (as %)
Rhodes 3	CGG CCC CTG T	350-900	15 (1)	11.80 (6-14)	307 (78.72)
Rhodes 7	TCA CGA TGC A	400-830	13 (0)	8.95 (5-12)	197 (68.88)
Rhodes 12	ATT GCG TCC A	350-950	29 (2)	17.09 (12-21)	376 (58.93%)
OPAI-05	GTC GTA GCG G	500-830	15 (3)	8.71 (4-11)	122 (58.10%)
OPAI-13	ACG CTG CGA C	350-950	26 (0)	13.37 (7-20)	401 (51.41%)
Rhodes 3,7,12, OPAI-13		350-950	83 (3)	51.70 (48-60)	517 (62.29%)

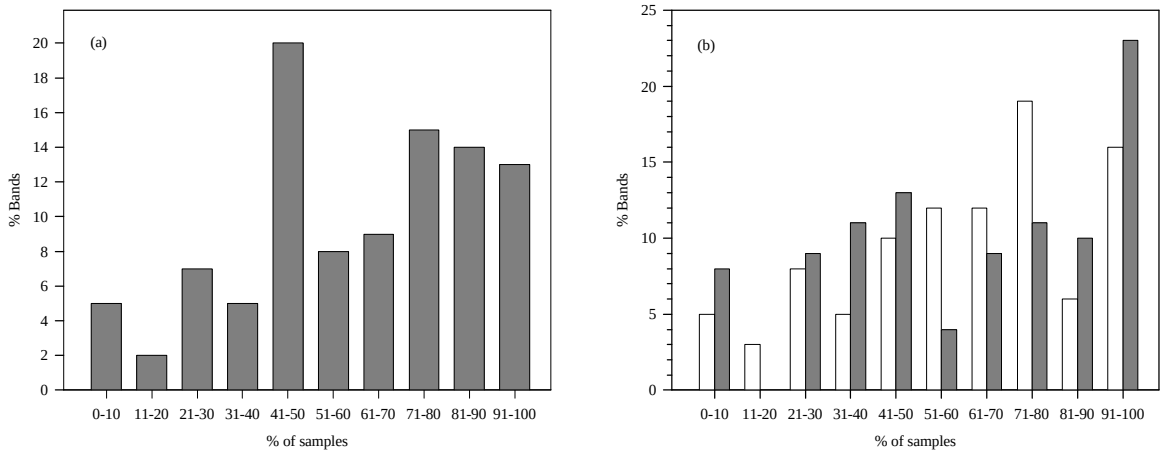
possible in the histograms in Fig. 4.2b. This shows the overall trends to be the same except that Population 1 showing a spike of 19 bands being present in 71-80% of the samples, whereas Population 2 only showed 11 bands being present in 71-80% of the samples. At the top end of the histogram, Population 2 shows 23 bands being present in 91-100% of the samples whereas Population 1 only exhibits 16 bands present in more than 90% of the samples.

Table 4.2:- Mean genetic variability (*V*) for the two populations of *Serruria roxburghii* as calculated for each of the five primers used and for the primers pooled (excluding OPAI-05). Genetic variability was calculated using the coefficient of Apostol *et al.* (1993) as $V = 1\text{-}S$, where $S = M/N_t$, M being the number of matches of shared bands, and shared absence of scored bands between plants within the population. N_t is the total number of bands identified for a particular primer or group of primers for the species (see Table 1). In the table, n is the number of individuals sampled in each population. It is followed by the number of unique genotypes in brackets. The last row shows genetic variability calculated from the coefficient of Nei & Li (1979), $1\text{-}F$ where $F = 2m_{xy} /(m_x + m_y)$. m_x and m_y are the number of bands in x and y respectively.

	Primer	Population 1	Population 2	Whole species
V	Rhodes 3	0.34 n=14(14)	0.26 n=12(10)	0.31 n=26(24)
V	Rhodes 7	0.40 n=11(11)	0.36 n=11(11)	0.38 n=22(21)
V	Rhodes 12	0.40 n=14(14)	0.35 n=8(8)	0.40 n=22(22)
V	OPAI-05	0.35 n=8(7)	0.30 n=6(6)	0.33 n=14(13)
V	OPAI-13	0.32 n=18(17)	0.36 n=12(12)	0.33 n=30(29)
V	Rhodes 3,7,12 & OPAI-13	0.36 n=5(5)	0.35 n=5(5)	0.36 n=10(10)
1-*F*	Rhodes 3,7,12 & OPAI-13	0.29 n=5(5)	0.28 n=5(5)	0.29 n=10(10)
1-*F*	OPAI-13	0.33 n=18(17)	0.34 n=12(12)	0.33 n=30(29)

When the coefficient of Nei & Li (1979) was applied to the pooled primer data and primer OPAI-13, the resultant mean genetic variation was lower than for the coefficient of Apostol *et al.* (1993), indicating that there were more shared absence of fragments than shared presence

(see Chapter 3). This suggested the presence of rare bands, a fact supported by the histograms in Fig. 4.2. These rare bands are present in both Population 1 and Population 2 (Fig.4.2b). For primer OPAI-13, however, the two coefficients produced comparable levels of genetic variation for Population 1, indicating that the levels of shared presence of fragments was approximately the same as the level of shared absence of fragments, whereas for Population



2 and the species as a whole, the coefficient of Nei & Li (1979) was lower.

Fig. 4.2 (a) Frequency distribution of individual RAPD bands for all five primers in the two *S. roxburghii* populations. The numbers of plants showing each band as a percentage of the number of plants sampled for that particular primer is plotted against the number of bands. The total number of bands scored is 98. **(b)** Band frequencies of Population 1 (open bars) and Population 2 (hatched bars). The number of bands remains 98.

Genetic Variability

Mean genetic distances were calculated for the populations and for the species as a whole using the different primers separately with the coefficient of Apostol *et al.* (1993) as a measure of genetic variation (Table 4.2). The mean genetic distance within Population 1 ranged from 0.32 to 0.40 across the five primers with an average of 0.36. For Population 2, the mean genetic distance was lower, ranging from 0.26 to 0.36 with an average of 0.33. Four of the individual primers and the pooled primer results showed Population 1 as having a higher level of genetic distance than Population 2, hence more variability. Only primer OPAI-13 showed Population 2 as being more variable than Population 1.

Potentially *S. roxburghii* is a candidate species for relocation should it become impossible to protect the natural habitat adequately. This would require the selection of individuals with high levels of genetic variation. To this end, it would become essential to have some measure of genetic variability within the populations. Thus it was useful to calculate the mean genetic distance of each of the individuals from others within the populations (Table 4.3).

Table 4.3:- Mean genetic variability calculated for individual plants screened with primer OPAI-13 and Rhodes 12.. These employ the coefficient of Apostol *et al.* (1993) *V*, calculated as 1-*S*, where $S = M/N_t$, *M* being the number of shared scored band presence as well as absence between plants in both populations. N_t, the total number of bands, is 26 for OPAI-13 and 29 for Rhodes 12. The genetic variability (*V*) for each plant is the average genetic distance of that plant from others in the resident population. AD replaces absent data.

plant	*V* Rhodes12	*V* OPAI-13	plant	*V* Rhodes12	*V* OPAI-13
R1-01	0.45	0.29	R1-23	0.37	AD
R1-02	AD	0.37	R1-24	0.38	0.40
R1-03	0.49	0.38	R1-25	AD	0.45
R1-04	0.38	0.32	R1-26	AD	0.39
R1-05	0.39	0.34	R2-01	0.35	0.32
R1-06	0.39	0.29	R2-02	0.34	0.36
R1-07	0.39	0.28	R2-03	AD	0.36
R1-08	0.42	0.34	R2-04	AD	0.31
R1-09	0.32	0.36	R2-05	AD	0.38
R1-10	0.42	0.28	R2-06	0.37	0.47
R1-11	0.42	0.30	R2-07	0.42	0.44
R1-13	AD	0.33	R2-08	0.40	0.34
R1-14	AD	0.35	R2-09	0.43	0.37
R1-15	AD	0.33	R2-10	0.39	0.39
R1-21	0.40	0.41	R2-11	AD	0.36
R1-22	0.36	AD	R2-12	0.36	0.38

This table was generated using primer OPAI-13 and primer Rhodes 12, both of which approximated the pooled data for *L. elimense*. It was felt that the pooled primer data for *S. roxburghii* was a much smaller sample size and so provided a less reliable comparison. This comparison of individuals showed that certain individuals are genetically more distant from others. Using primer OPAI-13, five individuals showed a mean genetic distance from other individuals of 0.40 or greater include R1-21, R1-24, R1-25, R2-06, R2-07. Results from primer Rhodes 12 were representative of the other primers used as the results generally approximated the results from the other three primers. Here individuals R1-01, R1-03, R1-08, R1-10, R1-11, R1-21, R2-07, R2-08, and R2-09 showed a genetic distance of 0.40 or more. These twelve individuals should have priority for translocation. As they represent 40% of the sample, it would seem that generally levels of genetic variation are high and the selection of particular individuals would not then be critical. It is preferable that as many plants as possible should be conserved.

Analysis of Molecular Variance

The AMOVA results are presented in Table 4.4. Four of the five primers showed over 89% of the variation lying within each of the two populations. The *P*-value for the variation was not statistically significant for any of the primers indicating that individuals from Population 1 might just as easily have come from Population 2. The two populations are not distinct.

Representative UPGMA trees are presented only for primer Rhodes 12 (Fig.4.3a) and primer OPAI-13 (Fig. 4.3b) with the coefficient of Nei & Li (1979). Here it is apparent that there is no clear division between the two populations with some individuals being more similar to others within the second population than the same. Clustering of the two populations was not distinct and a degree of overlap was evident, which supported the results obtained by the AMOVA. Although not shown here, this was seen in all five primers, the pooled primers, and with the coefficients of both Excoffier *et al.* (1992) and Nei & Li (1979). The correlation coefficient (r_{cs}), calculated for these coefficients, as well as for Apostol *et al.* (1993) and Jaccard (1901 cited in Armstrong *et al.* 1994), ranged from 0.7 to 0.9 which was acceptable to good.

Table 4.4 Analysis of molecular variance (AMOVA) for the two populations of *Serruria roxburgii*, using five primers. Statistics include the degrees of freedom (*df*), sum of squared deviations (SSD), mean squared deviations (MSD), variance component estimates (*Var*), the

percentage total variance (% Total), and the probability (*P*) of obtaining a more extreme component estimate by chance alone. 1000 permutations were used for each analysis.

Source of variation	*df*	SSD	MSD	*Var*	%Total	*P*-value
Pooled Primers						
Among populations	1	18.70	18.70	0.81	5.24	0.056
Within populations	8	117.20	14.65	14.65	94.76	
Primer OPAI-13						
Among populations	1	11.49	11.49	0.50	10.46	0.003
Within populations	28	119.94	4.29	4.28	89.54	
Primer OPAI-05						
Among populations	1	6.35	6.35	0.59	20.72	0.008
Within populations	12	27.29	2.27	2.27	79.28	
Primer Rhodes 3						
Among populations	1	3.32	3.32	0.08	3.49	0.140
Within populations	24	54.37	2.27	2.27	96.51	
Primer Rhodes 7						
Among populations	1	2.59	2.59	0.01	0.54	0.385
Within populations	21	48.91	2.45	2.45	99.46	
Primer Rhodes 12						
Among populations	1	10.19	10.19	0.46	7.54	0.022
Within populations	20	111.36	5.57	5.57	92.46	

The Principle Co-ordinate Analysis gives a three dimensional perspective of the relationship between the two populations (Fig. 4.4). In Fig. 4.4b some grouping is seen with Population 1 but in Fig. 4.4a the two populations are well mixed. The vertical elevation of the samples differs greatly even within populations. The use of different coefficients of dissimilarity did little to alter the overall pattern and showed a general absence of clustering. The pooled data was reduced to ten individuals that the primers amplified successfully. The dendrograms for this limited data set did not show marked separation of the populations and so is not presented.

a b

Fig. 4.3 UPGMA analysis of *S. roxburghii* plant relationships. Examples of dendrograms using the dissimilarity coefficient of Nei & Li (1979) with primer Rhodes 12(**a**) and primer OPAI-13 (**b**) show there is no distinction between the two populations.

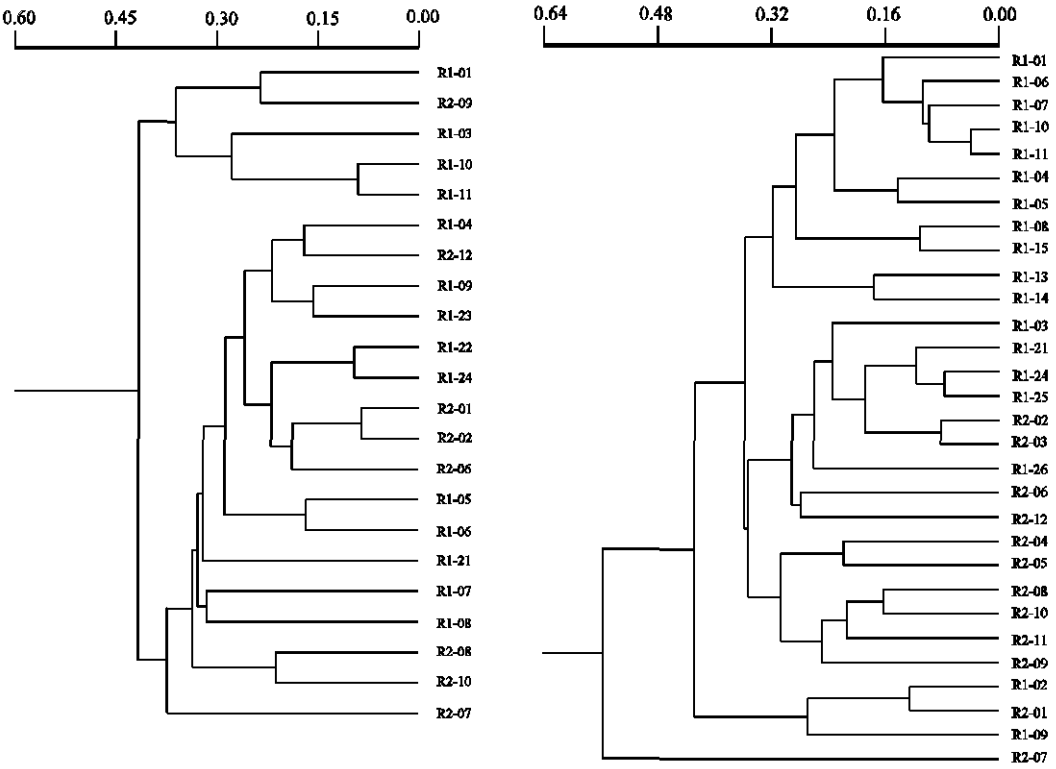


Fig. 4.4a

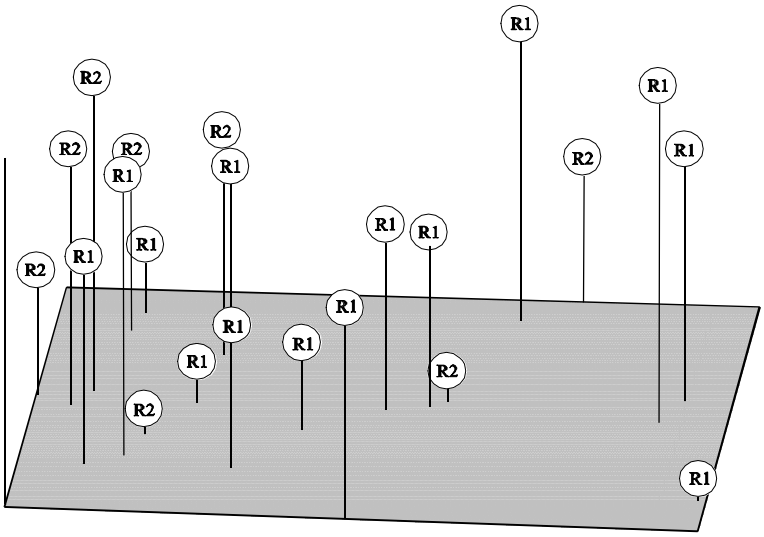


Fig. 4.4b

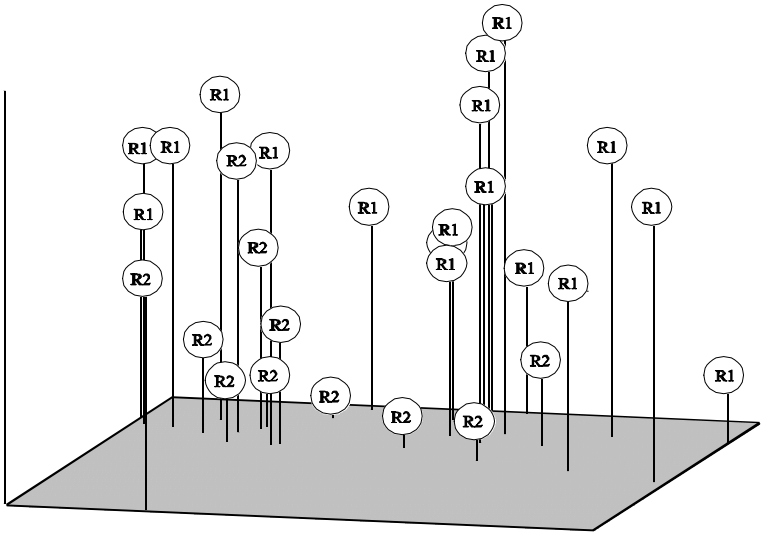


Fig. 4.4 Examples of Principle Co-ordinate Analysis of dissimilarity between the two populations of *S. roxburghii*, using the Euclidean distance (Excoffier *et al.* 1992) with primer 12 (**a**) and the dissimilarity coefficient of Nei & Li (1979) with primer OPAI-13 (**b**).

Discussion

The number of successful amplifications with *S. roxburghii* was not nearly as high as for *L. elimense*. The possibility that this might have resulted from the system and method not being optimised specifically for *S. roxburghii*, cannot be discounted. As there was general PCR failure with other unrelated species at this time, it was felt that it was more likely to have been a problem relating to *Taq* Polymerase.

Fragment polymorphism

Total band presence was seen to range from 51% to 78% (Table 4.1). This indicated that bands scored were generally common to more than half the samples and not unique to individuals, and that high levels of genetic variability were expected. The distribution of the fragments showed the same general trends as *L. elimense* with a double peak seen in Fig. 3.4a as well as Fig. 4.2a. One might have expected the pattern seen in Fig. 3.4b for *L.e. vyeboomense* with the high presence of rare bands if *S. roxburghii* is severely restricted and highly endangered. This was not seen in either population and is perhaps a function of the relatively large size of the *S. roxburghii* populations, despite the fact that they are the last two known populations. Less than 20% of the samples held rare bands and although low, this was not as low as for *L. elimense*. The fact that there were few rare bands has implications for genetic conservation (Graham *et al* 1997). If there were rare bands it would be important to ensure their continued success because the loss of rare alleles can cause genetic bottlenecks (Karron 1997). As this is not a factor, selection of individuals for translocation need not take this into account. The small sample size potentially might have missed unique individuals which were genetically greatly distant from others in the population. Ideally every individual should be screened.

Of the six bands scored as monomorphic in *S. roxburghii*, two of these were monomorphic in *L. elimense* and also *L. salignum* suggesting these two might be marker bands for all Proteaceae. Two bands were absent in all samples of *S. roxburghii* yet were present in *L. elimense*. There were an additional four bands monomorphic in *S. roxburghii* that were not monomorphic in *L. elimense*, suggesting that *S. roxburghii* had a slightly higher degree of distinctness. Further support for this individual distinctness was the absence of duplicate banding patterns within primers. Across the five primers there was a total of five (4% of samples) duplicate genotypes in *S. roxburghii* whereas there were 34 (9% of samples) in *L. elimense*. The low genotypic variation was particularly marked in *L. elimense* with primers

Rhodes 3 and Rhodes 7 in certain populations such as, for example, the population of *L.e. vyeboomense* with primer Rhodes 7. That this low genotypic within-population variation was not seen in either population of *S. roxburghii* with any primers, suggested that the low genotypic variation might have been a real result and not just a primer artifact. The use of primer Rhodes 7 would be worth further investigation.

Genetic variation

Genetic variability for *S. roxburghii* ranged from 0.31 to 0.40 with an average of 0.35 (Table 4.2). This was a smaller range than for *L. elimense*, where the range for the species as whole was from 0.29 to 0.43 resulting in an average of 0.35, identical to that of *S. roxburghii*. All these levels may be considered high for a species of rare Proteaceae (Rossetto *et al.* 1995). For both *L. elimense* and *S. roxburghii*, primer Rhodes 12 showed the highest levels of genetic variability of the whole species, suggesting that this primer was more sensitive to detecting variation than any of the other primers selected.

The average genetic variability for *S. roxburghii* was considerably higher at 0.35 than for the ubiquitous *L. salignum* where the mean variability was 0.30 (Table 3.2). Only one population of *L.salignum* was screened, which probably would have reduced this genetic variation measure from the expected level for the species as a whole. The population sampled was, however, part of a large continuous population that was widespread over many kilometres, suggesting the potential for free gene flow. This genetic variability within *L.salignum* was lower than any of the mean genetic variabilities estimated from any of the primers for *S. roxburghii* with the exception of Population 2 with primer Rhodes 3 where there were 10 genotypes in 12 individuals.

In comparing the genetic variation within the two populations, it was seen that, although Population 1 generally had a higher level of genetic distance than Population 2, both were considered to have an above expected level of genetic distance. These high levels of genetic distance support the contention of Baskauf *et al.* (1994) that rare species are often wrongly assumed to be genetically depauperate. In contrast, Gaston & Kunin (1997) state that rare species, with few exceptions, tend to have low genetic variability. The arguments of inbreeding depressions, genetic drift, founder effect, habitat loss, environmental catastrophes, loss of heterozygosity, loss of fitness, genetic load, and lethal recessives are commonly put forward

(Karron 1997). Rossetto *et al.* (1995) suggested that rare species are able to maintain unusually high levels of genetic variation. Evidence from *S. roxburghii* supported this.

It must be questioned whether there will be a difference between populations that are naturally rare and have presumably always been so, and populations which have been considerably reduced in size through anthropogenic activity or natural causes. The naturally rare populations could be adapted to cope successfully in small numbers. If a species had been widespread and numbers subsequently reduced, stress, loss of fitness and a reduction in genetic variability might result. Thus common taxa like *L. salignum*, which are still widespread but sparser now, may be genetically more depauperate than the rare taxa, as indicated by the generally low level of genetic variability of this species. Essentially *L. salignum* forms a continuum in its distribution, rather than discreet populations. This distribution stretches across the whole of the Cape Floral Kingdom, and covers a variety of different habitats. There are still large numbers of individuals in total, although these numbers are considerably less than those a few hundred years ago as a result of habitat loss.

Should it become necessary, candidate individuals can, on the basis of their high measure of genetic distance from other individuals, be nominated for relocation. It is suggested that as many individuals as possible are considered. *S. roxburghii* is known to grow well from cuttings (Rourke & Ward-Hilhorst 1997), so the original plants need not necessarily be uprooted and transplanted, an often unsuccessful process. The plants designated R1-21 to R1-26 were all young which is a positive factor that will assist in their successful translocation.

Possible Effects of Frequent Fires on Genetic Variability

The vegetation of the Cape Flora is fire-prone and the fire interval is between 4 and 45 years depending on the build up of fuel (van Wilgen *et al.* 1992). In more recent times, fires have been deliberately set to clear areas to maintain water runoff and in agricultural land and surrounds to clear bush, allowing a flush of grass for grazing. Such deliberate fires take little account of natural fire regimes. *S. roxburghii* is killed by fire (pers.obs.). It reaches sexual maturity at three years of age and in cultivation is seen to senesce and die after about five years of age (Rourke & Ward-Hilhorst 1997), although natural populations may be considerably older. This would imply that the fire interval would be critical and would be optimal at about five years. More frequent fires would kill populations before they could reach sexual maturity

or sufficient seed stores could accumulate. Bradstock *et al.* (1996) propose that patchiness of fire helps to allow some individuals to escape frequent fires and thus reach sexual maturity. In this context, it is interesting to note that sample R1-21 to R1-26 are young plants growing after fire. As these were the only individuals in the immediate vicinity, it is suggested that the soil held seed reserves were low although genetically diverse.

Observations of Population 2 at Vlakfontein in July 1998 by R. Pool (pers. comm.), showed a mosaic of different aged plants, apparently as a result of patch burning. Some areas held plants of over 30 years, which still bore flowers on plants of 1.5 m tall. Other areas held young seedlings growing on an area known to have been burnt in May 1994. Seeds in this area would have germinated in the autumn of 1995 at the earliest and would be up to three years in age. These plants were single stemmed individuals only 20 cm tall and every plant had the growing tip grazed away by sheep. Because at this stage each plant consists of a single stem without lateral branching, such removal of the delicate growth region caused all grazed plants to die. The only chance for the survival of young plants after fire is for the farmer to exclude sheep until the plants are well established with branching and with the young tips no longer palatable to sheep, a period of possibly up to five years. The observations at Population 2 suggest that not only is fire interval critical but protection of the young plants after fire in areas utilised for grazing of sheep.

Rossetto *et al.* (1995) suggest that species restricted to small populations have developed some adaptations for maintaining high level of variability. They found that *G. scapigera* has large, genetically well balanced, long-lived seed banks to survive natural disturbance events. The appearance of individuals of *S. roxbughii* after fire in part of Population 1 suggests a viable seed store. As these individuals had good levels of genetic variability, this further suggests that they are probably genetically well balanced thus supporting the theory and findings for *G scapigera*.

Possible Effects of Population Size on Genetic Variability

Restricted population size is characteristic of rare and threatened taxa of Cape Proteaceae (Tansley 1988). As many of the rare Cape Proteaceae have only ever been recorded from naturally small populations, this suggests that small population size is not necessarily associated with low levels of genetic variation. There are only two populations known for *S. roxburghii*.

These occupy restricted areas of only 60 and 100 ha. The two populations of *S. roxburghii* were small, numbering about 3000 and 5000 plants in population 1 and 2, respectively. Despite all these restrictions, genetic distance was high (average 0.35). These two populations essentially represent the species as a whole as they are they only extant populations. Despite this, the levels of genetic distance measured were considerably higher than for the widespread and common *L. salignum* (average 0.30) for all but primer Rhodes 3. The evidence of large genetic variation in the two small remaining populations of *S. roxburghii* is strong support for the potential success of small nature reserves.

Karron (1997) indicated that small populations frequently have high levels of homozygosity due to the effects of biparental inbreeding. Self compatible hermaphrodite individuals may self-fertilize, a phenomenon which will increase in small or low density populations. *S. roxburghii* has the potential for self-fertilization and biparental inbreeding, so this factor must also be considered as a potential problem for conservation management should the numbers decline further or only a few plants survive translocation.

Predictions about genetic variation in small populations would be useful for conservation managers. In the review by Ellstand & Elam (1993), although small populations are generally associated with less genetic variability as predicted by genetic theory, this is not always the case. An example includes *Senecio integrifolius*, where Widén & Andersson (1993) found more genetic variation in the small population of around 130 plants than in a large population of up to 3000 plants of the same species. The large population is of a comparable size to the 3000 and 4000 individuals estimated for the populations of *S. roxburghii* in 1994. The *S. integrifolius* is recorded from 40 populations previously although only eight were considered extant at the time of the study (Widén & Andersson 1993). Relatively speaking then, the populations of *S. roxburghii* might not be considered small, although they are restricted to two populations in total. It is important to assess the level of genetic variation in the individuals, populations and the entire species in order to evaluate fully the impact of rarity (Baskauf *et al* 1994).

It is well-known that pollinators can effectively isolate small populations. Ehrlich & Raven (1969) demonstrated that in insect pollinated plant species, 15m was sufficient to isolate two populations. The pollinator of *S. roxburghii* is uncertain but evidence suggests that it is a moth

(Rourke & Ward Hilhorst 1997) or a beetle (pers. obs.). The two populations are not, however, genetically distinct implying pollen transfer between them over a distances of some kilometres. This suggests that these populations might be part of a large disjunct single unit and should possibly not be treated as two small separate populations. The seeds of *S. roxburghii* are not palatable to birds but are dispersed by ants (See table 2.1.) so dispersal of seeds between the two populations is unlikely.

Hierarchical structure within S. roxburghii

As might be expected with two geographically close populations of the same taxon, most of the genetic variation lay between individuals within the populations and consequently hierarchical structure was lacking (Morell *et al.* 1995). It is also noted that the P-values were all non-significant indicating that the genetic differences were not significantly less within populations than between populations, and the difference between populations was also not significant. This lack of structure within the species suggested that the two populations lack distinctiveness and were genetically very similar. This was firm evidence to support the suggestion that the two populations were probably island remnants of what was previously a single large if disjunct population.

Conclusions and implications for conservation

The main aim of assessing the genetic variation in *S. roxburghii* was to allow conservation efforts to protect the species so as to maintain a constant level of genetic variability, deemed necessary by Frankel & Soulé (1981), and Simberloff (1988). In addition, *S. roxburghii* provided a test case for the applicability of the methods used for *L. elimense* for other small populations of rare Cape Proteaceae.

In general the results showed that *S. roxburghii* exhibited high levels of genetic variability in both the two known remaining populations (0.35 and 0.36 respectively). These levels were comparable with results from *G. scapigera* and *L.e. salteri*, higher than for *L.e. elimense* and considerably higher than for *L.e. vyeboomense*. The two populations both exhibited similar levels of genetic distance with all primers (except primer OPAI-13). The implications of the AMOVA results for conservation are that most of the genetic variation lay within populations The variation between the populations was not significantly different. Individuals of the two populations could consequently be mixed into a single population as they are genetically similar. Indeed, they may be relicts of an historically single larger population. This was further supported by the fact that the two populations were not easily separated using UPGMA trees or Principle Co-ordinate Analysis. These results are critical in the light that there are only these two populations remaining.

Population 1 generally had a slightly higher level of variability than Population 2. This suggested that it was preferable to conserve Population 1 over Population 2. However as the difference was so slight, other parameters such as age and threat of frequent fire must also be considered. Population size has no apparent effect on levels of genetic variation. Ideally part of both the two populations should be fenced and grazing excluded. A fire regime should be established and monitored. Selection of individuals should translocation become necessary could include any of the individuals sampled as all exhibited relatively high levels of genetic variability. The highest levels were seen to be R1-01, R1-03, R1-08, R1-10, R1-11, R1-21, R1-24, and R1-25 from population 1 and R2-06, R2-07, R2-08 and R2-09 and so these are suggested as potential candidate individuals for translocation. It is suggested that as many individuals as possible are translocated or, better still, cuttings are taken.

Peakall & Sydes (1996) point out that rare species are often few in number so that ideally all remaining populations should be preserved. This would exclude the need for genetic studies. Unfortunately this is not the case in the Cape Flora as it is seldom possible to conserve all populations of all highly endangered taxa. *S. roxburghii* is one such taxon. If there are many known populations of a rare species, genetic measurements will allow for selection of populations based on maximum genetic variation.

An important result from this specific study is that although these populations of *S. roxburghii* are extremely small and always have been, they had a high level of genetic variation suggesting that they would be more than adequately protected in a small area. This does not necessarily require an official reserve, merely protection from grazing when young. As this species has a narrow edaphic tolerance, soil type has to be selected accurately. There is evidence to support reserves as small as 1000 ha (Zuidema *et al.* 1996) although data on Cape Proteaceae suggest much smaller areas, such as for the *S. roxburghii* populations, may be viable (Tansley 1988). The above conclusions indicate that useful results have been obtained, which are directly applicable to conservation management, thus supporting the proposal that RAPD's is a usable tool for conservation of the small populations of rare Cape Proteaceae. RAPD's does not allow good comparisons between studies at present so is of little use in long term studies. In the future, this might be less of a problem as the RAPD technique becomes more refined or superior methods are developed .

CHAPTER 5

Population differentiation of the three subspecies of the rare *Leucadendron elimense* E. Phillips (Proteaceae) using polyacrylamide gel electrophoresis (PAGE) of total protein

Introduction

Molecular studies are proving increasingly valuable in providing input for conservation decisions. There is a basic need to assess some measure of genetic variability within and between populations and subspecies, so that areas of high variability are selected preferentially, resulting in the efficient and effective focussing of management and funding to where it will accrue the most dividends. The cheapest, simplest method that offers sufficient information for this purpose clearly has advantages. Although perhaps being one of the crudest methods of assessment of differentiation among populations, measurements of total protein by polyacrylamide gel electrophoresis (PAGE) meets these criteria.

Johnson & Hall (1965) used protein electrophoresis to analyse phylogenetic affinities in wheat and an early review paper by Fairbrothers (1968) lists a number of studies which used total protein for taxonomic studies in higher plants. Another example of the use of total protein electrophoresis in plants is seen in the study on seed proteins in *Trifolium* (Leguminosae) by Badr (1995). More recently, PAGE has been used extensively in taxonomic studies on micro-organisms (see Jackman & Pelezynska 1986, Qhobela & Clafin 1988, Qhobela *et al.* 1991). This method was also used by Nxomani *et al.* (1994) to determine genetic relatedness between isolated, allopatric populations of *Tilapia* fish. In the gymnosperm, *Pinus,* it has been used for genomic mapping (Plomion *et al.* 1995) and genetic differentiation (Petit *et al.* 1995). In South Africa, Thomas & Bond (1996) used the more refined, isozyme method of protein analysis to investigate genetic variation within populations of the rare cedar, *Widdringtonia cedarbergensis,* although they found the method unable to distinguish much variation and only two isozymes proved to be polymorphic for all populations studied. The information was thus of limited conservation value.

Plomion *et al.* (1995) point out that both proteins and isozymes correspond to coding DNA whereas the RAPD method identifies a large number of polymorphic DNA markers distributed throughout the genome, including both coding and non-coding regions. This means that RAPDs and total proteins could provide different, but perhaps complimentary, coverage of the genome. This is supported by Mitton (1994) who reports on a number of studies where the results from protein markers were dissimilar to results from DNA analysis and suggests that

the two sets of markers appear to reflect the predominance of different evolutionary forces. There are many studies where genetic variation is assessed using both protein-based markers, often allozymes, and a DNA-based marker, usually RAPDs. In general, there is more genetic variation detected by the DNA-based marker. Such animal studies include Soay sheep (Bancroft *et al.* 1995), the parasitic *Trypanosoma rangeli* (Steindel *et al.* 1994), the medfly (*Ceratitis capitata*) (Baruffi *et al.* 1995), the woodmouse (*Apodemus sylvaticus*) (Michaux *et al.* 1996), and such plant studies include the gymnosperm, *Amentotaxus formosana* (Wang *et al.* 1996), the pine (*Pinus pinaster*) (Petit *et al.* 1995), buffalograss (*Buchloë dactyloides*) (Peakall *et al.* 1995), wild rice (*Oryza glumaepatula*) (Buso *et al.* 1998), the narrow endemic *Wyethia reticulata* (Asteraceae) (Ayres & Ryan 1999), and the sea grass, *Posidonia australis* (Waycott 1998).

Total protein may provide information of taxonomic relevance. In *L. elimense*, the three subspecies are fairly well isolated physically from each other by mountain ranges. The seeds are wind dispersed although no information is available on the distance seeds travel from the parent plant for this species. Personal observations of seed dispersal suggests that most seeds, although winged, are large and so do not travel far from the parent plant. The integrity of the grouping of the subspecies together within one species can thus potentially be tested by the PAGE method if sufficient variation can be detected. The difference between populations within subspecies might also be detectable and populations selected preferentially for conservation.

In this chapter, PAGE was used to determine the relationships between the five study populations of the three subspecies of *L. elimense* in terms of their conservation management.

Materials and methods

Experimental procedure

Leaves were collected from *L.e. vyeboomense*, *L.e. salteri* Populations 1 and 2, and *L.e. elimense* Populations 1 and 3. A population of *L. salignum* was used for comparison as it is a different species as well as being widespread and common. Leaves were frozen immediately on collection and stored at -20^{o}C until protein extraction. This appears to be a critical factor

as no total proteins were detectable in leaves from *Serruria roxburghii*, which were not frozen immediately on picking from the plant. Boulter *et al.* (1966) warn that different proteins will be found in different parts of the plant, so care was taken to avoid flower and pollen debris on the leaves. A way of potentially increasing the number of polymorphic markers would be to use a number of different organs, each containing different proteins (De Vienne *et al.* 1996). As leaves were used in the RAPD analyses, it was essential to use leaf material rather than other parts of the plant, for example seed tissue, for the total protein analysis to increase the comparative value of this study.

It has been shown that metabolic processes differ within the leaf as it ages. Such differences can even be detected between sequential leaves. This potentially could influence the proteins detected should equivalent leaves not be compared between individuals. No literature could be found which supported this, and most studies do not indicate due care in selection of specific leaves beyond referring, for example, to young leaves (Machon *et al.* 1995).

Various methods of extraction of total protein were tried. There appeared to be high protease activity, which could not be inhibited with standard inhibitors and eventually steps from several different methods were used to minimise this activity.

Using the inner seal of a 1.5 ml Eppendorf microfuge tube lid, a disc of leaf tissue was punched out of each leaf, which had previously been washed in distilled water and ethanol. This leaf disc was ground in liquid nitrogen to a fine powder in a 1.5 ml microfuge tube using custom-made glass grinding rods. The samples were then suspended in 400µl lysis buffer (modified from Westermeier 1993 and Plomion *et al.* 1995), which contained 1 M $NaHPO_4$ and NaH_2PO_4 (pH 7.00), 9.5 M Urea, 0.5% Dithiothreotol (DTT), and 2% Triton X-100. The samples were centrifuged at 4ºC, for 10 min at 13 000 rpm, the supernatant (300µl) removed and added to three volumes of ice-cold acetone. This was incubated for 60 min on ice. The sample was centrifuged for 10 min at 13 000 rpm, the supernatant again removed and replaced with 400µl ice-cold acetone. This was incubated for 10 min on ice. After a final centrifugation of 10 min at 13 000rpm, the supernatant was discarded and replaced with 50µl loading buffer (Ausubel *et al.* 1992) containing 125 mM Tris-HCl (pH 6.8), 10% beta mercaptoethanol, 4% SDS, 0.1%

bromophenol blue, and 20% glycerol. The sample was then boiled for 10 min, after which it was loaded onto a 10% discontinuous polyacrylamide gel (Laemmli 1970). Electrophoresis was carried out at 150V and the gel was subsequently stained with Coomassie Brilliant Blue G-250 and destained in methanol. This left a clear gel with fairly distinct blue bands of proteins. A set of calibration proteins, (Combithek; Boehringer Mannheim), was used as size markers to allow between-gel comparisons.

The amount of protein in a group of test samples was quantified using Bradford's method of protein assay (Hammond & Kruger 1988). It was found that each sample consistently contained between 1 and 10Fg protein. As the samples deteriorated rapidly, it was not practical to quantify the protein in each sample before loading onto the gel. Furthermore, because the test set of samples showed a consistent protein content and a standard amount of leaf tissue was used routinely for all samples, the amount of protein was assumed to be fairly consistent. Despite this, there were a few samples which appeared to be more heavily loaded than others, presumably because of inconsistent loading (see, for example, lane 16 in Fig. 5.1).

Data analysis

A total of 17 bands were scored by eye as present or absent. No account was taken of the density of the bands. Bands with small molecular weights were excluded, and only bands with a molecular weight of 26.6 to 116 kDa were scored.

Four coefficients of dissimilarity were used to generate dendrograms of the Unweighted Pair Group Method (UPGMA) (Sneath & Sokal 1973). These were the coefficients of Nei & Li (1979), Jaccard (1901, cited in Armstrong *et al.* 1994), Apostol *et al.* (1993) and Excoffier *et al.* (1992). Details are provided in Chapter 3. Co-phenetic values were calculated for each dendrogram to test the goodness of fit. The data were visualized in three dimensions using Principle Co-ordinate Analysis. Both the UPGMA dendrograms and the Principle Co-ordinate Analysis was done using the computer programme NTSYS-pc (Version 1.80) of Rohlf (1993).

Results

An example of a SDS polyacrylamide gel is shown in Fig 5.1. The distinct banding patterns obtained are evident, although the individual bands often appeared slightly fuzzy. This is possibly due to enzyme activity because any delay in the different steps of the method resulted in total failure for those samples. The addition of inhibitors did not improve the resolution.

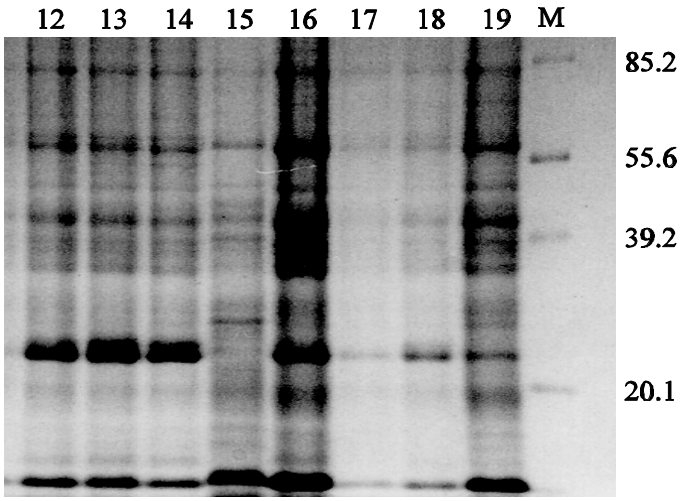


Figure 5.1 An example of a SDS- polyacrylamide gel for total protein banding patterns for *L.e. vyeboomense*. The numbers above each lane refer to the individuals sampled and the molecular weight markers are shown on the right lane.

There were few differences observed between samples. Of the 17 bands, only six were polymorphic. The pattern of distribution of these polymorphic bands is summarised in Table 5.1. No band presence or absence was restricted to a particular population of *L. elimense*. Bands which were exclusively present in or absent from a particular subspecies might be of value as markers.

Band position 7 was absent in all samples of all subspecies of *L. elimense* but present in *L. salignum*. Band position 2 was present in all samples of all subspecies of *L. elimense* but absent in *L. salignum*. Band position 3 was absent from all samples in both populations of *L.e.*

salteri but present in all samples in all populations in the other two subspecies and in most of *L. salignum* samples. Band position 5 was completely absent in *L.e. salteri*, but was mostly present in samples of *L.e. vyeboomense* and *L.e. elimense*, and was always present in *L. salignum*. Band positions 14 and 16 were mostly present in the populations but showed occasional absences throughout all the populations (Table 5.1).

Table 5.1: Polymorphic bands recorded for the total protein analysis for the study populations. *L.e. vyeboomense* is represented by (v), *L.e. salteri* Populations 1 and 2 by (s1) and (s2), respectively, *L.e. elimense* Populations 1 and 3 by (e1) and (e3), respectively,and *L. salignum* by (o). The number of individuals sampled is indicated by (n).

	v (n=9)	s1 (n=8)	s2 (n=10)	e1 (n=7)	e3 (n=9)	o (n=7)
band 2	100%	100%	100%	100%	100%	0%
band 3	100%	0%	0%	100%	100%	71%
band 5	78%	0%	0%	86%	100%	100%
band 7	0%	0%	0%	0%	0%	100%
band 14	89%	100%	90%	43%	100%	86%
band 16	67%	88%	100%	86%	100%	100%

Fig 5.2 shows the UPGMA tree generated from the coefficient of Excoffier *et al.* (1992). The co-phenetic value (r_{cs}) was between 0.86 and 0.88 for the four coefficients, which is considered statistically acceptable (Sneath & Sokal 1973). As the trees were identical for the four coefficients, only a single tree is presented. The total protein analysis was not able to separate populations within subspecies for either *L.e. elimense* or *L.e. salteri*. The two subspecies *L.e. vyeboomense* and *L.e. elimense* are not well separated, with many of the samples from the two populations of *L.e. elimense* and the population of *L.e. vyeboomense* exhibiting identical banding patterns. Below the group of identical samples of *L.e. vyeboomense* and *L.e. elimense* in Fig. 5.2 are a few individuals that appear to be different as they are on their own branch, separating out at a genetic distance of 1.47 (A) and 1.56 (B). Investigation of the banding patterns of these "non-conformist" samples revealed that they

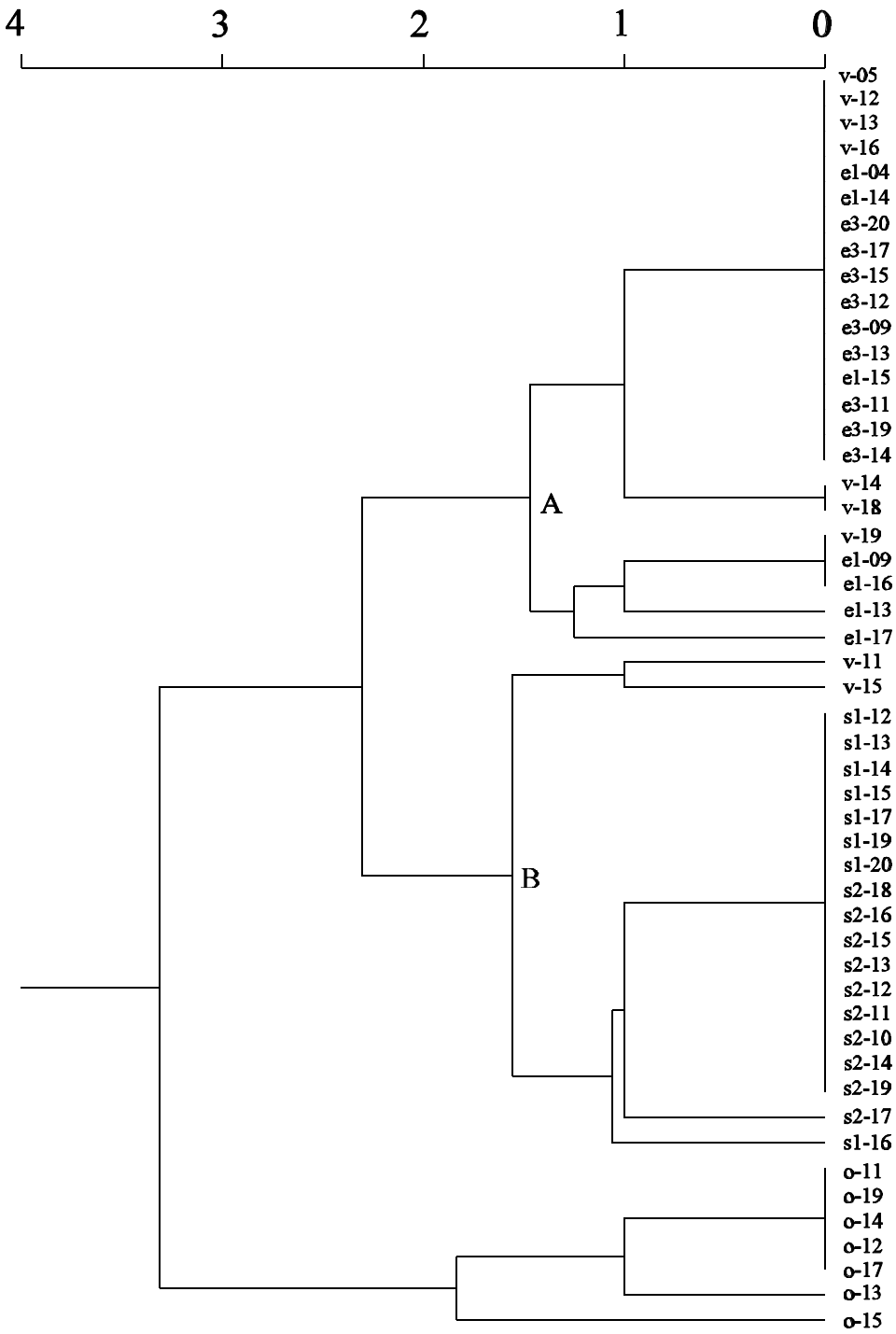


Figure 5.2 A UPGMA tree generated from the coefficient of Excoffier *et al.* (1992) for the total protein banding patterns. *L.e. vyeboomense* population is designated (v), *L.e. elimense* populations are designated e1 (Population 1), e3 (Population 3). s1 represents Population 1, *L.e. salteri*, s2 Population 2, *L.e. salteri*. o is for the common, widespread species, *L. salignum*. All four coefficients produced identical UPGMA trees.

differed in only one or two band positions from the large group of identical samples. *L.e. salteri* separated out from the other subspecies at a fairly high level of genetic distance (value 2.31). In this subspecies, there were two samples, S217 and S116, which separated markedly from the other *L.e. salteri* samples yet on investigation only differed by the absence of a single band. The common, unrelated species, *L. salignum* was shown to be well separated from all three subspecies of *L. elimense*, separating at a genetic distance of 3.31. A three dimensional visualization was possible using Principle Co-ordinate Analysis (Fig. 5.3). This distinguished *L. salignum* clearly, as well as separating out *L.e. salteri*. It appears that only a few samples were included in the Principle Co-ordinate Analysis, but large numbers of identical samples are hidden behind others. The positions containing duplicate samples could be determined from the dendrogram in Fig. 5.2.

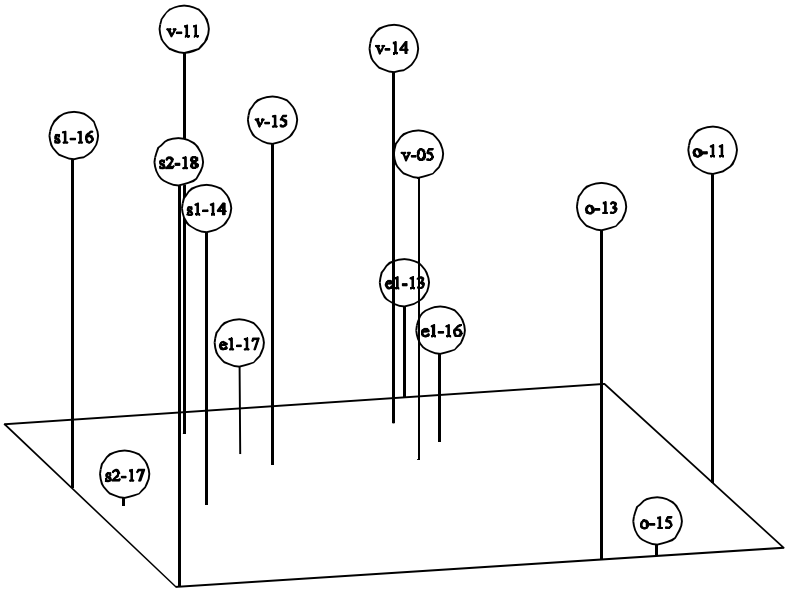


Figure 5.3 The Principle Co-ordinate Analysis from the coefficient of Excoffier *et al.* (1992) for the total protein banding patterns. *L.e. vyeboomense* population is designated (v), *L.e. elimense* populations are designated e1 (Population 1), e3 (Population 3). s1 represents Population 1, *L.e. salteri*, s2 Population 2, *L.e. salteri*. o is for the common, widespread species, *L. salignum*. The four coefficients produced identical three dimensional distributions of samples.

The groupings were not particularly tight and the three dimensional representation showed the distance of some *L.e. salteri* from each other to be no less that the distance of some *L.e.*

salteri from *L.e. elimense* or *L.e. vyeboomense*. *L. salignum* was shown to be well distanced from the *L. elimense* complex, as might be expected with prior knowledge of the existing taxonomy.

Discussion

Total proteins did produce groupings for the three subspecies of *L. elimense* in both the UPGMA trees and the Principle Co-ordinate Analysis. These groupings are based on six bands, which exhibited differences between the subspecies *L.e. salteri*, *L. salignum* and the other two subspecies of *L. elimense*. Some bands distinguished differences within the same population. If samples within a population differed only in one or two band positions, this was few in total, yet it represented a third of all polymorphic bands scored. Fairbrothers (1968) reports that differences in total proteins within populations or even between populations of the same taxon are generally not expected. Extensive within-population protein variability has, however, been exhibited in Soay sheep on the island of Hirta, St. Kilda (Bancroft *et al.* 1995).

The first and most important point in comparing these results with the RAPD banding patterns is the small number of polymorphic bands produced by the total protein analysis; only six of 17 bands scored for the three subspecies of *L. elimense* were polymorphic. In comparison, a total of 98 RAPD bands were scored for *L. elimense*, of which 96 were polymorphic.

The total protein analysis grouped the two populations of *L.e. elimense* and the population of *L.e. vyeboomense* together and yet distinguished the subspecies *L.e. salteri*, although the two *L.e. salteri* populations were grouped together. This raises the possibility that *L.e. salteri* might not be as closely related taxonomically to the other two subspecies. It is possible that it should be regarded at a different taxonomic level, sufficiently distant from the other two subspecies. Williams (1972) states that from his observations that *Leucadendron globosum* is closely related to *L.e. salteri*. This is somewhat strange an observation as he did not then include *L. globosum* as a subspecies within the species *L. elimense*. Williams (1972) states that *L. globosum* is easily distinguished from *L.e. salteri* by having "oval dark green leaves, as opposed to leaves elliptic", "pubescent stems as opposed to glabrous", and "recurved basal bracts as

somewhat recurved" (Williams 1972). All these minor variations could be accounted for by slight environmental differences experienced. Perhaps *L. globosum* is closely related taxonomically to the three subspecies of *L. elimense*, particularly to *L.e. salteri* or, alternatively, *L. globosum* and *L.e. salteri* should form a taxonomic group a little distant from the other two subspecies of *L. elimense*. Because it was not part of the aim of this project to investigate taxonomic affinities, further genetic studies will be necessary to confirm or refute this hypothesis and to determine the level of taxonomic distinctness. No suggestion of a marked difference between *L.e. salteri* and the other two subspecies is seen from the RAPD dendrograms in Fig. 3.6 or the RAPD Principle Co-ordinate Analysis in Fig.3.7 (but see Chapter 6). The UPGMA trees for the RAPD analysis generally grouped the individuals from within one population together and separated them from other populations, even within the same subspecies, although the tree shows within-population branching at fairly high levels of genetic distance relative to the distance that the populations separated from one another (See Fig. 3.6a). For example, in Fig. 3.6c where the coefficient of Apostol *et al* (1993) is used, individuals of *L.e.vyeboomense* separate from other individuals at a level of 0.32 whereas *L.e. vyeboomense*, as a subspecies only separates from the other subspecies at 0.39. Some RAPD primers showed identical genotypes between samples of *L.e. salteri* and *L.e. vyeboomense* or *L.e. elimense*. The RAPD analysis did, however, show some distinctions between *L.e. vyeboomense* and the other two subspecies which is definitely not reflected in the total protein analysis. In no way did the total protein electrophoresis support the results obtained from the RAPD analysis for *L. elimense*.

As with *L. elimense*, Boulter *et al.* (1966) used total protein band patterns to detect differences between species of legumes. They report that no between-population differences could be detected, although total protein bands differed between species in *Brassica* (Cruciferae). Boulter *et al.* (1966) pointed out that the study of total protein banding patterns assumes that the same band in different individuals represents the same protein. This is the concept of homology. The more closely related the taxa, the better the chance that such an assumption is correct and because *L. elimense* in this study is represented by three similar subspecies, this assumption should hold. In light of the lack of differences between individuals of the same species in Boulter *et al.*(1966), this would support the hypothesis that *L.e. salteri* should not

be part of *L. elimense* but rather a separate species or that the other two subspecies should be demoted in rank.

L. salignum was selected for comparison for the UPGMA analysis and it is shown as being well removed and quite distinct from the *L.e. elimense/vyeboomense* grouping. This is clearly visible in the principle co-ordination in three dimensions where *L. salignum* is distanced on more than one axis from the *L. elimense* subspecies.

A caution to consider in total protein analysis is that stress may alter the pattern of total protein electrophoresis. Burke *et al.* (1985) showed that heat shock proteins accumulated in water-stressed cotton leaves, which resulted in altered banding patterns. Previously it was thought that stress proteins occurred in such small amounts as to be visible only with autoradiography. Burke *et al.* (1985), however, observed heat protein accumulation using Coomassie blue. This is potentially a problem with the collection of leaf material which might not have been frozen quickly enough, time of day of collection, and season of collection. In this study all leaves were collected in the early morning when heat stress in unlikely to have been a factor and they were then frozen immediately. Other environmental stresses have also been found to induce the production of stress proteins (Ho & Sachs 1989), which might further complicate the method should adequate consideration not be given to this potential problem. Seasonal variation in proteins, particularly heat shock proteins, have also been recorded for some fish (Fader *et al.* 1994). If similar seasonal proteins occur in Cape Proteaceae, this would limit the validity of sampling at different times of the year. For this project, all samples were collected on the same day.

Implications for Conservation

Overall, total proteins did not readily distinguish differences between populations of *L. elimense* subspecies. This was largely due to the small number of polymorphic bands. This would not allow for any kind of priority ranking of populations for conservation, and certainly not of individuals, such as was carried out for *Serruria roxburghii*. In comparison to the RAPD analysis, total proteins can be considered a fairly crude measurement without sufficient detail for use in conservation management. In contrast, in their study on threatened *Tilapia* fish

(Nxomani *et al.* 1995) were able to distinguish differences between geographically isolated populations of *T. sparmanii*, which had conservation implications. In rare Cape Proteaceae total protein did, however, produce results that might have implications for the taxonomy of the *L. elimense* complex.

Conclusions

There has been much debate about the preferential use of different genetic techniques, the context of their particular application, and the supposed evolutionary forces affecting the observed distribution of genetic variation (Bancroft *et al.* 1995). It might be that selective pressures act differently on the different classes of markers (Petit *et al.* 1995). Total protein analysis only allowed for a crude measure of total protein differences within the individuals sampled in the present study. It did not measure variation in patterns of DNA sequences as did RAPD analysis, albeit cryptically as one does not know what the selected primer codes for or even if it codes for anything of significance. Protein analysis did, however, show marked differences between one of the subspecies, *L.e. salteri*, and the other two subspecies, which might have taxonomic implications. From the UPGMA tree, both the *L.e. salteri* and the common species, *L. salignum*, were grouped distinctly enough that should the samples have been unlabelled, it would have been clear that these two were separate groups, probably species, well distant from *L.e. vyeboomense* and *L.e. elimense*.

CHAPTER 6

A comparison of the DNA sequences of the Internal Transcribed Spacers in the three subspecies of *Leucadendron elimense* E. Phillips.(Proteaceae)

Introduction

Genetic variation, measured as polymorphisms at the DNA level, can be studied by several means, the most direct of which involves the reading of a particular sequence of nucleotides and comparing these between individuals. Such data have been used successfully for taxonomic classification and phylogenetic relationships. DNA sequencing has also been used in population studies (Weising *et al.* 1994). Richman *et al*. (1995) observed S-allele sequence diversity in populations of the horse nettle, *Solanum carolinense* (Solanaceae). Intraspecific Internal Transcribed Spacer (ITS) sequences have also been used to compare populations of the *Heuchera* group (a clade in the plant family Saxifragaceae) (Soltis & Kuzoff 1995) and disjunct allopatric populations of *Calycadenia* (Asteraceae) were investigated by Baldwin (1993) using the same method. In general, sequences evolve slowly over generations, so most populations of plants monitored over decades are not expected to show changes (Avise 1994) making sequencing inappropriate in population-based studies.

In plant studies, areas of the genome commonly sequenced include chloroplast genes such as *rbc*L and *rpo*C2 genes (See Barker 1997, Barker *et al.* 1995, Linder *et al.* 1997, Bergqvist *et al.* 1995) and ribosomal RNA such as 5S RNA gene (Kellogg & Appels 1995). The Internal Transcribed Spacer (ITS) region of the 18S-26S nuclear ribosomal DNA has been used increasingly for phylogenetic studies in angiosperms (Baldwin *et al.* 1995, Weising *et al.* 1994, Scott *et al*. 1997, Hsiao *et al.* 1998 and Soltis & Kuzoff 1995, Sang *et al.* 1995) and has proved useful in resolving relationships within families, between genera, within genera, and within species relationships . The ITS1 and ITS2 region in angiosperms is of a relatively small size, numbering under 700 base pairs including the 5.8S region. Variability is usually a result of point mutations /deletions /insertions (Baldwin *et al.* 1995). The function of this region appears to be the maturation of the nuclear RNA. A feature of the ITS region is that it is highly repeated in plant nuclear genome resulting in a high copy number, which in turn facilitates amplification, cloning and subsequent sequencing. This system has the advantage of requiring fewer samples. Baldwin *et al.* (1995) has suggested that within an interbreeding population, sexual recombination will promote nuclear DNA uniformity and hence reduce the need for repeated sampling within such a population. In addition, concerted evolution can homogenise parental genomes causing uniformity in offspring ITS sequences (Sang *et al.* 1995). This is

further assisted by the low error rate with the use of *Taq* polymerase. Baldwin *et al.* (1995) also found that in using a double stranded sequencing method (cycle sequencing) high quality ITS sequences were generated without evidence of divergent repeat-types within individuals.

In this study, the Internal Transcribed Spacer (ITS) region of the three subspecies of *L. elimense* was sequenced to assess and quantify any differences between the study populations, and between subspecies of *L. elimense*. Such differences would have implications for conservation in that the populations would then represent gene mutations in this region of the genome. This would suggest that should such differences be detected between two populations, both populations would need to be conserved to maximise and maintain the divergent genes. It might also have evolutionary significance in that such divergent populations might contain different genes yet appear morphologically similar enough to still be considered the same taxon.

Material and Methods

DNA extraction

The DNA was extracted from the samples according to the modified method of Doyle & Doyle (1990) as described in Chapter 3 for the RAPD analysis. Two samples were taken from each of the three subspecies. Where more than one study population was used, samples were taken from the different populations. It subsequently became necessary to sequence an additional two samples for *L.e. salteri* to confirm a point mutation.

DNA amplification and sequencing

A requirement for ITS sequencing is that pure DNA be used. This was obtained by using small amounts of DNA in combination with relatively few amplification cycles to reduce Polymerase Chain Reaction (PCR) drift (Baldwin *et al.* 1995). Amplification of the ITS region was carried out using a Perkin-Elmer GeneAmp, PCR System 9700 thermocycler at 96ºC for 5 minutes, followed by 30 cycles of 96ºC for 45 seconds, 50ºC for 2 minutes and 72ºC for 2.5 minutes, with a final extension of 72ºC for 5 minutes. The amplification reaction contained 10 Fmol of each of the two sequencing primers, 2.5 units Taq Polymerase (Advanced Biotechnologies), 2 mM MgCl, 50 mM KCL, 10 mM Tris-HCL (pH 8.8), 0.2mM dNTPs, and 50-60 ng DNA

in 50Fl volume. Thin walled tubes were used in combination with a heated lid on the thermocycler.

The primers, shown in Table 6.1, were selected on the advice of Dr N. Barker. The actual primers used for amplification were designed such that the 5' end sequences were the same as the Cy5 end-labelled (fluorescent) primers to be used later for the sequencing reaction. The forward primer (P1L) was synthesised with the 24mer-80 M13 forward primer at the 5'end. The reverse primer (P2R) was synthesised with the 17mer-40 M13 reverse primer at the 5' end (M.D. James pers. com.).

Table 6.1 The sequence of the primers used in the amplification.(5' - 3'). These were designed by P Weston (Royal Botanic Gardens, Sydney, Australia).

Primer	Sequence
Primer P1L	5'- CTG TAG GTG AAC CTG CGG AAG GAT C -3'
Primer P2R	5'- CTT TTC CTC CGC TTA TTG ATA -3'

Only the bases of the ITS primers are used for annealing during the initial PCR reaction, but the products then have the sequencing primers attached to the 5' ends. A low concentration of genomic DNA was used for PCR (50-60ng) so that the products would form templates and the complimentary sequences of each sequencing primer would be amplified. It is to these complimentary sequences that the Cy5 end-labelled sequencing primers (Synthetic DNA Laboratory, Department of Biochemistry, University of Cape Town) anneal during the sequencing reaction.

The PCR product was purified using a High Pure PCR Product Purification Kit (Boehringer Mannheim). Sequencing was carried out using the thermosequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech UK Ltd using the Sanger dideoxy chain termination technique with 20-50ng PCR product and 2pMol fluorescent primer. The Pharmacia ALFexpress Automated DNA Sequencer was used with 0.5 mm

spacers on 5% gels poured with Long Range gel solution (FMC Products) and run as per manufacturer's instructions.

Sequences from the two primers were assembled, aligned and compared using the Wisconsin Package (version 9.1)(Genetics Computer Group, Madison, Wisconsin). Multiple sequence analysis was performed using the PILEUP feature of this package.

Leucadendron chamelaea is grouped taxonomically in the section *Ventricosa* alongside *L. elimense* (Williams 1972) and so provided a comparison of a closely related taxon with species status. Dr N. Barker kindly supplied an additional sequence for *Leucadendron chamelaea* from his own database for comparison with those in this study.

Results

An accurate reading for up to 513 base pairs was obtained for most of the samples, of which 477 were able to be read clearly across all eight samples (Table 6.2). The sequence was identical for the two samples from the population of *L.e. vyeboomense* and the two samples from Population 1 and Population 3 of *L.e. elimense*. The two samples (S1-10, S2-47) for Population 1 and Population 2 of *L.e. salteri* were identical but showed a single base pair difference from the other two subspecies. This difference was at base 137 (Table 6.2) where cytosine was replaced by a thymine or a "probable" thymine. Two additional samples, one from each of the study populations of *L.e. salteri*, were subsequently sequenced to verify this and showed the same substitution at base 137. In most of the *L.e. salteri* samples, the forward reading of the sequence indicated a thymine but the reverse reading indicated a possible thymine or adenine. This area is difficult to interpret because of the effect of a particularly large adenine peak preceding this region. There are multiple copies of the ITS region which are not necessarily identical and which might produces a doubtful reading of a particular base. The exact reading of this base pair is not critical, suffice to accept that it is definitely not a cytosine as in *L.e. elimense* and *L.e. vyeboomense*.

Table 6.2 Comparison of the DNA sequences of the ITS region for *L. elimense* using the multiple sequence analysis from PILEUP (Genetics Computer Group). The samples are coded on the left. E2-20 is sample 20 from Population 2, *L.e. elimense*, E3-16 is sample 16 from Population 3, *L.e. elimense*, V-19 and V-16 are sample 19 and 16 from the population of *L.e. vyeboomense*. The four bases are A = adenine, C = cytosine, G = guanine, T = thymine. S1-45 and S1-10 are samples 45 and 10 from Population 1, *L.e.salteri*, S2-47 and S2-46 are sample 47 and 46 from Population 2, *L.e. salteri*. CHAM is the sequence from the *L.chamelaea* sample (N Barker, unpubl. data). The "t" represents a probable thymine at base pair 137. The * represents a deletion in the *L. chamelaea* sequence at 467, or an insertion in *L. elimense*. The missing bases at the end of the sequences are areas which could no longer be read accurately. The numbering of the bases is arbitrary for the purposes of this study and appears above each block of 50.

	1				50
E2-20	GTGATCGGGG	TGGGGTGCGG	TTGGCTCACA	CTGAGCCCCC	TGCATACCCT
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

	51				100
E2-20	TCACCCCTTT	ATGGTCGGTC	GGTCCCCGTG	GCCTCCGACC	CAACACAACA
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

Table 6.2 continued on next page...

.... Table 6.2 continued

	101				150
E2-20	TCACCCCTTT	ATGGTCGGTC	GGTCCCCGTG	GCCTCCGACC	CAACACAACA
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~ t ~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~ t ~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~T ~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~ t ~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

	151				200
E2-20	CCGTGACTTG	TGCATGGCGT	GTGCCATCCC	AAAATCCAAA	TCTTATTAAC
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~ G ~	~~~~~~~~~~

	201				250
E2-20	GACTCTCGGC	AACGGATATC	TCGGCTCTCG	CATCGATGAA	GAACGTAGCG
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

Table 6.2 continued on next page ....

.... Table 6.2 continued

	251				300
E2-20	AAATGCGATA	CTTGGTGTGA	ATTGCAGAAT	CCCGTGAACC	ATCGAGTCTT
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

	301				350
E2-20	TGAACGCAAG	TTGCGCCCTA	GGCCATCGGG	CCGAGGGCAC	GTCTGCCTGG
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

	351				400
E2-20	GCGTCACGCA	TCGCGTCGCC	CTCCCTCGAA	CACCATTGTG	GCGGTCGAGT
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

Table 6.2 continued on next page .....

Table 6.2 continued

	401				450
E2-20	GTGGAGCGGA	GATTGGCCCC	CCATTCCCCC	TGTTGGGGTG	TGGTCGGCCT
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
Cham	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

	451				500
E2-20	AAAAGGAATG	GCCCCCCGTG	TGACGAGCAC	CACGACGAGA	GGTGGTTGTA
E3-16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~
V19	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~	
V16	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-45	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	
S1-10	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~
S2-47	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~
S1-46	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~	
Cham	~~~~~~~~~~	~~~~~~~ * ~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~

Discussion

There is no difference between the ITS sequence for *L.e. vyeboomense* and *L.e. elimense* despite their geographic isolation. With ITS sequences, it is unfortunately not possible to estimate with any confidence at what time intervals point mutations are likely to occur. We do know that the coastal plain occupied by *L.e. elimense* and some of the *L.e. salteri* sites, was below sea level until the late Pleistocene, about 20 000 years ago when there was a drop in sea level of some 120m (Deacon 1979). Therefore we know that these two subspecies are no older than this, and probably fit the term "neo-endemics" as suggested by Taylor (1979). Since the

earliest collections over the past three hundred years, herbarium records show that *L.e. vyeboomense* and *L.e. elimense* were distinct, both morphologically and geographically.

The single base difference (one out of 470 base pairs) between *L.e. salteri* and the other two subspecies represents a 0.2% difference between *L.e. vyeboomense* and *L.e. elimense* on the one hand and *L.e. salteri* on the other. A comparison of the two similar subspecies, *L.e. elimense* and *L.e. vyeboomense*, with *L. chamelaea*, across the same set of 470 base pairs, showed a point substitution at base 188 (relative to Table 6.2) of adenine by guanine and a point deletion at base 467 (relative to Table 6.2). These two differences represent some 0.4% variation at the species level. In the comparison of *L.e. salteri* with *L. chamelaea*, this variation rises to 0.6% with the addition of a further substitution at base 137. Geographically, *L. chamelaea* lies as far north of *L.e. vyeboomense* as *L.e. vyeboomense*, itself, lies north of *L.e. salteri* so geographical isolation is not a consideration.

In three subspecies of *Leucadendron spissifolium* found in the Cape Flora, N. Barker (pers. com.) found no differences in the 500 to 700 base pairs for the ITS region. Here the subspecies form a geographical continuum with variations in leaf breadth (see Williams 1972). Williams (1972) suggests that *Leucadendron spissifolium* subsp. *spissifolium* is variable in morphology and that it is closely related to both *Leucadendron spissifolium* subsp. *fragrans* and *Leucadendron spissifolium* subsp. *phillipsii*. The geographical situation of *L. spissifolium* is similar to *L. elimense* yet in *L. elimense* it is seen that the subspecies, *L.e. salteri*, which lies intermediate to the other two subspecies both morphologically and geographically, shows a single base difference in a highly conserved sequence. Given the conservative nature of this region of the genome, it then becomes a taxonomic decision as to what level of difference justifies subspecies or species distinction.

Considering the total lack of variation in ITS sequences in the three subspecies of *L. spissifolium*, and the 0.4% difference between the taxonomically closely related species, *L.e. elimense*, *L.e. vyeboomense*, and *L. chamelaea*, the taxonomic status of *L.e. salteri* may need revision. The sequence differences of 0.2% between *L.e. salteri* and the other two subspecies of *L. elimense*, and of 0.6% with *L. chamelaea* are small. In terms of the rate of evolution of

ITS sequences specifically, these differences become meaningful (N.Barker pers. com.) as ITS sequences are seen to be slow to change relative to morphological species differences. From the present results it appears that *L.e. salteri* should be of intermediate species/subspecies status - some difference is shown but not as much as between other good species of Cape Proteaceae.

The difference in the ITS sequences is unlikely to be of stochastic origin as the sequence is repeated many times within the genome (King 1993). Therefore it is not the result of a sudden random event. It might, however, be hypothesised that at some time in the history of *L. elimense*, a stochastic event did result in the alteration of a single base from guanine to thymine in the ITS region in an individual of *L.e. salteri*. Despite the high copy number of both the spacers, the near uniformity of ITS paralogues would have resulted from rapid concerted evolution, charactersitic of this region (Baldwin *et al.* 1995). Thus a point mutation like this guanine/thymine change would have become fixed. Should this single base pair change have been restricted to a single individual, it might well represent a random event. Therefore it is important to note that this mutation was present in all four of the samples of *L.e. salteri* from two populations. This suggests that the altered base pair appears to be fixed in *L.e. salteri* and represents a real change with accompanying evolutionary and taxonomic implications.

In the light of the above, the subspecies *L. e. salteri*, should be viewed as a "evolutionarily significant unit". Moritz (1994) defines "Evolutionarily significant units" as being reciprocally monophyletic for mitochondrial DNA alleles and show significant divergence of allele frequencies at nuclear loci (Moritz 1994). In practise, populations might show only some of these characteristics. This has led to the term "management units" which are defined as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of the alleles (Moritz 1994). This is the more useful definition in terms of population conservation management in the short term. The evolutionarily significant unit is similar to the phylogenetic species concept of Cracraft & Prum (1988). Potential genealogies of markers could contain information from which inferences about long-term demographics could be made (Milligan *et al.* 1994). This would be of particular use in directing conservation efforts effectively."

To put the sequence variation within *L. elimense* in a family perspective, Barker (pers. com.) has indicated that within Cape members of the Proteaceae, ITS sequence differences between some genera range from about 10% between *Leucospermum* and *Leucadendron* to just under 4% between the more similar genera, *Serruria* and *Paranomus*. In contrast, ITS sequence divergence between individuals from conspecific, allopatric populations of *Calycadenia* was shown to be 3.7% (Baldwin 1993). An investigation of the ITS1 region of *Lantana camara* and *Lantana urtifolia* (Verbenaceae) showed four (out of 258) bases as variable (Scott *et al.* 1997). This represents a 1.6% between-species difference within the same genus from different continents. These two examples would suggest that the degree of sequence divergence varies from one plant group to another and is likely to be specific to each family or even each genus. Although such differences appear small and are possibly stochastic in origin, if they are constant in a taxonomic unit and are considered fixed, they might justly be considered as genetic markers.

Evidence from the sequence data that suggest that *L.e. salteri* is different from the other two subspecies is supported by the protein electrophoresis data (Chapter 5). Similarly, Soltis & Kuzoff (1995) showed that ITS sequence-based phylogenies correlated well with allozyme-based phylogenies in the *Heuchera* group. Although only crude total protein extracts (as opposed to allozymes) were examined for *L. elimense*, this still shows a strong correlation with the ITS sequence results here. In contrast, RAPD analysis (Chapter 3) shows *L.e. vyeboomense* to have the greater genetic distance from the other two subspecies. Scott *et al.* (1997) found that RAPD profiles, analysed using UPGMA and AMOVA analysis, were successful in characterising genetic variation not otherwise detected using ITS1 sequence data for varieties of *L. camara*.

Conclusions

The ITS sequence data for *L. elimense* does not directly provide any useful information for conservation. It does, however, show a small difference between *L.e.salteri* and the other two subspecies which might have implications with regard to the taxonomic status of *L.e.salteri*. The use of DNA sequence data in assessing taxa of uncertain status can have an important indirect role in conservation in that it can be valuable in adding clarity to a taxon's definition.

In general terms, phylogenetic appraisal of a threatened species can provide an understanding of the underlying evolutionary relationships (Avise 1994). In this context, Vane-Wright *et al.*(1991) suggest conservation priority be given to taxa which have made a greater evolutionary contribution in terms of present day diversity in preference to taxa which have contributed little or are of apparently recent origin.

CHAPTER 7

Summary and conclusions

Summary

The Cape flora, the vegetation of the South Western Cape Province, South Africa, is characterised by an inordinate diversity of species, many of which are endemic (Oliver *et al.* 1983). A characteristic of many of these endemic species is their restricted geographical distribution and small population numbers. This has resulted in many of them being ascribed the IUCN status "naturally rare". This flora is increasingly threatened by both agricultural and urban development, which has brought about a change of status of a large number of species from "naturally rare" to "vulnerable", "endangered" or even "extinct". The Cape Proteaceae, a characteristic component of the flora, has a particularly high number of species which apparently have always occurred naturally in small numbers and which, as a result of escalating disturbances, are under increasing threat of extinction.

Anthropogenic disturbances potentially reduce numbers in all natural plant populations, both common and rare (Leijs *et al.* 1999). The impact on the rare species is, however, often more catastrophic because they have fewer individuals initially. The probability of extinction is consequently much more likely in rare species than in common species and continued disturbances will artificially select against numerically small species (Rapoport *et al.* 1986). Conservation effort should therefore focus primarily on rare or threatened species. There are exceptionally high numbers of rare species in the Cape flora and studies on rare species are thus particularly important if any attempt is to be made to maintain its natural diversity. These naturally small populations of rare Cape Proteaceae could potentially be adequately and relatively cheaply protected by small nature reserves if it can be shown that there is no loss of genetic diversity in such populations. This provided the motivation for this thesis.

The overall aim of this thesis was to investigate levels of genetic diversity within and between populations of some rare Cape Proteaceae in comparison to a common widespread species, and to assess the implications of the results for conservation management of such genetic diversity. This represents the first such study on Cape Proteaceae.

Taxa for the study were selected to provide a representative cross section of species of different conservation status. In addition, it was felt that closely related taxa would be

preferable as this would reduce the possibility of any between-taxa differences merely being ascribed to taxonomic differences. A suite of subspecies of *Leucadendron elimense* provided suitable candidates. *L.e. vyeboomense* is a taxon restricted to a single population of about 50 plants with the IUCN status of "endangered". *L.e. salteri* is a taxon with about six populations totalling some 1 000 plants with the IUCN status of "endangered" and *L.e. elimense* is a taxon covering an area of some 10 km^2 with about 7 000 plants and is ascribed the IUCN status of "vulnerable". A population of *L. salignum*, a ubiquitous taxon found in a more or less continuous distribution throughout the Cape flora, was included for comparison as a common taxon. The only two known populations of *Serruria roxburghii*, a highly endangered taxon from a very restricted habitat is urgently in need of conservation and provided a further taxon for testing.

Morphology

A comparison of ecological and morphological characteristics of the study taxa showed few differences except in leaf size as shown in Tables 2.2 and 2.3. Measurements of leaf size ratios revealed statistically significant sexual dimorphism in all three subspecies. Largest leaf size ratios were found in male plants of *L.e. vyeboomense* and smallest leaf size ratios in female plants of *L.e. elimense*. In terms of absolute size, largest leaves were found in *L.e. salteri* male plants where the average length was 67.87 mm (Table 2.4) for leaves 6 cm from the growing tip. Leaf size ratios were found to be significantly different between subspecies and the different sexes but not between populations of the same subspecies. This result is directly applicable to the taxonomy of the species because it provides a clear morphological character, which is diagnostic in distinguishing the three subspecies.

RAPD analysis

L. elimense

RAPD analysis was successfully used to measure genetic variation within and between populations of the three subspecies of rare *L. elimense* as well the two populations of *S. roxburghii*. This is the first study in rare Cape Proteaceae to provide a quantitative measure which can be used to rank both individuals and populations in terms of conservation priority. Using five primers, genetic variation was measured as genetic distance in six study populations

of the three subspecies of *L. elimense*. The levels of genetic variability were seen to differ from subspecies to subspecies and also between populations within a subspecies. The highest level of genetic variation within *L. elimense,* measured as average genetic distance for all primers, was 0.35 in a population of *L.e. salteri*. This was above that considered high by Rossetto *et al.* (1995) in their *Grevillea* study.

Using the same formula, the lowest level of genetic variation (0.24) was seen in the only population of *L.e. vyeboomense* where the genetic variation was 30% less than for the species as a whole and certainly represents a lower level of genetic diversity. Rare bands may be of biological significance in that they may indicate the presence of rare genes, which might have considerable positive or negative implications. They might, for example, confer beneficial characteristics like disease resistance (Petit *et al.* 1997) and then should consequently be weighted in conservation considerations (Sampson *et al.* 1988). On the other hand, it cannot be discounted that rare bands might represent PCR artifacts or reflect poor quality template DNA.

Using Analysis of Molecular Variance (AMOVA) to partition the hierarchically measured variation, it was seen that most variation (75%) lay within each population, as might be expected in an obligative outcrossing species. The total band presence was high, ranging from 48% to >78% depending on the primer used.

Genetic distances between the three subspecies indicate that *L.e. vyeboomense* is most distant from *L.e. salteri* (0.39) and *L.e. elimense* (0.40). This reflects the geographical isolation of *L.e. vyeboomense*. The levels and patterns of genetic variation in the populations of *L.e. elimense* do not point to any particular population receiving preferential treatment as the mean genetic variation from all the primers was similar. In general, for *L.e. salteri* higher levels of genetic variation were seen in Population 2 than in Population 1 (See Table 3.2). Population 2 is smaller than Population 1 but contained older plants and possibly has plants from more than one age cohort.

The interpretation of the RAPD results for *L. elimense* is that there is a high level of genetic variation in the three subspecies of *L. elimense*. This means than the small populations *L.elimense*, and possibly also of other rare Cape Proteaceae, should not be regarded as genetically depauperate simply because they occur in small populations, are few in number, and/or have limited distributions. This result was unexpected in light of island biogeographical theory and recent literature. High levels of genetic diversity in rare species of plants have, however, also been found in other studies such as that of Rossetto *et al.* (1995). These, and data from the present study, largely serve to underline the fact that many ecological management decisions are based on theory rather than on empirical data. This extends to the genetic aspects of rare plant conservation and management. It also emphasizes the importance of genetic data in the study of rare plants, particularly when these relate to long term management and protection.

S. roxburghii

Having optimised the RAPD technique for *L. elimense*, it was useful to test this technique on another species. The "endangered" and restricted *S. roxburghii* provided such a test species. Levels of genetic variation were measured in the two remaining known populations and found to be comparable at 0.36 (average for five primers for Population 1) and 0.33 (average for five primers for Population 2). This is similar to the genetic variation seen in *L.e. salteri* and again, is considered high for an endangered species occurring in only two known populations on a specific substratum. The greatest significance of this result is, firstly, that it was possible to use the RAPD technique optimised for one genus on another genus successfully and, secondly, that although this species is found in only two populations in a restricted habitat, it contained high levels of genetic variation. This supports the theory that rare taxa of Cape Proteaceae are able to maintain genetic variation despite their small numbers, few populations and limited distributional ranges.

Analysis of molecular variance (AMOVA) indicated that over 90% of the variation lay between individuals within populations. A genetic distance matrix was generated between individuals within each population to assist with selection of individuals with relatively high levels of genetic variation for possible translocation should this become necessary for their conservation.

Interestingly enough these results, along with the UPGMA trees, indicated that individuals from the two populations did not exhibit RAPD profiles that were distinguishable. This suggests that in terms of genetics, there is probably gene flow between the populations via pollen or seed vectors. Either population would thus be suitable for conservation as most individuals hold sufficient genetic variation to be representative of the species. Indeed the two populations could well be regarded genetically as a single entity or population.

The successful measurement of genetic variation in *S. roxburghii* showed that RAPD technique is directly applicable to other members of the family. This offers the opportunity of comparing results from a wide range of species and also different genera by using identical reaction conditions. It is recommended that measurements be made of levels of genetic variation in a selection of other rare and common Cape Proteaceae. In addition, the comparison of the RAPD results with microsatellites, should suitable primers become available, would give addition credibility to these results.

L. salignum

A comparison between the rare study taxa and the common *L. salignum* revealed that in the specific study population of *L. salignum* the mean level of genetic variation at 0.30, was within the range exhibited by the three subspecies of *L. elimense* and below the levels measured for *S. roxburghii*. The following three assumptions need to be made at this point; that the study population is representative of the genetic variation found in *L. salignum* as a whole; that this level of genetic variation is typical for a common Cape Proteaceae with a widespread distribution; and that this level of variation represents an adequate level of genetic variation for long term fitness. If these assumptions hold, the levels of genetic variation measured in all but *L.e. vyeboomense* might be considered high.

Conclusions pertaining to RAPD methodology

From this study, a number of pertinent comments and conclusions can be made pertaining to the RAPD technique. It proved relatively simple to learn this method without any supervision or technical guidance. It was also relatively cheap and quick to produce empirical, quantitative results.

The use of the three different extraction techniques showed, however, that it is important to select one appropriate for the organism under consideration. Only the method of Doyle & Doyle (1990) produced sufficient relatively clean DNA that allowed amplification of samples from the Proteaceae screened. It would thus be expected that the different extraction techniques would influence the quality of the PCR product, possibly to the extent that different bands might appear for the same sample. It was also observed that magnesium chloride concentration influenced the PCR reaction, with different banding patterns resulting from different concentrations. This potentially has implications for ensuring precision in dispensing aliquots in multiple PCR reactions. It also argues in favour of the use of a master mix, as was used in the present study.

Throughout the study, reaction conditions were stringently controlled and faithfully reproduced, resulting in a high degree of uniformity in replicate banding patterns (See Fig. 3.1). This uniformity was restricted to the banding pattern, not banding intensity. Banding intensity is affected by the amount of template DNA and the amount of amplified sample loaded onto the gel. This dispels the idea that RAPD results are not reproducible (as indicated by Elsworth *et al.* 1993), merely that they require accurate handling (as supported by Weeden *et al.* 1993). Different primers produced very different banding patterns in terms of the number of bands, arrangement of bands and, most importantly, degree of variability of banding patterns among samples. In comparing the three populations of *L.e. elimense*, the mean genetic variation from all the primers was similar. An assumption in RAPD studies is that the RAPD profiles are a representative sample of the genome. Similar results across more than one primer lent confidence to this assumption. It was, however, shown that for one primer only four genotypes could be distinguished as opposed to 15 for the same population by all the other primers. It would, therefore, be useful to verify whether such a primer is indeed reflecting low variability and that this is not merely a function of the particular sequence of that random primer. Ideally, as large a number of primers as possible should be screened to identify some for high and some for low variability.

Along the same line of argument is the selection of the coefficient of genetic distance, which is used as the measure of genetic variation. The term "high level of genetic variability" is

essentially a measure of genetic dissimilarity. This, to some extent, is dependant on the selected coefficient of dissimilarity, which limits its usefulness in comparison between studies. For the purposes of this study, coefficients were selected to conform to those used by Rossetto *et al.* (1995). Even with the two coefficients used here, it was possible to manipulate the results, depending on the number of common bands versus the number of unique bands. The choice of RAPD primer in combination with the choice of coefficient of RAPD genetic variation can consequently produce significantly different results. This serves to emphasise that RAPD results must be interpreted in the correct context and that relative values should be ascribed rather than absolute measurements of genetic variation. This also limits the value of comparisons between studies.

In the fruitfly, *Drosophila,* there is a good correlation between genetic distance and the degree of isolation of populations (Harrison 1991). This has been extended to provide a threshold value above which populations are then considered separate species. This correlation is, however, not seen to hold for all species. In pocket gophers, *Thomomys,* speciation (measured as reproductive isolation) is unrelated to levels of genetic differentiation (Hafner *et al.* 1987). In cultivation, *Leucadendron spp.* readily form hybrids suggesting no well developed reproductive isolation. This suggests that the use of genetic distance should be confined to measuring genetic variation and should not be used to make inferences about reproductive isolation in Cape Proteaceae.

While this study contributes to the understanding of the genetic variation of such small populations of rare Cape Proteaceae, the genetic variation represented in each small population might not be representative of the species as a whole (Peakall & Sydes 1996). This is less of a problem where there are only few populations or few remaining plants and the number sampled then approaches the whole species. It is, however, still important that as many of the small populations as possible within a taxon be conserved.

Total Proteins

Total protein gel electrophoresis of *L. elimense,* presented as UPGMA dendrograms, showed a close similarity between samples from *L.e. elimense* and *L.e. vyeboomense*. Indeed, many of

the banding patterns were identical. The banding patterns for *L.e. salteri*, however, grouped away from the other two subspecies, separating at a level of dissimilarity of 2.31. *L. salignum*, a different species separated at a level of 3.31. Although total protein results are generally considered crude, these results do have taxonomic implications. While the protein results do not have any use directly for conservation management they suggest that *L.e. salteri* should possibly not be grouped at the subspecies level with *L.e. vyeboomense* and *L.e. elimense*.

DNA Sequencing

Sequencing of the Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA for the three subspecies of *L. elimense* supported the results based on total proteins. The ITS sequences between samples from populations of *L.e. elimense* and *L.e. vyeboomense* were identical, whereas a point mutation in both populations of *L.e. salteri*, made for a 0.2% difference between this taxon and *L.e. elimense* and *L.e. vyeboomense*. Generally within other species of Proteaceae, the ITS region is highly conserved so one might expect subspecies to exhibit the same sequence. The implications of a 0.2% difference in *L.e. salteri* suggests that it should possibly be elevated to a status greater than subspecies level. The two similar *L. elimense* subspecies sequences, however, differed from another related species, *L. chamelaea* by 0.4%. If subspecies exhibit identical sequences and differences of 0.4% represent different species, it is then questionable as to the exact position of *L.e. salteri*, which falls between the two.

Whether *L.e. salteri* is elevated in status or alternatively, the other two subspecies are reduced to varieties or a single subspecies, is a taxonomic decision beyond the scope of this thesis. In contradiction of the protein and sequence results, the RAPD results indicate a smaller genetic distance between *L.e. elimense* and *L.e. salteri* than between these two taxa and *L.e. vyeboomense*. It is possible that the RAPD technique is simply measuring anonymous, arbitrary sequences subjected to ecological pressures whereas the ITS sequences and proteins are restricted to specific, well defined areas of the genome. The RAPD markers might well be complimentary to the ITS sequence data. The different molecular markers are known to vary and to display different amounts of genetic variation and different modes of inheritance (Ouborg *et al*. 1999).

The information obtained from ITS sequencing, along with the total protein results, did not provide a measure of genetic variation nor could these methods be used to rank populations in any way that was directly relevant to their conservation management. The information did, however, highlight taxonomic anomalies that might be indirectly applicable to conservation management (Avise 1995). ITS sequences could also be of use in clarifying genetic species where more than one morph occurs. Although it is important to identify genetic or evolutionary species, morphological variation within such a unit is important and deserves recognition and conservation as Karron (1997) warns that resident phenotypes show adaptive advantages in distinct habitats and do not compete successfully in habitats where markedly different phenotypes dominate.

Maintaining genetic variation in small populations of rare Cape Proteaceae.

The desirability of a single large reserve versus several small reserves has been debated for a number of years with no definitive conclusions except that each case should be assessed independently. The debate drew largely on the theory of island biogeography of MacArthur & Wilson (1967), where it was hypothesised that a minimum viable population size criterion had to be met to sustain long term fecundity. This hypothesis consequently resulted in general recommendations for larger reserves. A weakness with such ecological recommendations is that they are based on theoretical assumptions which do not necessarily hold in practice. Critical appraisal of the issues involved is well covered in standard textbooks (See, for example, Primack 1998). Jansen (1983) emphasized that small islands of vegetation are poorly analogous to oceanic islands which undermined the value of using island biogeographical theory or its application. In addition, there has been increasing empirical evidence to show that the theory favouring large reserves is not necessarily the best answer for conservation (Simberloff & Abele, 1982) and that for a variety of taxa, for a number of different habitats, and for a wide range of sizes of biota, either there is no clear best strategy, or several small sites are better than one large site. Such an example is the conservation of limestone pavements in the Yorkshire Dales (Higgs & Usher 1980) where many small sites would better preserve diversity. The preservation of many small ancient remnants of woodlands in central England (Game & Peterken 1984), both in and out of official reserves, has successfully conserved representative examples of this woodland with a variety of environmental variables. In a study

on habitat insularisation and nature reserves, Simberloff & Gotelli (1984) concluded that groups of small sites tended to contain more species than a single large site, no species were excluded from a small site, relatively speaking small sites contained more species than large sites, and most importantly in terms of this thesis, rare species occur more often in small sites. Very small areas of less than 1 000 ha have been shown to be adequate for preservation of lowland tropical rain forests (Turner & Corlett 1996) while intermediate sized reserves also have their place and are, for example, proposed for forest patches by Zuidema *et al.*(1996).Using computer simulation models to determine critical population size, Shaffer & Samson (1985) showed that there was no simple, universal critical population size that, if achieved, removed area requirements or the effects of subdivision. These examples support the need for reserves of different sizes, depending on the target species. As rare species are often the target or priority species in the formation of reserves, it is worth observing the success reported by Miller & Bratton (1987) who used the distribution of rare and endangered species to design a system of effective and viable nature reserves in which size was not the foremost criterion.

From my previous work (Tansley 1988), there was strong evidence that many rare Cape Proteaceae were restricted in geographical distribution and commonly had few populations or numerically few plants. Large reserves would capture very few such taxa whereas several small reserves would capture more. The best scenario might be many very small reserves (even unofficial reserves) targeting key taxa. Petit *et al.* (1997) pointed out in their study on argan trees in Morocco, that officially designated reserves were not the major component in conservation strategy but rather small areas in private ownership. In the Cape too, protection by private land owners is a potential option for rare Cape Proteaceae. If an adequate system of small private nature reserves is drawn up, most could function in private ownership with minimal management input. A working example of this is seen on the farm Eensaamheid, near Paarl in the southwestern Cape where the farmer has fenced some 10 ha alongside an existing reserve of 8 ha to further increase the protection of the rare geometric tortoise, *Psammobatis geometricus*, and in so doing has successfully protected small populations of three rare Cape Proteaceae, *Serruria incrassata, Leucadendron verticillatum* and *Serruria gracilis* (pers. obs.). A number of rare species from other families have subsequently also been found on this

site. Education of farmers and other such land owners consequently becomes a priority. This approach should be two fold; first to impress on land owners the importance of preventing the loss of the small populations of rare species, and second, educating them to help manage the rare species optimally. Education for management has many advantages. As the owner becomes the custodian, sense of pride, concern and protectiveness are developed for the resident rare species. Furthermore, as the owner is usually on site most of the time, they are in a position to monitor populations frequently and observe changes or potential threats. An additional benefit is that, in the long term, the fiscal responsibility to the state, with its limited resources, becomes minimal.

While it has in the past generally been accepted that small populations will contain low levels of genetic variation, there is increasing evidence to the contrary. Within the Cape Proteaceae there are many rare taxa (152), almost all of which are restricted in at least one of population numbers, geographical distribution, or total plant count. Although I have only measured genetic variation in a few rare taxa, these all show levels of genetic variation similar to that of a common species despite their known historically restricted distribution and numerically few populations and numbers. These high levels of genetic variation in small populations of rare Cape Proteaceae is important both in terms of their conservation and how they achieve and maintain such unexpectedly high level of genetic variation.

If a population has always occurred in small numbers, it may be adapted to its rare condition (Milligan *et al.* 1994). How small populations maintain high levels of genetic variation equivalent to those seen in common taxa is, however, not clear. It would seem that there are a number of features of small rare populations that might play a role. These include cyclic chaotic fluctuations in numbers, reduced inbreeding, edge effect, and large, long-lived seed stores. These are considered below.

Are rare Cape Proteaceae chaotic species?

Chaotic populations have been defined as rare populations which flush occasionally and then crash back to a low level (Vandermeer 1982). Populations used in the present study have been shown to exhibit a history of chaotic, dynamic fluctuations over time. This is not restricted to

the study taxa, but is a feature of many rare Cape Proteaceae. Boucher (1981) observed identical fluctuations in the rare and very restricted Proteaceae, *Orothamnus zeyheri*. Many Cape Proteaceae have been shown to meet the requirements for endogenous chaos, particularly those which survive fire only as seeds in single cohort populations, with flowers borne terminally on a single seasons growth, growing in large, relatively uniform stands with limited dispersal (Bond *et al*. 1995). *L. elimense* and *S. roxburghii* meet all of these criteria. It has been suggested that chaos may engender adaptations that coincidentally protect chronically rare species from extinction (Rosenzweig & Lomolino 1997). It might even serve to maintain levels of genetic variation above those generally expected in small populations. Whether in fact this influences levels of genetic diversity positively or, in contrast, whether genetic bottlenecks result when populations crash is open to question. Genetic bottlenecks are thought to reduce allelic diversity in small populations (Karron 1997) and elevate the inbreeding coefficient (Merilä *et al.* 1996) yet actual evidence from a simulation study on *Drosophila* showed that a single severe bottleneck resulted in an increase in genetic variation (Carson 1990). Another example is seen in the now rare one-horned rhino in Nepal which was reduced from thousands to what might be considered a bottleneck of 80 individuals, all of which exhibited exceptionally high levels of allozyme heterozygosity (Avise 1994). As this is a recent event in evolutionary time, there have not been enough generations at these low numbers to allow the levels of genetic variation to drop. In contrast, many rare Cape Proteaceae have been recorded with restricted numbers and distributions since the arrival of the European settlers, 400 years ago.

Marked cyclic fluctuations in population numbers are well documented for *L.e. salteri*, as discussed in Chapter 2. This becomes significant in light of the fact that levels of genetic variation were highest at 0.39 in *L.e. salteri* when compared to other taxa studied. The management of such fluctuating taxa would require considerable careful planning as different genetic systems might respond differently to fluctuations in population size (Harrison 1991).

Breeding systems in rare Cape Proteaceae

One of the most important considerations affecting genetic variability is inbreeding, which might decrease genetic variation. Small populations have long been thought to be associated with increased inbreeding relative to large populations (Milligan *et al.* 1994). Kunin (1997a)

provides many arguments supporting the increased self-compatibility or asexual breeding system in rare plant species Self-compatible species have been associated with lower allozyme variation than obligative outcrossing species (Babbel & Selander 1974). Is inbreeding seen in rare Cape Proteaceae or how is it avoided? If they are able to avoid inbreeding, this would assist in maintaining the high levels of genetic variation. Steiner (1987) estimates that 77.6% of the Cape flora are hermaphrodite, and 6.6% are dioecious. This is an exceptionally high rate of dioecy, far greater than is seen for any other mainland flora, particularly relative to other Mediterranean type floras. The high hermaphrodite levels support Kunin (1997a) in his theory that narrowly endemic species, such as those typifying the Cape flora, contain a disproportionate number of self-compatible species. In the genus *Protea*, with their bisexual flowers, 10 out of 11 species tested, proved to be self compatible. *Leucospermum*, also hermaphrodite, appears to be predominately self compatible and self pollination is possible in most species (Steiner 1987). It is only the breeding system in *Leucadendron spp.* that avoids self fertilisation by dioecy. Such a completely outcrossing mating system can only serve to promote gene flow (Krauss 1994, 1997). *Leucadendron* makes up 14% of the dioecious taxa in the Cape flora (Steiner 1987). An assessment of rare or threatened Proteaceae taxa genus by genus, however, does not show any correlation between rarity and dioecy. The genus *Leucadendron* shows 45% of the taxa being rare or threatened while the bisexual genera *Mimetes* shows 77% and *Sorocephalus* 82% at the top end of the scale, and *Protea* shows 17% at the other extreme. This does not exclude the possibility that small populations of rare taxa in bisexual genera do manage to avoid self-pollination by mechanisms such as protandry.

Ellstrand & Elam (1993) consider that an increase in population size is not necessarily linked to a decrease in the chance of inbreeding and that other factors such as the environment also play a role in preventing inbreeding. The dissected and varied environment of the Cape floral kingdom could well play an influential role here. In *Acer saccarum* (Aceraceae), forest fragmentation has reduced population sizes yet there is no evidence of any loss of within-population genetic variation (Young *et al.* 1993). It is thought that there is an accompanying increase in inter-population gene flow. It is possible that pollen and seed vectors are maintaining gene flow similarly in *L. elimense*. This is further supported by the dominant presence of *L. elimense* in all three subspecies in all populations. This is not expected of rare

plants which are typically cryptic and hard to see. In order for the plant to be seen by pollinators it is necessary for the plant to be highly visible for pollinator visitation and effect (Kunin 1997a). This is the case for most of the rare Cape *Leucadendron spp*. which are showy, highly visible, and usually brightly coloured.

Small populations have large edges

Mathematics deems that a small volume has a perimeter which is large relative to a large volume. Small populations of rare plants typically occupy small areas and so have a relatively large edge which is associated with increased interaction with the surrounding environment and disturbances relative to larger populations (Jansen 1983). In addition, the impact of any outside disturbance will be more marked and will penetrate into a greater percentage of the area of the population than would have occurred for the same disturbance in a larger population. This edge effect, with its greater diversity of environmental conditions (Kunin 1997b) could preferentially select for plants with high levels of genetic variation and which are able to cope with environmental fluctuations. This could be a strong factor for preserving genetic variation in small populations. The alternative would simply be local extinction.

Seed stores in rare Cape Proteaceae

In the Proteaceae, large seed reserves accumulate under the soil in a population. This is particularly apparent in species killed by fire, which then are replaced simultaneously by thousands of seedlings from soil-stored seed reserves. Extant living plants are thus only part of the gene pool and a large genetic reserve lies in buried seed stores, the size and long term viability of which is unknown. Evidence for this was provided by populations of *L.e. salteri* which were known to be exterminated by burning and grazing, and yet three years later were replaced by a flush of young two-year-old seedlings, often numbering more than the original mature population (Pool & Smuts 1993, pers. obs.). Evidence from a rare chaparral shrub, *Fremontodendron decumbens* (Sterculiaceae), suggested that it is possible for seed banks to retain a 50% viability level for more than 21 years (Boyd & Serafini 1992). This species is also killed by fire and a lack of fire is cited as a possible cause of lack of recruitment via sexual reproduction. *L. elimense* also is killed by fire, followed by recruitment of new genetically different individuals. Seeds can accumulate over a number of years, all with a unique genome

until a fire clears the adult plants and then a new cohort will germinate. This is potentially a method of maintaining high levels of genetic variability in what appears to be a system of small populations.

Conclusion

In an African context, Siegfried (1984) suggests that conservation priority and resources be directed towards species that play key functional roles in ecological processes or are of economic importance. The Cape Proteaceae meet both these criteria in that they are a dominant element in fynbos ecosystems and are extensively exploited in the wild by the cut flower industry. This thesis has shown for the first time that the RAPD technique can be used to assess the genetic variation in rare Cape Proteaceae and that it can be a valuable tool in the selection for conservation of plants or populations with high levels of genetic variation.

The single most important result from this thesis is that the rare taxa examined, exhibited unexpectedly high levels of genetic variation that were comparable to a common taxon, *L. salignum*, and higher than levels measured in a rare Australian Proteaceae. *L.e. vyeboomense*, a highly endangered and restricted taxon, exhibited a significant loss of variation relative to the other two subspecies. This taxon is in need of immediate management if it is to survive in its natural habitat.

The high levels of genetic variation recorded is strong evidence to support the theory that present day rare Cape Proteaceae were historically restricted, hence they have probably always been naturally rare, narrow endemic species. They are characterised by few populations, relatively few plants in total, and a limited geographical range. It is apparent that these small populations are somehow able maintain high levels of genetic variation with a minimum viable population size at extremely low numbers by employing strategies other than simply being numerous. As such, a system of small nature reserves is unlikely to deplete the gene pool and small nature reserves, even in private ownership are thus considered to be a viable solution for the conservation of rare Proteaceae.

Although at this stage only the three subspecies of *L. elimense*, *L. salignum* and *S. roxburghii* have been investigated, it is strongly recommended that other representatives from both rare and common taxa of all the genera of the Proteaceae be investigated to provide some empirical measurements of their genetic variation. This applies particularly those taxa with different breeding systems in which mechanisms that promote gene flow might be less.

Finally, sadly, it remains that rare and endangered plants under threat of extinction are a symptom of an environment in distress and the need for generally improved conservation management measures. This is the underlying message from the rare Cape Proteaceae.

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