SPECIES DELIMITATION AND ASSESSMENT OF GENETIC VARIABILITY IN *CARPOBROTUS* (AIZOACEAE): EVIDENCE FROM AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biodiversity and Conservation Biology, University of the Western Cape.

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ABSTRACT

SPECIES DELIMITATION AND ASSESSMENT OF GENETIC VARIABILITY IN *CARPOBROTUS* (AIZOACEAE): EVIDENCE FROM AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

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M.Sc. thesis, Department of Biodiversity and Conservation Biology, University of the Western Cape.

Species of *Carpobrotus* (Aizoaceae: Ruschioideae) are widely spread throughout South Africa and are commonly used for their medicinal properties. The genus consists of 13 species of which seven occur in South Africa, five in Australia and one in Chile. A number of investigations on South African species have demonstrated that some taxa exhibit anti-microbial activity. Using AFLPs (amplified fragment length polymorphisms) this study was undertaken with the primary aim of resolving relationships between *Carpobrotus* species in South Africa, and determining species limits by assessing their genetic distinctness. In addition, levels of genetic variability among populations of two South African species, *C. acinaciformis* and *C. edulis* were assessed in more detail. AFLPs were not able to resolve conflict surrounding species boundaries, perhaps due to extensive hybridization. However, major groupings within South African species were identified, although no geographical structuring of these populations was evident. From a bio-prospecting perspective caution needs to be taken when sampling *Carpobrotus* due to difficulty in identifying species possibly as a result of hybridization.

May 2004

DECLARATION

I declare that "*Species delimitation and assessment of genetic variability in Carpobrotus (Aizoaceae): evidence from amplified fragment length polymorphisms*" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Angeline Khunou

May 2004

Signed:

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Chapter 1 - Introduction

South African plant diversity:

South Africa is world renowned for its diversity of plant life. Comprising some 18 000 species, more plant species occur here than in any other region of comparable size. Within South Africa an estimated 9 000 vascular plant species (of which ca. 70% are endemic) are found in the Cape Floristic Region (CFR). The species richness of this region is remarkable for a temperate zone and compares favourably with areas of similar size in the wet neotropics (Goldblatt & Manning 2000). The composition of the Cape flora is unusual compared with floras of other parts of the world, the most species-rich families include Aizoaceae, Ericaceae, Iridaceae, Proteaceae and Restionaceae, which do not contribute substantially to any other flora on earth (Goldblatt & Manning 2000).

Succulent diversity - In South Africa succulents make up approximately 20 per cent of all recorded plant species (Van Jaarsveld *et al.* 2000). The areas most rich in succulent diversity are the western, south-central and eastern parts of the country (Van Jaarsveld *et al.* 2000). However, the highest concentrations of succulents are found in the semi-arid regions subject to winter rainfall, where families like Aizoaceae, Asclepiadaceae and Crassulaceae are well represented. The areas of winter rainfall include the Bushmanland, Fynbos, Knersvlakte, Namaqualand, Richtersveld, Sandveld and Tanqua Karoo regions (Van Jaarsveld *et al.* 2000).

The succulent diversity in South Africa is comprised of succulents from two distinct floral kingdoms: the temperate Cape flora in the south, and the palaeotropical flora in the north. It is believed that the current rich diversity of succulents in South Africa is due to a long history of aridity, enhanced by topographic and geological complexity, resulting in locally dry sites and a variety of soil types (Van Jaarsveld *et al.* 2000). One other important reason for high succulent plant diversity is the seasonality of rainfall. Sixty five per cent of all South Africa's succulent plants occur in the winter-rainfall region. With rainfall in excess of 400 mm per annum the region is dominated by fynbos, a unique vegetation type characterised by fine-leaved sclerophyllous

shrubs. Where annual rainfall is less than 400 mm (the Succulent Karoo specifically) the region is dominated by dwarf succulent shrubland (Van Jaarsveld *et al.* 2000).

Aizoaceae - Comprising 127 genera and ca. 2500 species (Hartmann 1991), Aizoaceae are the second largest family in the southern African flora (Goldblatt 1978), and the fourth largest family in the CFR (Goldblatt & Manning 2000). The family includes both leaf and stem succulents and it is most abundant in the Namaqualand/south-western Namibia region that lies to the north of the CFR. The family consists predominantly of succulent, annual to perennial herbs, subshrubs or shrubs and is distributed in arid parts of the tropical and subtropical zones of both hemispheres, with the main centre of endemism in southern Africa (Hartmann 1991). Although species of Aizoaceae are found in both the winter and summer rainfall regions, the majority are found in the winter rainfall region.

Characterised by the possession of epidermal bladder cells, Aizoaceae are one of 29 families belonging to the order Caryophyllales (Cuénoud *et al.* 2002; APG 2003). Other major families in this order include Cactaceae, Caryophyllaceae. Molluginaceae, Nyctaginaceae and Phytolaccaceae. The most recent classification of Aizoaceae by Hartmann (2001) recognised five subfamilies namely, Aizooideae, Sesuvioideae, Tetragonioideae, Mesembryanthemoideae and Ruschioideae. Phylogenetic reconstruction of the family using DNA sequence data from two plastid data sets for 91 taxa (Klak *et al.* 2003) has shown three of the five subfamilies to be monophyletic (Mesembryanthemoideae, Ruschioideae and Sesuvioideae). However, Tetragonioideae are polyphyletic with one of its constituent genera placed as sister to the Sesuvioideae and the remainder of its representatives included in the study are embedded within the Aizooideae. With regards to relationships among the subfamilies, Sesuvioideae were resolved, as sister to the remainder of the family; Tetragonioideae and Aizoodeae together were monophyletic (without *Tribulocarpus* S. Moore) and sister to a clade comprised of the Ruschioideae and Mesembryanthemoideae. The latter two subfamilies were each monophyletic and together comprised the terminal clade (Klak *et al.* 2003; see Figure 1.1).

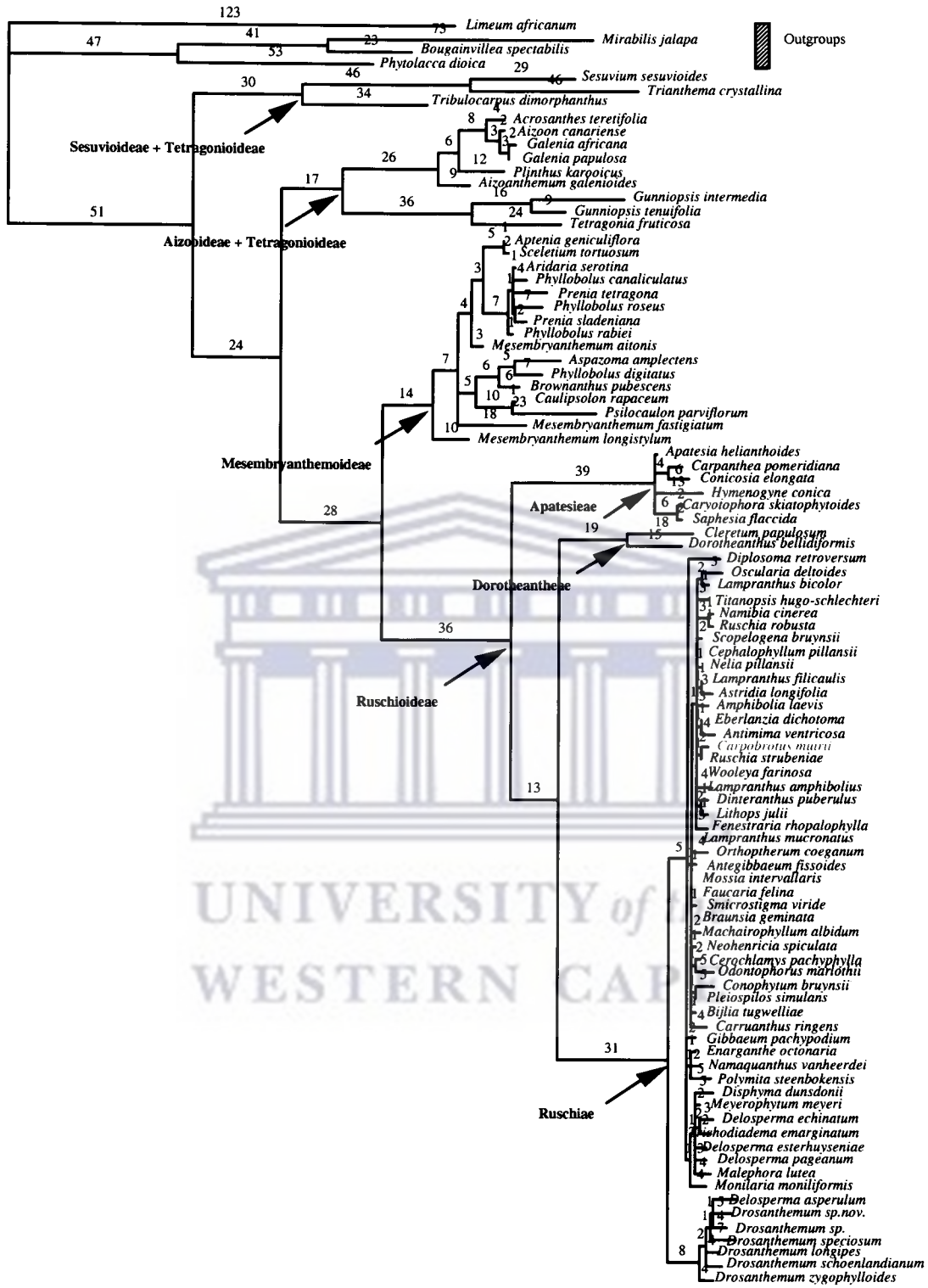


Figure 1.1 Phylogenetic reconstruction of the family Aizoaceae based on two plastid data sets (after Klak *et al.* 2003) with *Carpobrotus* highlighted. Numbers shown above the branches correspond to branch lengths.

Ruschioideae - The Ruschioideae are by far the most species-rich subfamily of Aizoaceae, with 111 genera and *ca.* 1585 described species (Chesselet *et al.* 2002). Most of the members are leafy succulent subshrubs, but a diversity of succulent growth forms occurs including 'stone plants' to tree like shrubs (Chesselet *et al.* 2002). Hartmann (1988, 1991 and 1998) and Chesselet *et al.* (2002) have proposed generic affinities within the Ruschioideae. Hartmann (1988, 1991 and 1998) subdivided the subfamily into 11 informal groups based upon fruit structure. These 11 groups are named Apatesia, Bergeranthus, Cleretum, Delosperma, Dracophilus, Lampranthus, Leipoldtia, Mitrophyllum, Ruschia, Stomatium and Titanopsis. Chesselet *et al.* (2002) proposed a more conservative, but formal tribal classification for the subfamily based upon floral nectary characteristics (Table 1). Apatesieae was proposed in place of the Apatesia group, Dorotheantheae was proposed instead of the Cleretum group, and Delospermeae was proposed to include the Delosperma group, Stomatium group and Bergeranthus group. The last tribe, Ruschieae, was proposed to accommodate the remaining groups classified by Hartmann. The most recent tribal classification within the Ruschioideae by Klak *et al.* (2003) was based on DNA sequence data, and differed little from that of Chesselet *et al.* (2002). Limited resolution was achieved within the Ruschioideae, and thus only three clades were identified and recognised as formal tribes. Two of the four tribes (Apatesieae, Dorotheantheae) as circumscribed by Chesselet *et al.* (2002) were recognised. The third tribe, Ruschiae, *sensu* Klak *et al.* (2003), was enlarged to incorporate both Ruschieae and Delospermeae of Chesselet *et al.* (2002). These tribal arrangements within the Ruschioideae are summarised in Table 1.1.

Table 1.1 Comparison of groupings and tribes within subfamily Ruschioideae *sensu* Hartmann (1988, 1991 and 1998), Chesselet (2002) and Klak *et al.* (2003).

Proposed groupings *sensu* Hartmann (1988, 1991 and 1998)	Proposed tribal circumscription *sensu* Chesselet (2002)	Proposed tribal circumscription *sensu* Klak *et al.* (2003)
Apatesia	Apatesieae	Apatesieae
Cleretum	Dorotheantheae	Dorotheantheae
Bergeranthus Delosperma Stomatium	Delospermeae	Ruschiae
Dracophilus Eberlanzia Lampranthus Leipoldtia Mitrophyllum Titanopsis	Ruschieae	

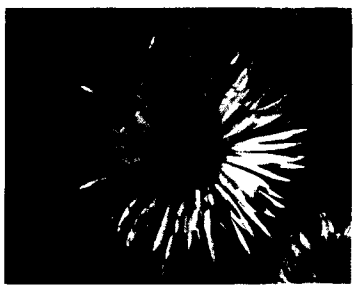
Many taxa belonging to the Ruschiae (*sensu* Klak *et al.* 2003) received considerable attention due to their horticultural and bioprospecting potential. This thesis concentrates on one such genus belonging to this tribe – *Carpobrotus* N.E.Br. The genus will form the focus of this study and is discussed in more detail below.

Carpobrotus:

Geographical distribution - The genus *Carpobrotus* consists of 13 species of which seven occur in Southern Africa. These indigenous taxa are *C. edulis* (L.) L. Bolus subsp. *edulis*, *C. acinaciformis* (L.) L. Bolus, *C. deliciosus* (L. Bolus) L. Bolus, *C. dimidiatus* (Haw.) L. Bolus, *C. mellei* (L. Bolus) L. Bolus, *C. muirii* (L. Bolus) L. Bolus and *C. quadrifidus* L. Bolus (photographs of representatives of the genus are shown in Figure 1.2). Within South Africa *C. edulis* spans both the Eastern and Western Cape. *Carpobrotus deliciosus* and *C. dimidiatus* are found in the Eastern Cape with *C. dimidiatus* extending into Mozambique, whereas the remaining species

are found only in the Western Cape. Most species of *Carpobrotus* occur along the coastline of South Africa with the exception of *C. acinaciformis* and *C. edulis,* which both occur inland and along the coast.

C. dimidiatus



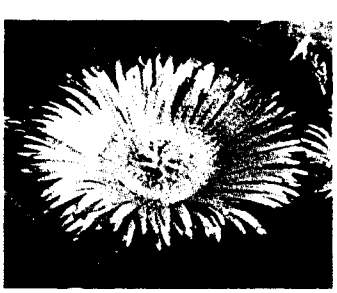
C. quadrifidus

C. acinaciformis

C. edulis

C. edulis X *C. mellei*

C. deliciosus



C. mellei

Figure 1.2 South Africa representatives of the genus *Carpobrotus*

Carpobrotus species are generally found in sandveld and fynbos habitats. The sandveld region includes the coastal flats between the Atlantic coast and the Namaqualand Hills. It stretches from Alexander Bay in the north to the mouth of the Olifants River in the South (Hilton-Taylor 1996). This habitat is very sandy and flat, interrupted by rocky ridges. The sandveld sands are nutrient poor, and are dominated by scattered low shrubs and small trees. *Carpobrotus acinaciformis* and *C. edulis* dominate the vegetation here, which is typically low and open. The rainfall is mainly in winter receiving less than 50 mm per annum in the north to 150 mm per annum in the south (Hilton-Taylor 1996). The fynbos region stretches from Nieuwoudtville in the North, along the Cederberg, southwards to the Hottentots Holland mountains and the Cape Peninsula (Van Jaarsveld *et al.* 2000). Within the coastal fynbos and strandveld vegetation, species like *C. edulis*, *C. acinaciformis*, *C. muirii* and *C. quadrifidus* are particularly abundant. The fynbos habitat has a mineral-deprived quartzitic sandstone soil derived from Table Mountain sandstone. The rainfall varies considerably as a result of the mountainous terrain and topographic diversity. Winters are relatively cold and wet (Van Jaarsveld *et al.* 2000).

Five indigenous species (*C. aequilaterus* (Haw.) N. E. Br., *C. glaucescens* (Haw.) Schwantes, *C. modestus* S. T. Blake, *C. rossii* (Haw.) Schwantes *and C. virescens* (Haw.) Schwantes) occur in Australia, New Zealand and Tasmania, and one species, *C. chilensis* (Mol.) N. E. Br, is found in Chile and California. The global distribution of the genus is shown in Figure 1.3 below. The genus has been naturalised in many parts of the world beside South Africa mainly in south-eastern France and California. *Carpobrotus acinaciformis* and *C. edulis* have invaded the Island of Bagaud within the Hyères archipelago of southern France (Suehs *et al.* 2004) where the two species have also been found to hybridise with each other. The same problem is also experienced between *C. edulis* and *C. chilensis* in California, where the two species hybridise readily (Weber & D'Antonia 1999).

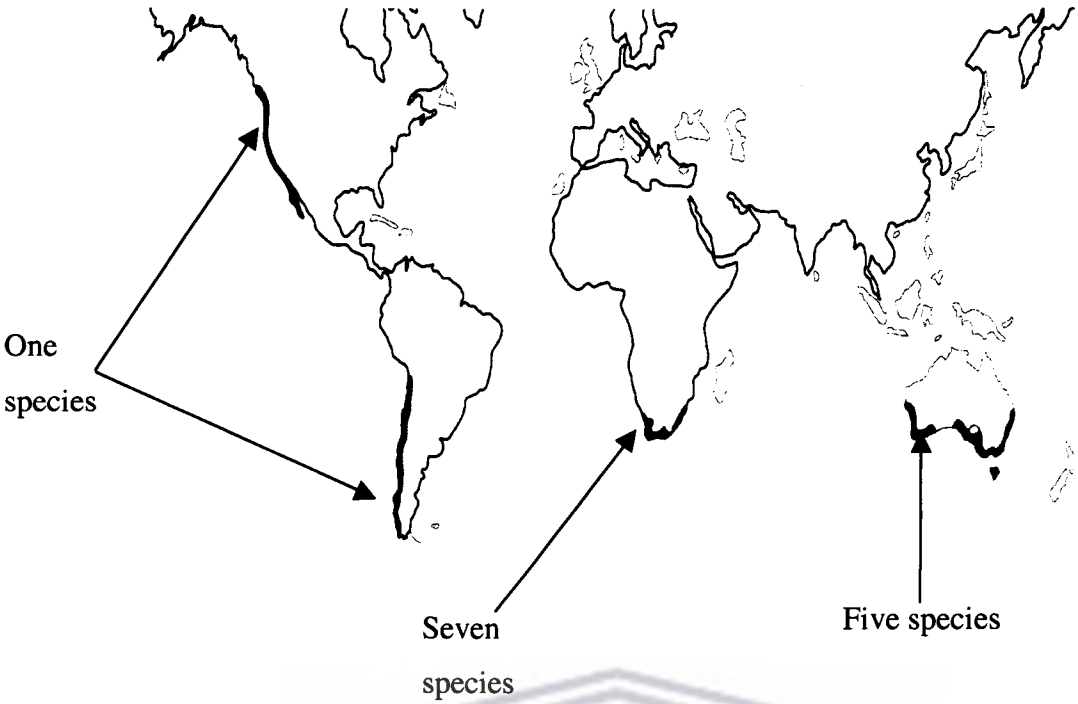


Figure 1.3 Worldwide distribution of the genus *Carpobrotus*.

Australian Carpobrotus and related genera - *Carpobrotus* has five native and two naturalised species in Australia. The native species are *C. aequilaterus, C. glaucescens*, *C. modestus*, *C. rossii* and *C. virescens* and the two naturalised species are *C. edulis* and *C. chilensis*. The genus *Sarcozona*, which is indigenous to Australia, is considered to be very closely related to *Carpobrotus*. The two genera both possess fleshy, indehiscent, berry-like fruits. However, *Sarcozona* has small, erect leaves and its flowers are less showy than *Carpobrotus* (Smith *et al.* 1998). *Sarcozona* has two species, *S. praecox* and *S. bicaritana,* which both occur in the southern parts of Australia. *Sarcozona praecox* is scattered along the south coast and further inland, whereas *S. bicaritana* has a more restricted distribution (Smith *et al.* 1998).

Disphyma N.E.Br. is widely distributed in Australia, New Zealand and South Africa. The plants lie flat on the ground and form dense mats, which root at the nodes. The leaves are three-edged and fringed at the base (Smith *et al.* 1998). The genus tends to hybridize naturally with *Carpobrotus* in Australia and comprises four species. These taxa are *D. crassifolium*, *D. dunsdonii*, *D. papillatum* and *D. australe*. The genus was included in the study because it has been found to hybridize with *Carpobrotus* in

Australia. Although it also occurs in South Africa there is no record of the genus hydridizing with *Carpobrotus* taxa in South Africa.

Identification - Hermann first described the genus *Carpobrotus* in 1687, and in 1789 and 1795 both Aiton and Haworth respectively studied the two commonest species, *C. edulis* and *C. acinaciformis*. Later in 1969, Blake studied five of the Australian species based on morphological data, and Wisura and Glen (1993) revised seven South African species of *Carpobrotus* also using morphological data.

The genus is known informally by the common names "sour fig" and "Hottentot fig", and is recognised by its trailing habit and long stem that forms large mats, carpeting flat ground and slopes or draping over rocks (Jonkers 2001). This fleshy succulent is a perennial and has scimitar-shaped leaves that are triangular in cross section. The leaf size varies with ecological factors such as availability of water and nutrients (Glen and Wisura 1993). The leaves of *C. dimidiatus* are smaller than other members of the genus in Southern Africa, whereas *C. quadrifidus* has the largest leaves in the genus (Glen and Wisura 1993). The leaf colour may be red, grey or emerald green depending largely on ecological conditions and long-term response to cold and hot temperatures. The flowers of *Carpobrotus* are on average 60 mm in diameter, and pink to purple in colour. *Carpobrotus edulis* is the exception with yellow flowers, but this species can also have white to pink coloured flowers. *Carpobrotus quadrifidus* has flowers over 120 mm in diameter, representing the largest flowers in the genus. All species in the genus have fleshy fruits that develop a sour-sweet taste.

Ethnobotanical Value:

Traditional medicinal uses - Species of *Carpobrotus* are commonly utilised by humans for their medicinal properties. The main species used for this purpose are *C. edulis*, *C. acinaciformis* and *C. dimidiatus*. The leaf juice has mild antiseptic properties and is highly astringent, and it is therefore used as a gargle for throat and mouth infections (Watt & Breyer-Brandwijk 1962). The leaf juices of *Carpobrotus* have also been used in the treatment of diarrhoea, dysentery, and applied to burns, insect and blue-bottle stings and mild skin conditions. *Carpobrotus dimidiatus* is said to be a good remedy for jellyfish stings (Hardy 1992).

Biochemical characteristics - Very little is known about the biochemical characteristics of *Carpobrotus*. However, there is evidence that the genus exhibits strong anti-microbial activities. Van der Watt & Pretorius (2001) identified tannins and five bioactive flavanoid compounds in *C. edulis* namely: rutin, neohesperidin, hyperoside, cactichin and ferulic acid. These five compounds have anti-microbial activity and they also protect the skin layers against fluid losses. Van Wyk *et al.* (1997) also identified three other compounds in the leaves of *Carpobrotus* species namely: tannic, malic and citric acid. Two other species of *Carpobrotus*, *C. muirii* and *C. quadrifidus*, were also investigated by Springfield *et al.* (2003), and were shown to demonstrate strong anti-microbial activities. The species were tested against *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Mycobacterium smegmatis.*

Food resource - The genus is not only used medicinally, but also considered as an edible fruit. The fruits of the genus are berries and harvested commercially for local markets. The commercial species are *C. edulis*, *C. acinaciformis* and *C. deliciosus*. The fruits of *C. acinaciformis* are used once the desiccated berry assumes a brown and leathery appearance. However, the fruits of *C. edulis* and *C. deliciosus* are also eaten fresh, and sour-tasting fruits can be eaten preserved, desiccated or as a jam (Du Preez & Esler 1997). They are very popular in the Western Cape for use in curry dishes and jam. Syrup can also be made from these species and, when eaten raw and slightly warmed by the sun, can act as a powerful laxative (Du Preez & Esler 1997).

Commercial value - *Carpobrotus edulis* has been cultivated worldwide as an ornamental and to control erosion (Du Preez & Esler 1997). It has also been used to act as a firebreak groundcover and as a result some of these species have become invasive (Du Preez & Esler 1997). *Carpobrotus* has increasily been exported for these purposes because it grows quickly.

This study was undertaken with the primary aim of resolving relationships between *Carpobrotus* species in South Africa, and determining species limits by assessing their genetic distinctness. In addition, levels of genetic variability among populations of two South African species, *C. acinaciformis* and *C. edulis*, used traditionally for their medicinal properties, were assessed in more detail. A host of molecular tools are

now readily available to investigate genetic diversity in plants at a variety of hierarchical levels. These techniques are discussed in more detail below.

The use of molecular tools in species-level reconstruction and population studies in plants:

Molecular tools are now commonly used to determine phylogenetic relationships at various taxonomic levels and to provide measures of genetic diversity in natural populations. The following sections will describe the tools and methods available for such studies and their utility and merits.

DNA sequencing - DNA sequence data gives insight into the order of nucleotides in a sequence of a given gene region (Qamaruz-Zaman *et al.* 1998a), and many studies have successfully utilised DNA sequence data to resolve relationships among taxa at various hierarchical levels (e.g. Chase *et al.* 1993, Savolainen *et al.* 2000, and Klak *et al.* 2003). However, it is difficult to determine *a priori* which genes or gene regions will provide sufficient variability for the group under investigation. In many cases DNA sequence variation at the species-level is limited, and many studies have struggled to recover well-supported species-level relationships with DNA sequence data. For example, within the Aizoaceae, Klak *et al.* (2003) were unable to resolve relationships among genera of the subfamily Ruschioideae, despite using DNA sequence data from four plastid non-coding regions. These authors have gone on to show that this clade has radiated recently with an estimated clade age of 3.5 – 8.7 million years (Klak *et al.* 2004). If indeed this clade has diversified relatively recently then this may provide an explanation as to why DNA sequence data has not been useful for reconstructing relationships in this group. Thus, this has implications for the efficacy of DNA sequence data to resolve species-level relationships within *Carpobrotus*.

DNA fingerprinting techniques - Within populations, the development of fingerprinting techniques has had a great impact on our understanding of the processes that determine population structure at a genetic level (Provan *et al.* 2001). These tools include allozymes and Restriction Fragment Length Polymorphisms (RFLPs) along with PCR-based techniques such as Randomly Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs) and

microsatellites. Each of these techniques will be briefly discussed below along with their respective advantages and disadvantages.

Allozymes - Allozymes have been widely used to assess genetic variation within and among natural populations (Aman 1995). The technique involves extraction of proteins from tissue, and separation on a polyacrylamide gel according to the net charge and size. The gel is then stained for a particular enzyme by adding a substrate and a dye under appropriate reaction conditions. This results in a band at the position to which the enzyme has migrated. Depending on the number of loci, several bands can be visualised. The pattern of these bands can be very polymorphic and informative regarding evolutionary relationships (Qamaruz-Zaman *et al.* 1998a). The technique is a single-locus marker and requires fresh or frozen material (frozen material requires liquid nitrogen) to prevent the protein from degrading. This may lead to difficulties when collecting on field trips.

Microsatellites or simple sequence repeats (SSRs) - Microsatellites or SSRs consist of short tandemly repeated nucleotides scattered throughout the genome of eukaryotic organisms. The most common are dinucleotide repeats e.g. $(CA)_n$, $(CT)_n$, and $(AT)_n$ (Wolfe & Liston 1998). Within plants these sequences are mostly found in introns and 5' flanking regions (Morgante & Oliviers 1993). Microsatellites are non-coding and evolve faster than surrounding DNA, and as a result they are generally highly polymorphic (Qamaruz-Zaman *et al.* 1998a). They are co-dominant markers, making it possible to identify homozygote and heterozygote alleles. One disadvantage is that in most cases the sequence of the SSRs must be known to design appropriate primers for PCR, which can be time consuming and expensive.

Restriction fragment length polymorphisms (RFLPs) - RFLPs were one of the first methods developed for genetic fingerprinting (Tanksley *et al.* 1989). The technique involves the digestion of genomic DNA with restriction enzymes. The fragments are then separated on a gel, followed by Southern blotting onto a membrane and hybridisation with appropriate multilocus labelled probes to make them visible. Hybridised fragments are finally visualised by autoradiography or non-radioactive detecting methods (Qamaruz-Zaman *et al.* 1998a). The technique is highly reproducible and requires no sequence specific information. However, it requires

large quantities of good quality DNA, which makes it unsuitable for rare and endangered species. The technique of Southern blotting and hybridisation can also be time consuming.

Randomly amplified polymorphic DNA (RAPDs) - RAPDs were one of the first fingerprinting methods to be developed based on PCR (Williams *et al.* 1990). The technique requires small amounts of DNA and also requires no sequence information for the design of specific primers (Qamaruz-Zaman *et al.* 1998a). It involves random primers to amplify DNA fragments and it is quick and easy. The marker is dominant and multilocus, screening many loci in the genome. Bands are then scored as either present or absent. However, there are limitations as the technique suffers from lack of reproducibility and is very sensitive to different reaction conditions.

Amplified fragment length polymorphisms (AFLPs) - AFLPs were developed to combine the best characteristics of other techniques (RAPDs and RFLPs) whilst avoiding their disadvantages (Vos *et al.* 1995). The technique involve three major steps: (1) restriction of total genomic DNA with two enzymes (EcoR1 and Mse1) and in the same reaction, ligation of double stranded adaptors to the restriction sites, (2) pre-selective amplification of a subset of fragments containing the primer binding site and restriction site and (3) selective amplification involving amplification of a subset of fragments with fluorescently labelled primers (see Figure 1.4).

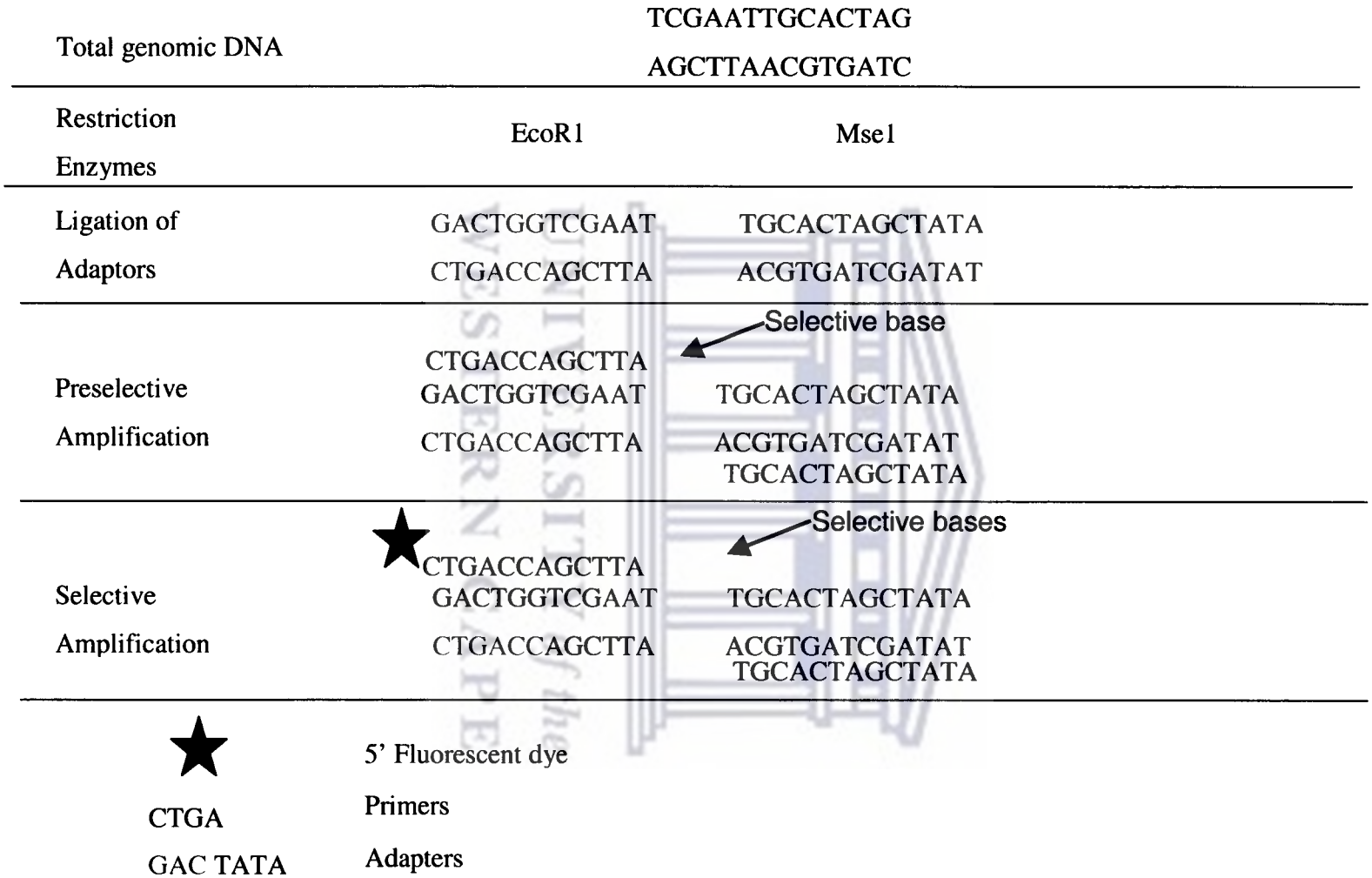


Figure 1.4 Schematic diagram of the AFLP technique

AFLPs have several advantages over other fingerprinting techniques. The technique requires less DNA compared to RFLPs (for "regular" genomes 500 ng per reaction and for "small" genomes 50 ng per reaction). This makes the technique suitable for rare and endangered species since only small quantities of starting material are required. The technique has now been automated making it fast, and it provides 10-100 times more markers than RFLPs and RAPDs. It is also highly reproducible due to high stringency PCR conditions. Another advantage over other techniques is the ability to detect genetic variation over the whole nuclear genome.

AFLPs have been used in a number of investigations, mainly to assess genetic distance, genetic variation, phylogenetic relationships, populations structure and for identification of hybridized individuals (e.g. Qamaruz-Zaman *et al.* 1998b, Palacios *et al.* 1999, Hodkinson *et al.* 2000, Schmidt & Jensen 2000, Parson & Shaw 2001, Bottini *et al.* 2002, Coulibaly *et al.* 2002, Saarela *et al.* 2003 and Chen *et al.* 2004). Qamaruz-Zaman *et al.* (1998b) used AFLPs to determine the genetic structure of populations of *Orchis simia* Lam. (Orchidinae: Orchidaceae), considered to be a rare and endangered species in the UK. Within Britain the species only occurs in Kent and Oxfordshire, but is widespread in continental Europe. The species started to decline in numbers in the mid-18th century due to over collecting. The Hartslock, Oxfordshire populations of *O. simia* were sampled in 1997 for genetic fingerprinting studies. Other plants of *O. simia* were sampled from the Living Collections of the Royal Botanic Gardens, Kew (originating from Italy and Greece). The results of Qamaruz-Zaman *et al.* (1998) revealed that the Hartslock populations were genetically distinct from the other plants sampled, but within the population low levels of genetic variation were detected. The recommendation resulting from this study was that the Hartslock population should be treated as a separate genetically distinct population and every effort made to increase its population size.

AFLPs were used to examine levels of genetic variation in two wild species of *Stylosanthes* Sw. (Fabaceae) and to determine whether the variation was geographically structured (Sawkins *et al.* 2001). *Stylosanthes* comprises 30-40 species found throughout the tropics and subtropics of America, Africa and South-East Asia. The two species considered in this study were *Stylosanthes humilis* Kunth and *Stylosanthes viscosa*. The two species have similar distributions and are mainly used

as cattle fodder, as a cover crop and a green manure. Over 900 accessions of both species were collected and are held by the gene banks of the Centro Internacional de Agricultura Tropical (CIAT). The majority of the accessions were collected from Brazil and Venezuela. In this case, Sawkins *et al.* (2001) used AFLPs to show that *S. humilis* and *S. viscosa* have different degrees of genetic variation, but showed similar patterns of geographical clustering, and that *S. humilis* was less genetically variable than *S. viscosa.* AFLPs did not only detect genetic diversity within species of *Stylosanthes*, but identified individuals that were misclassified and misidentified.

Another study undertaken by Richardson *et al.* (2003) used AFLPs to resolve relationships between island group species of *Phylica* L. (Rhamnaceae) and to determine species limits by assessing their genotypic distinctness. The island groups include *P. polifolia* from St Helena, *P. arborea* from Tristan da Cunha and New Amsterdam, *P. nitida* from Mauritius and Réunion and the widely distributed mainland species *P. paniculata* from southern Africa. The island species together with a species (*P. paniculata)* from the mainland formed a monophyletic group based upon DNA sequences from the plastid *trnL-F* region, and the internal transcribed spacer (ITS) of nuclear ribosomal DNA. The AFLP data indicated that *Phylica arborea*, *P. polifolia*, and *P. paniculata* formed distinct groups relative to *P. nitida*. AFLPs also indicated that *Phylica arborea*, *P. polifolia*, and *P. paniculata* were derived independently from a common ancestor. AFLPs were successfully utilised in this study to reconstruct species-level relationships and indicated that the species of *Phylica* were genotypically distinct and that current species delimitation in this group was congruent with genetic evidence.

The AFLP technique generates dominant markers making it difficult to identify homologous alleles (dominant markers are markers that fail to distinguish AA from Aa genotypes). They are thus less useful for studies that require heterozygosity analyses and therefore precise assignment of allelic states. The technique also becomes problematic when applied to higher taxonomic levels because similarity between taxa is not necessarily due to common ancestry, but may be due to chance (Ulrich & Wolfenbarger 1999).

AFLPs are the chosen genetic markers for this study of genetic variability within and among populations of *Carpobrotus*. This choice was based upon the robustness of the method and its efficiency for systematic studies in plant groups. Because of its medicinal and commercial application, the purpose of this investigation is to evaluate the robustness of species delimitation so as to assist targeted selection of taxa for bio-prospecting. Furthermore, levels of genetic variability in those South African taxa known to demonstrate anti-microbial activity will also be investigated. Therefore the aims of this study are as follows:

1. To successfully identify AFLP primer combinations for use with species of the genus *Carpobrotus*.
2. To investigate the efficacy of AFLP markers in defining species boundaries within *Carpobrotus*.
3. To assess the levels of genetic variation among populations of South African *Carpobrotus* taxa, specifically those with known anti-microbial properties i.e. *C. edulis* and *C. acinaciformis*.

The chapter outline of this thesis is as follows:

- Chapter 2 presents the materials and methods.
- Chapter 3 contains the results of both DNA sequence and AFLP studies.
- Chapter 4 discusses the results obtained and general conclusions.

Sampling of plant material:

Plant material used in this study and corresponding voucher specimens are listed in Appendix 1. African specimens of *Carpobrotus* were collected from the Cape Peninsula, West coast, South coast and Mozambique (refer to Figures 2.1, 2.2, and 2.3 respectively). In total, 26 individuals of *C. edulis*, 22 individuals of *C. acinaciformis*, three individuals of *C. deliciosus*, two individuals of *C. dimidiatus*, two individuals of *C. muirii*, two individuals of *C. quadrifidus* and one individual of *C. mellei* were sampled from South Africa. Additional samples from Mozambique, Corsica and Australia were also included. From Australia, these comprised one individual of *C. chilensis,* two individuals of *C. edulis,* two individuals of *C. modestus*, one individual thought to be hybrid between *C. rossii* and *C. modestus* and three individuals of *C. rossii.* From Mozambique, one naturalised individual of *C. acinaciformis* was collected. Six individuals of *C. edulis* and six individuals of *C. affine acinaciformis* from the French island of Baguad were also sampled (*C. acinaciformis* specimens from Baguad are thought to be of hybrid origin - hence the assignment of *C. affine acinaciformis* (Suehs *et al.* 2001)). The following Australian taxa were also sampled as outgroups: two individuals of *Disphyma australe*, one individual of *Disphyma crassifolium*, one individual of *Disphyma papillatum*, one individual of *Sarcozona bicarinata* and two individuals of *Sarcozona praecox.* At least one representative of each South African species was collected.

DNA extraction:

Genomic DNA was extracted from 1-2 g of fresh leaf material using the 2x CTAB method (Doyle & Doyle 1987) with 1% PVP (polyvinylpyrrolidone). DNA extracts were further purified using QIAquick silica columns (Qiagen Inc.). Purified total DNA was resuspended in 1x TE and stored at 4° C.

DNA sequencing of psbA-trnH intergenic spacer:

DNA was amplified using 100 µl reactions containing Promega magnesium free thermophilic buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 3 mM

$MgCl_2$, 0.004% BSA (Savolainen *et al.* 1995), 0.2 mM each dNTP (dGTP, dATP, dCTP, dTTP), 100 ng of each primer and 2.5 U Taq polymerase. Amplification of the *psbA-trnH* intergenic spacer was achieved using primers *psbA* (5'-GTT ATG CAT GAA AAT GCT C-3') and *trnH* (5'-CGC GCA TGG ATT CAC AAA TC-3'; Sang *et al.* 1997) and the following program: denaturation, 94°C, one minute; annealing, 52°C, one minute; extension, 72°C, one minute for 30 cycles. Amplified fragments separated by electrophoresis on 1% agarose gel and then visualised. Successfully amplified fragments were purified using QIAquick silica columns (Qiagen Inc.) and directly sequenced on an ABI 377 automated sequencer using PCR primers as sequencing primers. Assembly and editing of the complimentary strands was carried out using Sequencher 4.1 (Gene Codes). Assembled sequences were transferred into the software package PAUP* version 4.02b (Swofford 2000) and aligned by eye.

Generation of AFLPs profiles:

AFLPs were generated following the AFLP plant mapping protocol of Applied Biosystems (Vos *et al.* 1995). This protocol distinguishes between taxa that have 'small' versus 'regular' sized genomes, where small genome kits are for use with plant taxa with a 1C genome size from 50-500 Mb. (The 1C value is equivalent to the nuclear DNA content of the unreplicated haploid genome). The 1C-value of *Carpobrotus* is 0.43 pg (http://www.rbgkew.org.uk/cval), which is considered to be small according to the criteria applied by Applied Biosystems. The AFLP procedure for small genomes thus requires 50 ng of DNA per reaction. DNA concentrations were determined for each of the total genomic DNA extracts using a spectrophotometer at λ 260 nm. In cases where DNA extracts were not sufficiently concentrated the volume containing 50 ng was calculated. This volume was then evaporated and the pellet resuspended in 5.5 µl of double distilled water (the maximum volume allowed by the protocol). The AFLP procedure is summarised below and in Figure 1.4 (Chapter 1):

Step 1: Restriction-ligation - Total genomic DNA was cut with two restriction enzymes EcoR1 (a rare six base cutter) and Mse1 (a frequent four base cutter) to

generate DNA fragments. In the same reaction double stranded DNA adapters were ligated to the 3' end of the restriction fragments. These adaptor pairs thereby generated a template for subsequent amplification.

Step 2: Preselective amplification - Two primers, complimentary to the adapter-ligated ends with one extra nucleotide at the 3'end, were used to amplify fragments containing both the primers binding site and restriction site. This led to an approximate 16-fold reduction in the number of amplified fragments (Vos *et al.* 1995).

Step 3: Selective amplification - Selective primers with two extra nucleotides at the 3' end (three nucleotides are added for regular sized genomes) were employed to amplify the pre-selective amplification templates. At this stage there were 64 fluorescently labelled primer combinations to choose from. A primer trial was carried out with 35 primer combinations to identify suitable selective primers. Two combinations were subsequently chosen as selective primers to proceed with this study: CAC + AA (JOE), and CAT + AC (FAM) (from here on these are referred to as G1 and B1 respectively). The triplet is attached to the Mse1 based primer, and the dinucleotide is attached to the EcoR1 primer, which is fluorescently labelled. This resulted in a further reduction in the number of amplified fragments. The products were then mixed with 1.2 µl of loading dye (containing deionized formamide, loading buffer and Genescan 500 ROX size standard), and denatured at 94°C for 3 min. A total of 1.2 µl of the selective amplification products were then separated on a 5% denaturing polyacrylamide gel according to manufacturer's protocols on an ABI 377 automated sequencer.

Scoring of AFLP fragments:

AFLP fragments were analysed using Genescan version 3.1.2 and Genotyper version 2.5 (Applied Biosystems Inc.). To verify correct assignment of the presence or absence of a band, all fragment profiles were also edited by eye. Bands were scored as either present (1) or absent (0), and tabulated to produce a binary matrix. Low frequency bands (present in < 5% of individuals), and those present in all samples

(fixed) were excluded from the subsequent analysis. This assumption appeared to be justified because low frequency bands are likely to contribute noise to the data due to outcrossing among individuals within a species.

Measurements of genetic diversity:

A number of different measures of genetic diversity for African and Mediterranean *Carpobrotus* taxa were calculated from the subsets of data listed below.

1. 254 AFLP markers generated using primer combination B1 for 56 individuals.
2. 230 AFLP markers generated using primer combination G1 for 51 individuals.
3. 480 AFLP markers in a combined analysis of bands generated using primer combinations B1 and G1 for 39 individuals.

Percentage polymorphic loci and heterozygosity were calculated using allelic frequencies in TFPGA v1.3 (Tools for Population Genetic Analysis; Miller 2000). The first measure calculates the proportion or percentage of loci in a population that are not fixed for a single allele. Heterozygosity is more difficult to calculate from dominant data (as is the case for AFLP data) than co-dominant data (such as microsatellite data). Expected heterozygosity is calculated in TFPGA by using the absence of a fragment to calculate the frequency of the recessive allele (a). This measure can then be used to calculate the frequency of the dominant allele (A) (since the frequency of alleles A + a = 1). Assuming that the population is in Hardy-Weinberg equilibrium the frequency of heterozygotes (Aa genotypes) can then be calculated.

Shannon's index of diversity (1949) and Nei's gene diversity (1978) were calculated in PopGene v1.31 (Yeh *et al.* 1997) from allele frequency data. Shannon's index of diversity measures the allelic diversity within populations, taking into account the abundance of a particular allele in the total data set, whereas Nei's gene diversity calculates the average proportion of heterozygotes per locus in a population. These measures were calculated only for the African and Mediterranean taxa included in this study due to the fact that the Australian taxa were represented by too few

individuals. *Carpobrotus quadrifidus* was also omitted due to the fact that it was represented by only one individual.

Principle co-ordinate analyses:

Principle co-ordinate (PCO) analysis was performed using Jaccard's coefficient of similarity in order to determine genetic affinities within and among species of *Carpobrotus, Disphyma and Sarcozona.* In PCO analysis the variance in the data is reduced to a number of principal co-ordinates; I report structuring among *Carpobrotus, Disphyma and Sarcozona* taxa at the first two principal co-ordinates. The following combinations of data for all *Carpobrotus, Disphyma and Sarcozona* taxa included in this study were subjected to PCO analysis implemented in NTSYSpc (Rohlf 1997), with pairwise similarities calculated using Jaccard's coefficient (Jaccard 1908):

1. 254 AFLP markers generated using primer combination B1 for 70 *Carpobrotus, Disphyma and Sarcozona* individuals representing taxa from Africa, Australia and the Mediterranean Basin.
2. 199 AFLP markers generated using primer combination G1 for 66 *Carpobrotus, Disphyma and Sarcozona* individuals representing taxa from Africa, Australia and the Mediterranean Basin.
3. Combined analysis of 480 AFLP markers generated using primer combinations B1 and G1 for 52 *Carpobrotus, Disphyma and Sarcozona* individuals representing taxa from Africa, Australia and the Mediterranean Basin.

Principle co-ordinate analysis was also carried out on the following subsets of data representing African taxa only:

1. Analysis of 254 AFLP markers generated using primer combination B1 for 47 *Carpobrotus* individuals representing African taxa only.
2. Analysis of 230 AFLP markers generated using primer combination G1 for 46 *Carpobrotus* individuals representing African taxa only.

3. Analysis of 482 AFLP markers generated using primer combinations B1 and G1 for 35 *Carpobrotus* individuals representing African taxa only.

Cluster analysis:

Relationships among lineages of *Carpobrotus* from Africa were also investigated by means of cluster analysis. Cluster analysis is a family of techniques for representing similarity or distance data, which act to reduce variance by categorising or grouping similar data together. The most commonly used methods are based on arithmetic, geometric, and graphic-theoretic constructs (Legendre & Legendre 1998) following a set of steps (an algorithm) to arrive at a tree (Page & Holmes 1998). The technique has proved to be good and fast at analysing large numbers of taxa, and producing a single tree. The most widely used clustering methods are unweighted pair-group method using arithmetic averages (UPGMA; Sneath & Sokal 1973) and neighbour joining (NJ; Saitou & Nei 1987).

NJ and UPGMA analyses were performed on only one data set; corresponding to 254 markers (generated from primer combination B1) for 47 South African taxa. The NJ method was implemented in PAUP* v.4.0b10 (Phylogenetic Analysis Using Parsimony; Swofford 2000) using Nei's pairwise similarities for restriction site data (although Jaccard's coefficient is more suitable for these kind of data it is not possible to implement this option in PAUP*). UPGMA was performed in NTSYSpc (Rohlf 1997) with pairwise similarities calculated using Jaccard's coefficient (Jaccard 1908).

The UPGMA algorithm (Sneath & Sokal 1973) reconstructs a tree by clustering the most similar pair/smallest distance taxa and unites them into a single taxon for subsequent rounds (Penny & Hendy 2000). The optimality criterion is to select and join the closest (most similar) pairs of taxa. UPGMA is one of the few tree-building methods that reconstructs an ultrametric tree (Page & Holmes 1998), but this strategy is only effective when a molecular clock is a good description of the data. UPGMA is no longer consistent when there are differences in the rate of evolution among lineages (Penny & Hendy 2000).

Neighbour Joining (Saitou & Nei 1987) identifies the closest pair, or neighbour, to minimize the total length of a tree (Weir 1996). It attempts to correct the UPGMA method for its assumption that the same rate of evolution applies to all branches. The method keeps track of the nodes on the tree rather than clusters of taxa. The tree is then constructed by linking the least-distant pair of nodes (Swofford *et al.* 1996).

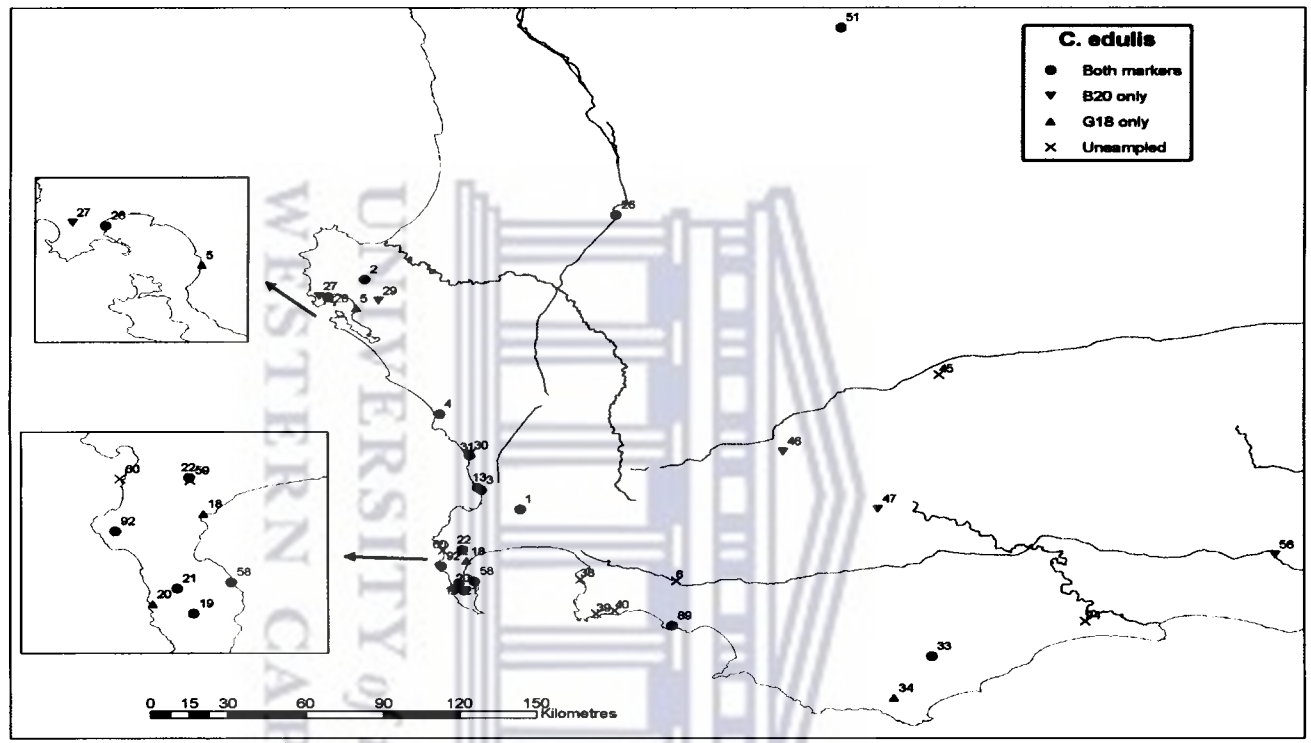


Figure 2.1 Map of the Western Cape of South Africa showing the localities of *Carpobrotus edulis* collected for this study. The unsampled points indicate taxa for which AFLP data were not successfully generated.

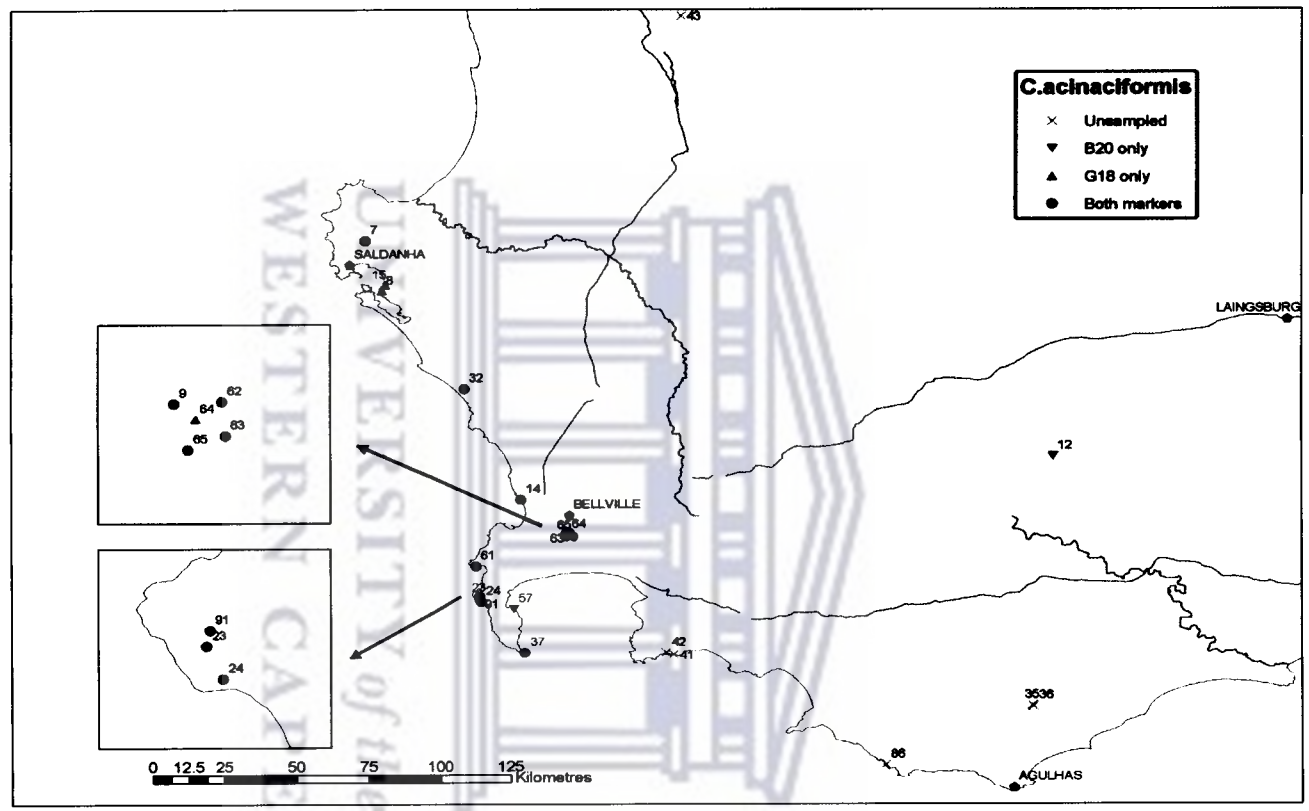


Figure 2.2 Map of the Western Cape of South Africa showing the localities of *Carpobrotus acinaciformis* collected for this study. The unsampled points indicate taxa for which AFLP data were not successfully generated.

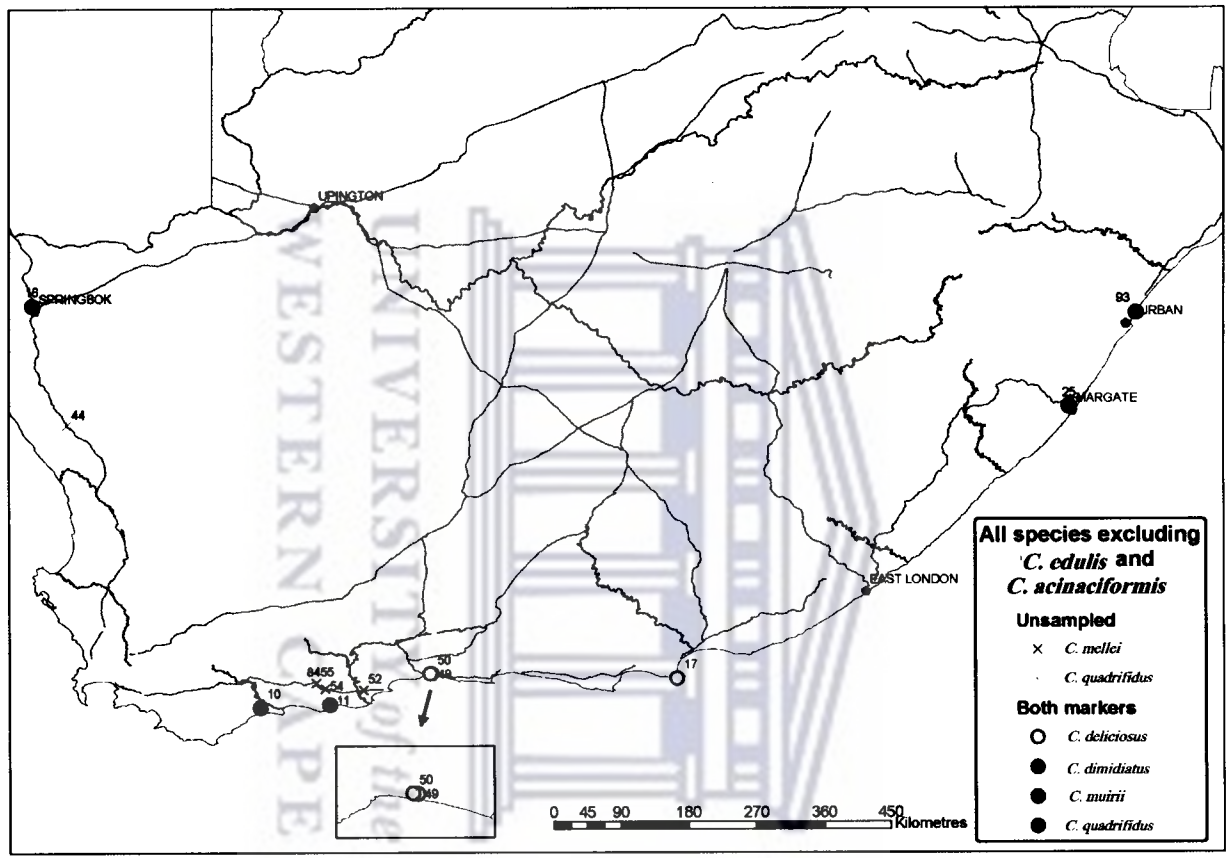


Figure 2.3 Map of the Western Cape of South Africa showing the localities for all South African species excluding *C. edulis* and *C. acinaciformis* collected for this study

DNA Sequencing of psbA – trnH region:

The *psbA-trnH* intergenic spacer was sequenced for 16 *Carpobrotus* taxa representative of both South African and Australian species, along with taxa collected from the Mediterranean. The *psbA-trnH* region included 280 nucleotide characters. All sequences were identical with the exception of one indel present in *C. quadrifidus* and a second indel in two of the *C. acinaciformis* individuals from the Mediterranean (see Appendix 2 for complete sequences). Owing to the lack of DNA sequence divergence within the *psbA-trnH* intergenic spacer of these taxa phylogenetic analysis of these data was not possible.

Analysis of AFLP profiles:

One individual of *C. quadrifidus* and one individual of *C. mellei* were not included in the AFLP study because these two South African taxa did not yield sufficient quantities of DNA for the AFLP procedure. AFLP profiles were therefore generated for 82 individuals of *Carpobrotus*. From a total of 35 primer combinations two AFLP primer combinations (Mse1CAT + EcoR1AC (FAM) and Mse1CAC + EcoR1AA (JOE)) were found to be suitable to the *Carpobrotus* taxa included in this study, due to the high degree of variability among the AFLP profiles obtained (Figure 3.1). From here on these primer combinations are referred to as B1 and G1 respectively. AFLP profiles were successfully generated for 70 *Carpobrotus*, *Disphyma and Sarcozona* individuals for primer combination B1, and 66 individuals for primer combination G1. Both primer combinations were generated for 52 individuals. For all the individuals included in this study, the total number of bands scored from primer combinations B1 and G1 were 515 and 502 respectively (Table 3.3). Fixed and low frequency bands were not included in the analysis and therefore 254, 199 and 480 loci were retained for analysis from B1, G1 and the combined data sets respectively.

Genetic diversity measurements:

Estimates of Nei's gene diversity and Shannon's diversity index of the Africa and the Mediterranean taxa were calculated along with percentage polymorphic loci and heterozygosity for primer combination B1 with 56 individuals, and G1 with 51 individuals. Both primer combinations were generated for 39 individuals. The number of loci retained for the analysis were 254, 230 and 480 for B1, G1 and the combined data set respectively (Table 3.1). The Australian taxa were excluded from these calculations. One individual of *C. acinaciformis* from Mozambique was included in the calculations with *C. acinaciformis* from South Africa. The measures obtained for B1, G1 and the combined data are shown in Tables 3.2a, b, and c respectively.

Table 3.1 Number of individuals sampled, and number of loci retained for measurements of genetic diversity.

AFLP primers	Number of individuals sampled	Total number of bands scored	Numbers of loci retained for analysis
B1: MseI-CAT EcoRI-AC	56	515	254
G1: MseI-CAC EcoRI-AA	51	502	230
Combined B1 and G1	39	1017	480

Table 3.2a Genetic diversity measurements for African and Mediterranean *Carpobrotus* taxa using primer combination B1. SA = South African and M = Mediterranean.

Taxon	Number of individuals	Hetero-zygosity	Polymorphic loci (%)	Shannon's diversity index	Nei's gene diversity
C. acinaciformis SA + Mozambique	18	0.15	70.9	0.252	0.156
C. edulis SA	21	0.14	63.8	0.230	0.145
C. deliciosus SA	3	0.06	19.3	0.110	0.074
C. dimidiatus SA	2	0.03	7.5	0.045	0.031
C. muirii SA	2	0.08	16.5	0.100	0.069
C. affine acinaciformis M	6	0.15	33.9	0.201	0.139
C. edulis M	4	0.12	34.3	0.176	0.116

In Table 3.2a, individuals of *C. acinaciformis* from Africa have the highest genetic diversity compared to the other South African species (reflected in both Shannon's diversity index and Nei's gene diversity), followed by South African *C. edulis* individuals. *Carpobrotus acinaciformis* from Africa and *C. affine acinaciformis* from the Mediterranean have the highest measure of heterozygosity both with 0.15, followed by *C. edulis* from South Africa and *C. edulis* from the Mediterranean with 0.14 and 0.12 respectively. *Carpobrotus acinaciformis* from Africa has the highest percentage of polymorphic loci of 70.9%, followed by *C. edulis* from South Africa with 63.8%. *Carpobrotus dimidiatus* has both the lowest percentage of polymorphic loci (7.5%) and heterozygosity (0.03).

Table 3.2b Genetic diversity measurements for African and Mediterranean *Carpobrotus* taxa using primer combination G1. SA = South African and M = Mediterranean. (Values are not available for *C. affine acinaciformis* from the Mediterranean, as data for only one individual of this species was available).

Taxon	Number of individuals	Hetero-zygosity	Polymorphic loci (%)	Shannon's diversity index	Nei's gene diversity
C. acinaciformis SA + Mozambique	18	0.25	99.5	0.399	0.215
C. edulis SA	21	0.24	97.5	0.394	0.250
C. deliciosus SA	2	0.08	17.0	0.103	0.070
C. dimidiatus SA	2	0.15	31.5	0.191	0.131
C. muirii SA	2	0.16	31.5	0.191	0.131
C. affine acinaciformis M	1	-	-	-	-
C. edulis M	5	0.21	64.0	0.306	0.197

Values of genetic diversity calculated from loci generated using primer G1 (Table 3.2b), increased compared to those generated from primer B1 (Table 3.2a), whilst still retaining the same number of individuals for *C. acinaciformis* and *C. edulis* from South Africa and Mozambique. *Carpobrotus acinaciformis* from Africa displayed the highest levels of heterozygosity, percentage polymorphic loci as well as Shannon's diversity index; with *C. deliciosus* having the lowest levels of Nei's gene and Shannon's diversity index. Both *C. muirii* and *C. dimidiatus* are represented by the same number of individuals as *C. deliciosus*, but have almost twice as many polymorphic loci.

Table 3.2c Genetic diversity measurements for African and Mediterranean *Carpobrotus* species using both B1 and G1 primer combinations. SA = South African and M = Mediterranean. Values are not available for *C. affine acinaciformis* from the Mediterranean, as data for only one individual of this species was available.

Taxon	Number of individuals	Hetero-zygosity	Polymorphic loci (%)	Shannon's diversity index	Nei's gene diversity
C. acinaciformis SA + Mozambique	14	0.21	79.4	0.322	0.203
C. edulis SA	15	0.20	75.2	0.306	0.195
C. deliciosus SA	2	0.06	14.0	0.084	0.058
C. dimidiatus SA	2	0.09	19.0	0.115	0.079
C. muirii SA	2	0.12	25.0	0.151	0.104
C. affine acinaciformis M	1	-	-	-	-
C. edulis M	3	0.16	40.2	0.215	0.142

In the combined data set (Table 3.2c) *C. deliciosus* displayed the lowest Shannon's diversity and Nei's gene diversity. *C. acinaciformis* from Africa harboured the highest heterozygosity and percentage polymorphic bands when compared with other South African taxa followed closely by *C. edulis* from South Africa.

Principle co-ordinate analyses:

Table 3.3 Number of individuals sampled, and number of loci retained for PCO analyses of all included *Carpobrotus*, *Disphyma* and *Sarcozona* individuals.

AFLP primers	Number of individuals sampled	Total number of bands scored	Numbers of loci retained for analysis
B1: MseI-CAT EcoRI-AC	70	515	254
G1: MseI-CAC EcoRI-AA	66	502	199
Combined B1 and G1	52	1017	480

Table 3.4 Number of individuals sampled, and number of loci retained for PCO analyses of African taxa only.

AFLP primers	Number of individuals sampled	Total number of bands scored	Numbers of loci retained for analysis
B1: MseI-CAT EcoRI-AC	47	515	254
G1: MseI-CAC EcoRI-AA	46	502	230
Combined B1 and G1	35	1017	482

Principle co-ordinate analysis of 70 individuals of Carpobrotus, Disphyma and Sarcozona using primer combination B1 - PCO analysis of AFLP markers generated using primer combination B1 for 70 *Carpobrotus*, *Disphyma* and *Sarcozona* individuals broadly displayed the following pattern. In Figure 3.2, a group comprised of 13 of 21 included representatives of *C. edulis* was identified (Group A; Figure 3.2). One individual of *C. edulis* collected from the Mediterranean and one individual of *C. acinaciformis* from South Africa also grouped with these *C. edulis* individuals.

A second group comprised of five of the six included *C. affine acinaciformis* individuals from the Mediterranean was also identified (Group B; Figure 3.2). Group B was placed between group A and group C. A third group consisting of eight of 17 included representatives of *C. acinaciformis* from Africa and one individual of *C. acinaciformis* from Mozambique was also identified (Group C; Figure 3.2). One individual of *C. edulis* from South Africa and one individual of *C. muirii* also grouped with these individuals of *C. acinaciformis*.

A fourth group comprised of three individuals of *C. deliciosus*, two individuals of *C. dimidiatus*, one individual of *C. modestus* and one individual of *C. rossii* was also retrieved (Group D; Figure 3.2). A fifth group consisted of five of 13 included representatives of the Australian taxa (Group E; Figure 3.2). These Australian taxa comprised of two individuals of *Disphyma*, two individuals of *Sarcozona* and one individual of *C. chilensis*. One individual of *C. quadrifidus*, one individual of *C. affine acinaciformis* collected from the Mediterranean, one individual of *C. edulis* from the Mediterranean, two individuals of *C. acinaciformis* from South Africa and one individual of *C. edulis* also grouped with these Australian taxa.

The final group comprised of another group of Australian taxa (Group F; Figure 3.2). These Australian taxa comprised of one individual of *C. modestus*, one individual of *C. rossii*, two individuals of *Disphyma*, one individual of *Sarcozona* and one individual thought to be a hybrid between *C. chilensis* and *C. rossii*. Three individuals of *C. acinaciformis* from South Africa, one individual of *C. edulis* from South Africa and two individuals of *C. edulis* collected from the Mediterranean also grouped with these Australian taxa.

Other individuals of *C. edulis*, *C. acinaciformis* and *C. muirii* were also scattered between the six groups. The two individuals of *C. muirii* included in this analysis did not group together. Three scattered individuals of *C. edulis* (#33, #47 & #58) were positioned in close proximity to group A (made up of *C. edulis*), along with one individual of *C. acinaciformis* from South Africa (24).

Principle Co-ordinate analysis of 66 individuals of Carpobrotus, Disphyma and Sarcozona using primer combination G1 - PCO analysis of AFLP markers generated using primer combination G1 for 66 *Carpobrotus*, *Disphyma* and *Sarcozona* individuals (Figure 3.3) displayed a similar pattern to analysis of AFLPs generated using primer combination B1, although the same individuals were not always sampled. In Figure 3.3, a group comprised of eight of 21 included representatives of *C. edulis* was identified (Group A; Figure 3.3). One individual of *C. acinaciformis* from South Africa also grouped with these *C. edulis* individuals.

A second group comprised of seven of the 14 included representatives of the Australian taxa was identified (Group B; Figure 3.3). These Australian taxa comprised of two individuals of *C. rossii*, one individual of *C. chilensis*, one individual thought to be a hybrid between *C. chilensis* and *C. rossii*, one individual of *C. modestus*, one individual of *Disphyma* and one individual of *Sarcozona.* One individual of *C. quadrifidus*, two individuals of *C. edulis* from South Africa and two individuals of *C. acinaciformis* also grouped with these Australian taxa.

A third group comprised of the remaining seven of the 14 included representatives of the Australian taxa (Group C; Figure 3.3). These Australian taxa comprised of two individuals of *Disphyma,* two individuals of *Sarcozona*, one individual of *C. modestus*, one individual of *C. rossii* and one individual of *C. edulis*. Four individuals of *C. edulis* from South Africa, two individuals of *C. acinaciformis* from South Africa and one individual of *C. edulis* from the Mediterranean also grouped with these Australian taxa.

The final group consisted of 6 of 18 included representatives of *C. acinaciformis* from South Africa and one individual of *C. acinaciformis* from Mozambique (Group D; Figure 3.3). Two individuals of *C. deliciosus*, one individual of *C. dimidiatus*, one individual of *C. muirii* and two individuals of *C. edulis* also grouped with the *C. acinacifomis* individuals.

Other individuals of *C. affine acinaciformis* from the Mediterranean, *C. edulis* from South Africa, *C. acinaciformis* from South Africa, *C. muirii*, *C. dimidiatus* and *C. edulis* from the Mediterranean were also scattered between the four groups. One individual of *C. edulis* from the Mediterranean (#83), which grouped with the individuals of *C. edulis* from South Africa in the previous analysis (Group A; Figure 3.2), does not group with any of the major groups here. The two individuals of *C. muirii* (#10 & #11) still do not group together in this analysis.

Principle Co-ordinate analysis of 52 individuals of Carpobrotus, Disphyma and Sarcozona using primer combinations B1 & G1 combined - A further PCO analysis of AFLP markers generated using a combination of both primers B1 and G1 for 52 *Carpobrotus*, *Disphyma* and *Sarcozona* individuals displayed the following pattern. In Figure 3.4, a group comprised of 13 representatives of Australian taxa was identified (Group A; Figure 3.4). These Australian taxa comprised of two individuals of *C. rossii*, two individuals of *C. modestus*, three individuals of *Disphyma*, three individuals of *Sarcozona*, one individual of *C. chilensis* and one individual thought to be hybrid between *C. chilensis* and *C. rossii* hybrid. The included individuals of *Disphyma* (#104, #113 & #110) did not group together separately from *Carpobrotus* and *Sarcozona*. Two individuals of *Sarcozona praecox* (#115 & #98) are positioned close together, but *Sarcozona bicarinata* (#112) is not placed together with these two individuals. *Carpobrotus chilensis* (#103) and the *C. chilensis x rossii* hybrid (#108) are positioned close together.

A second group comprised of 13 of 15 included representatives of *C. edulis* was identified (Group B; Figure 3.4). Six individuals of *C. acinaciformis* from South Africa, two individuals of *C. edulis* from the Mediterranean and one individual of *C. muirii* also grouped with these *C. acinaciformis* individuals.

A third group consisting of four of 14 included representatives of *C. acinaciformis* and one individual of *C. acinaciformis* from Mozambique was identified (Group C; Figure 3.4). One individual of *C. edulis* collected from South Africa also grouped with the *C. acinaciformis* individuals.

A final group comprised of *C. deliciosus* and *C. dimidiatus* individuals was also identified (Group D; Figure 3.4). One individual of *C. muirii* and one individual of *C. acinaciformis* from South Africa also grouped with the *C. deliciosus* and *C. dimidiatus* individuals. Other individuals of *C. affine acinaciformis* from the Mediterranean, *C. edulis* from the Mediterranean, *C. acinaciformis* from South Africa, *C. edulis* from South Africa and *C. quadrifidus* were scattered between group B and group D.

Principle Co-ordinate analysis of the African taxa using primer combination B1 - PCO analysis of 254 AFLP markers using primer combination B1 displayed the following pattern. In Figure 3.5, a group comprised of 13 of 21 included representatives of *C. edulis* was identified (Group A; Figure 3.5). Two individuals of *C. acinaciformis* grouped with these individuals. A second group, comprised of three representatives of *C. deliciosus* and two representatives of *C. dimidiatus*, was also identified (Group B; Figure 3.5). Two individuals of *C. acinaciformis* also grouped with Group B. The final group comprised of six of 18 included representatives of *C. acinaciformis* from South Africa and one individual of *C. acinaciformis* from Mozambique was identified (Group C; Figure 3.5). One individual of *C. edulis* from South Africa was also grouped with these *C. acinaciformis*. Other individuals of *C. edulis*, *C. acinaciformis*, *C. quadrifidus* and *C. muirii* were scattered between the three groups. With the exception of *C. deliciosus* and *C. dimidiatus* the manner in which the individuals were grouped did not indicate any major structuring based on geographical proximity

Principle Co-ordinate analysis of the African taxa using primer combination G1 - PCO analysis of 230 AFLP markers using primer combination G1 also identified a similar pattern to analysis of AFLPs generated using primer combination B1. In figure 3.6, a group comprised of six of 18 included representatives of *C. acinaciformis* from South Africa and one individual of *C. acinaciformis* from Mozambique was identified (Group A; Figure 3.6). One individual of *C. muirii* and two individuals of *C. edulis* also grouped with these *C. acinaciformis* individuals. A second group comprised of one individual of *C. deliciosus* and two individuals of *C.*

dimidiatus was identified (Group B; Figure 3.6). The second individual of *C. deliciosus* was positioned between groups A and B. A final group, comprised of nine representatives of *C. edulis* from South Africa was also identified (Group C; Figure 3.6). Other individuals of *C. edulis*, *C. acinaciformis*, *C. quadrifidus, C. deliciosus* and *C. muirii* were scattered between the three groups. Again the manner in which the individuals were grouped did not indicate any major structuring based on geographical proximity, with the exception of *C. deliciosus* and *C. dimidiatus.*

Principle Co-ordinate analysis of the African taxa using primer combinations G1 & B1 combined - A combined PCO analysis of 482 AFLP markers using primers B1 and G1 also showed a similar pattern. In Figure 3.7, a group comprised of seven of 15 included representatives of *C. edulis* was identified (Group A; Figure 3.7). A second group comprised of two individuals of *C. deliciosus* and two individuals of *C. dimidiatus* was identified (Group B; Figure 3.7). One individual of *C. muirii* also grouped with group B. A final group comprised of four of 14 representatives of *C. acinaciformis* from South Africa and one individual of *C. acinaciformis* from Mozambique was identified (Group C; Figure 3.7). One individual of *C. edulis* from South Africa also grouped with these *C. acinaciformis* individuals. Other individuals of *C. edulis*, *C. acinaciformis*, and *C. muirii* were scattered between the three groups.

Cluster analyses:

UPGMA and N-J trees of the African taxa based on primer combination B1 - Jaccard's similarity co-efficient was used to generate a UPGMA tree of the African taxa only using primer combination B1 (Figure 3.8). The resulting tree broadly recovered three groups, as in the PCO analyses. The first group comprised of *C. acinaciformis* individuals (Group A; Figure 3.8). One individual of *C. edulis* (#92) and one individual of *C. muirii* (#10) grouped with these individuals. One individual from Mozambique also grouped with *C. acinaciformis* from South Africa, as in the PCO analyses. A second group comprised of *C. edulis* individuals (Group B; Figure 3.8). One individual of *C. acinaciformis* (#32) from South Africa grouped with these individuals. Group B formed a sister group to two individuals of *C. acinaciformis* from South Africa (#24 & #61), and this group in turn was placed as sister to *C.*

acinaciformis comprising Group A. A final group comprised of both *C. deliciosus* and *C. dimidiatus* individuals was placed as sister to Group A and Group B combined. The remaining individuals of *C. acinaciformis, C. muirii, C. quadrifidus* and *C. edulis* were placed in a paraphyletic grade at the base of the tree. The broad geographic location of the areas from which these taxa were collected is indicated by colour codes in Figure 3.8. This shows that the groupings retrieved do not display any major structuring based on geographical proximity.

A N-J tree was also generated for the African taxa using primer combination B1 (Figure 3.9). Three groups were identified as depicted in Figure 3.8. The first group comprised of *C. edulis* individuals (Group A; Figure 3.9). Two individuals of *C. acinaciformis* grouped with these individuals. A second group comprised of both *C. deliciosus* and *C. dimidiatus* individuals (Group B; Figure 3.9). One individual of *C. acinaciformis* grouped with these individuals. A final group comprised of *C. acinaciformis* individuals (Group C; Figure 3.9). Three individuals of *C. edulis* and two individuals of *C. muirii* grouped with these individuals. The remaining individuals of *C. deliciosus*, *C. acinaciformis*, *C. edulis* and *C. quadrifidus* were placed between Group A and Group C. As from the UPGMA analysis these groups also did not display any major structuring based on geographical proximity.

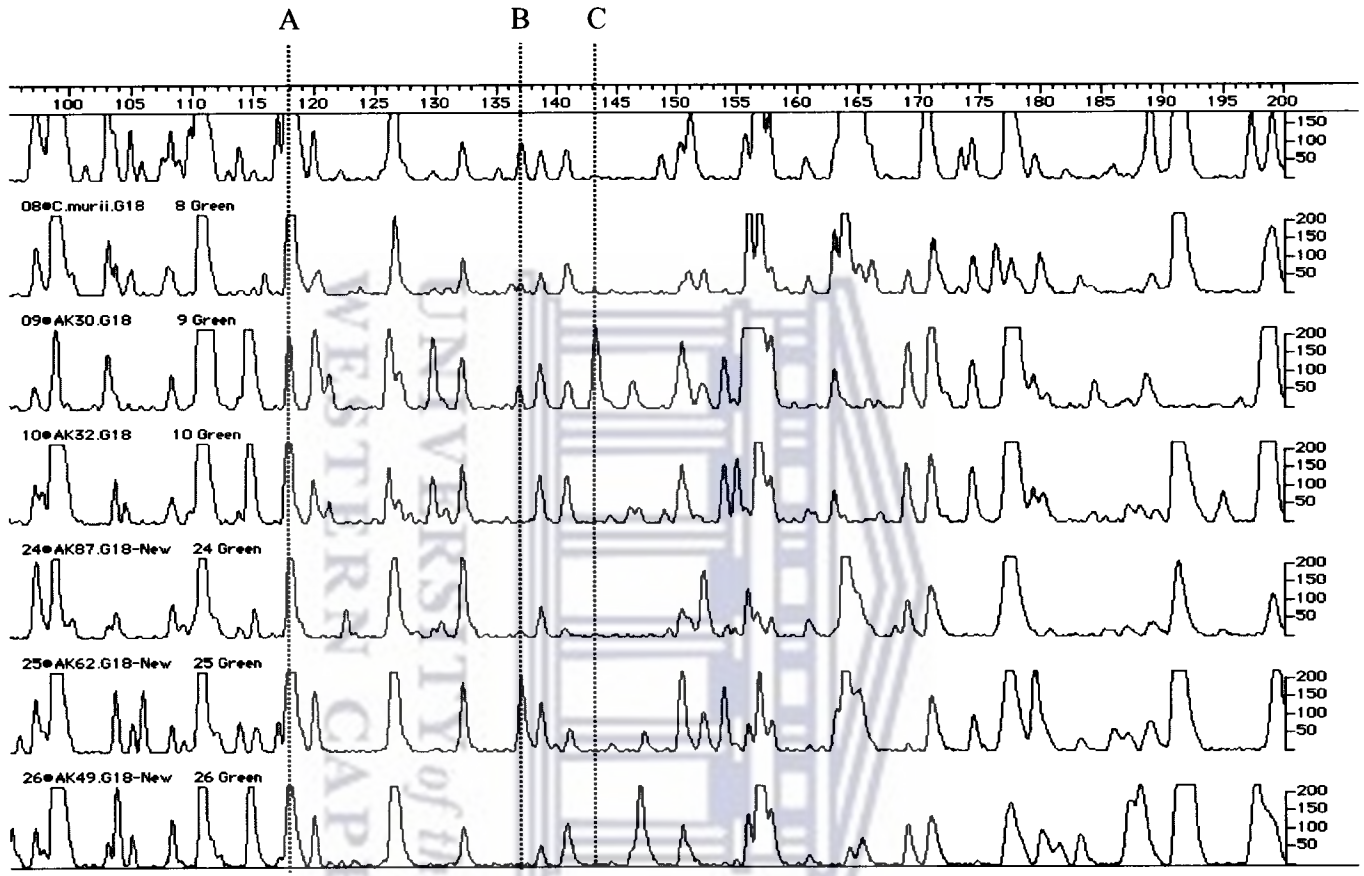


Figure 3.1 Representative traces of *Carpobrotus* AFLP profiles generated using primer EcoRI-CAC MseI-AA (referred to as primer G1 in text). A: represents a fixed band present in all individuals shown here; B: represents a polymorphic band present in a subset of individuals shown here; C: represents a unique (autapomorphic band).

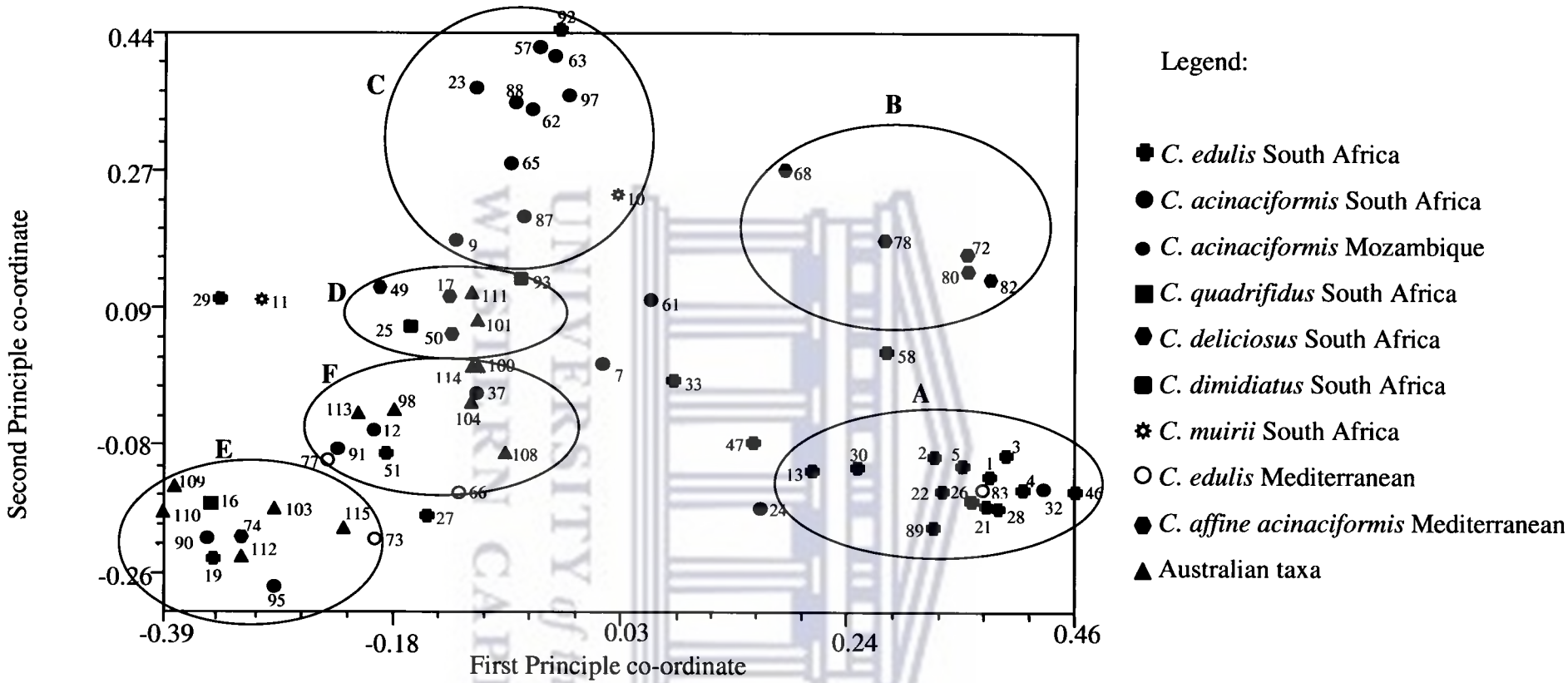


Figure 3.2 Principle co-ordinate analysis using Jaccard's co-efficient of similarity of African, Australian and Mediterranean *Carpobrotus* taxa based upon 254 AFLP markers generated using primer combination B1 with a percentage variance of 20.1 for both axes. The numbers refer to the voucher information in Appendix 1. Six clusters are broadly displayed comprising of Group A, Group B, Group C, Group D, Group E and Group F.

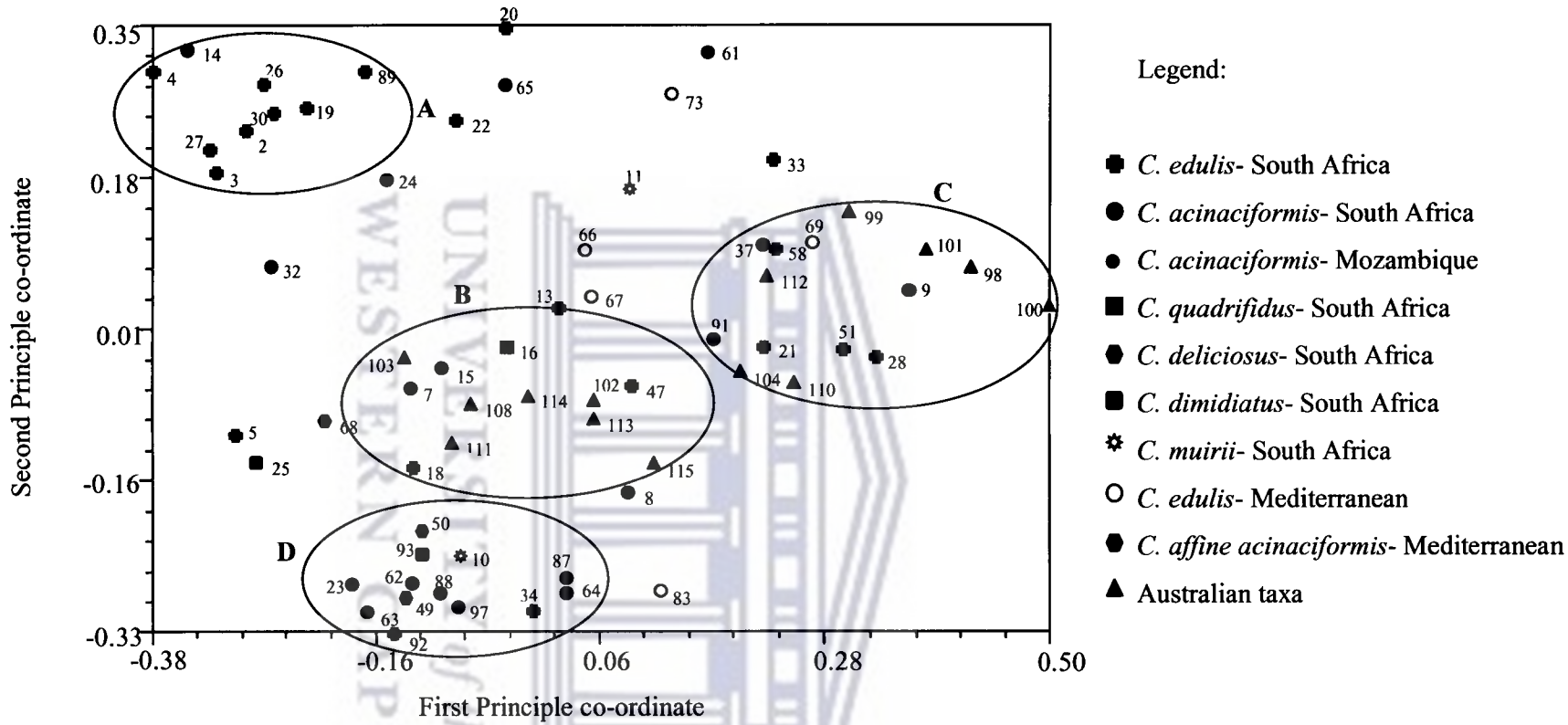


Figure 3.3 Principle co-ordinate analysis using Jaccard's co-efficient of similarity of African, Australian and Mediterranean *Carpobrotus* taxa based upon 199 AFLP markers generated using primer combination G1 with a percentage variance of 16.1 for both axes. The numbers refer to the voucher information in Appendix 1. Four clusters are displayed comprising of Group A, Group B, Group C and Group D.

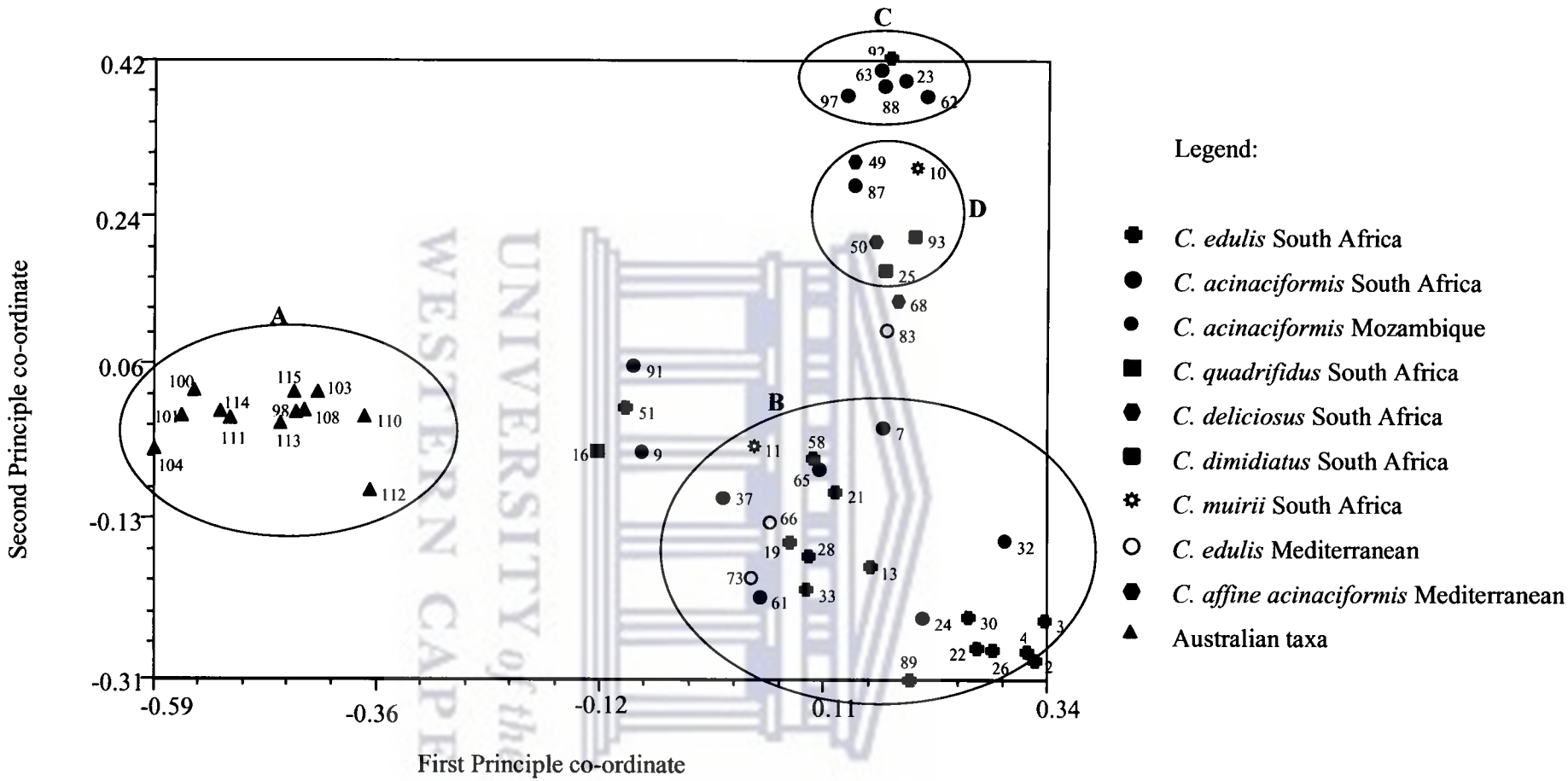


Figure 3.4 Principle co-ordinate analysis using Jaccard's co-efficient of similarity of African, Australian and Mediterranean *Carpobrotus* taxa based upon 480 AFLP markers generated using both B1 and G1 with a percentage variance of 20.7 for both axes. The numbers refer to the voucher information in Appendix 1. Four clusters are displayed comprising of Group A, Group B, Group C and Group D.

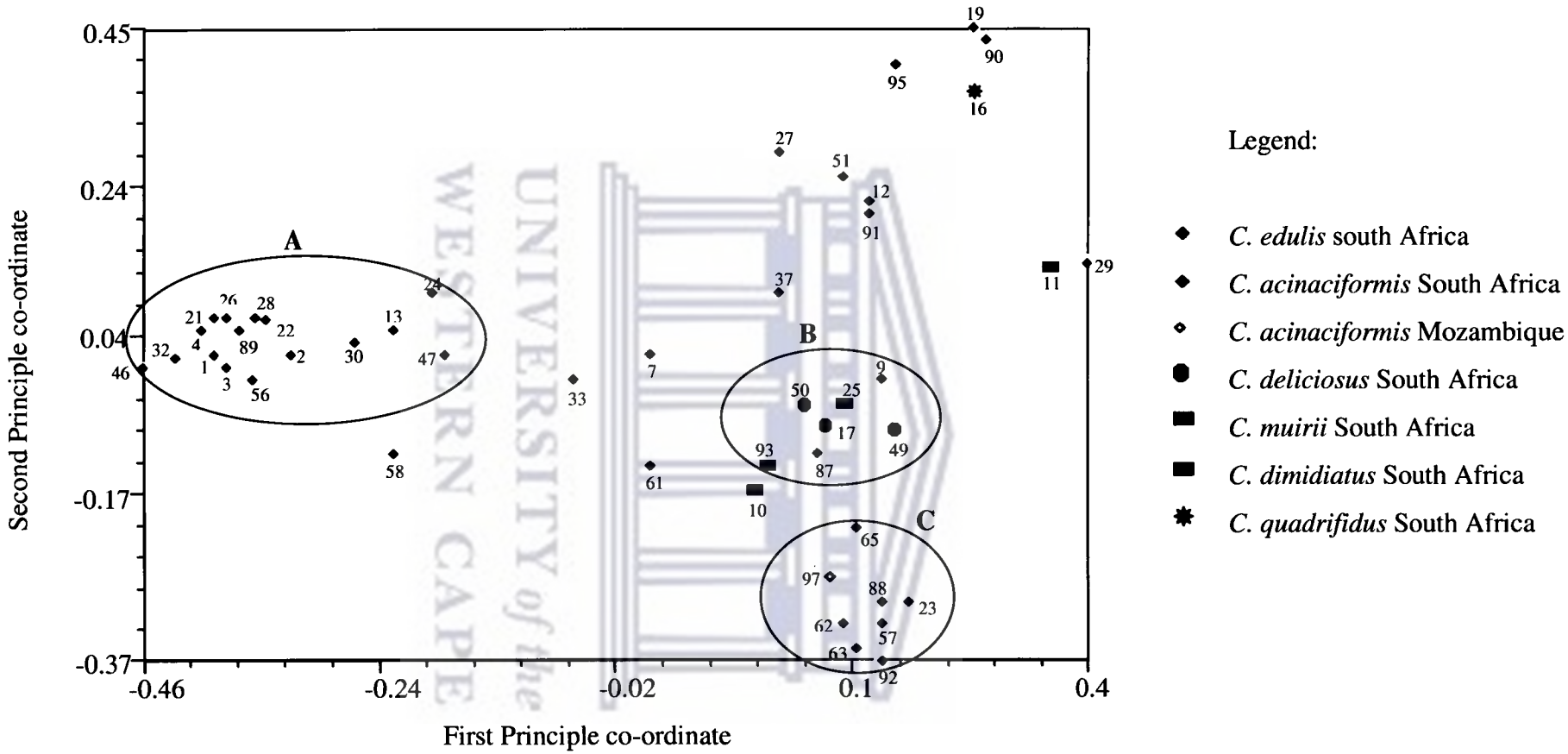


Figure 3.5 Principle co-ordinate analysis using Jaccard's co-efficient of similarity of African *Carpobrotus* taxa based upon 254 AFLP markers generated using primer combination B1 with a percentage variance of 24 for both axes. The numbers refer to the voucher information in Appendix 1. Three clusters are identified comprising of Group A, Group B and Group C.

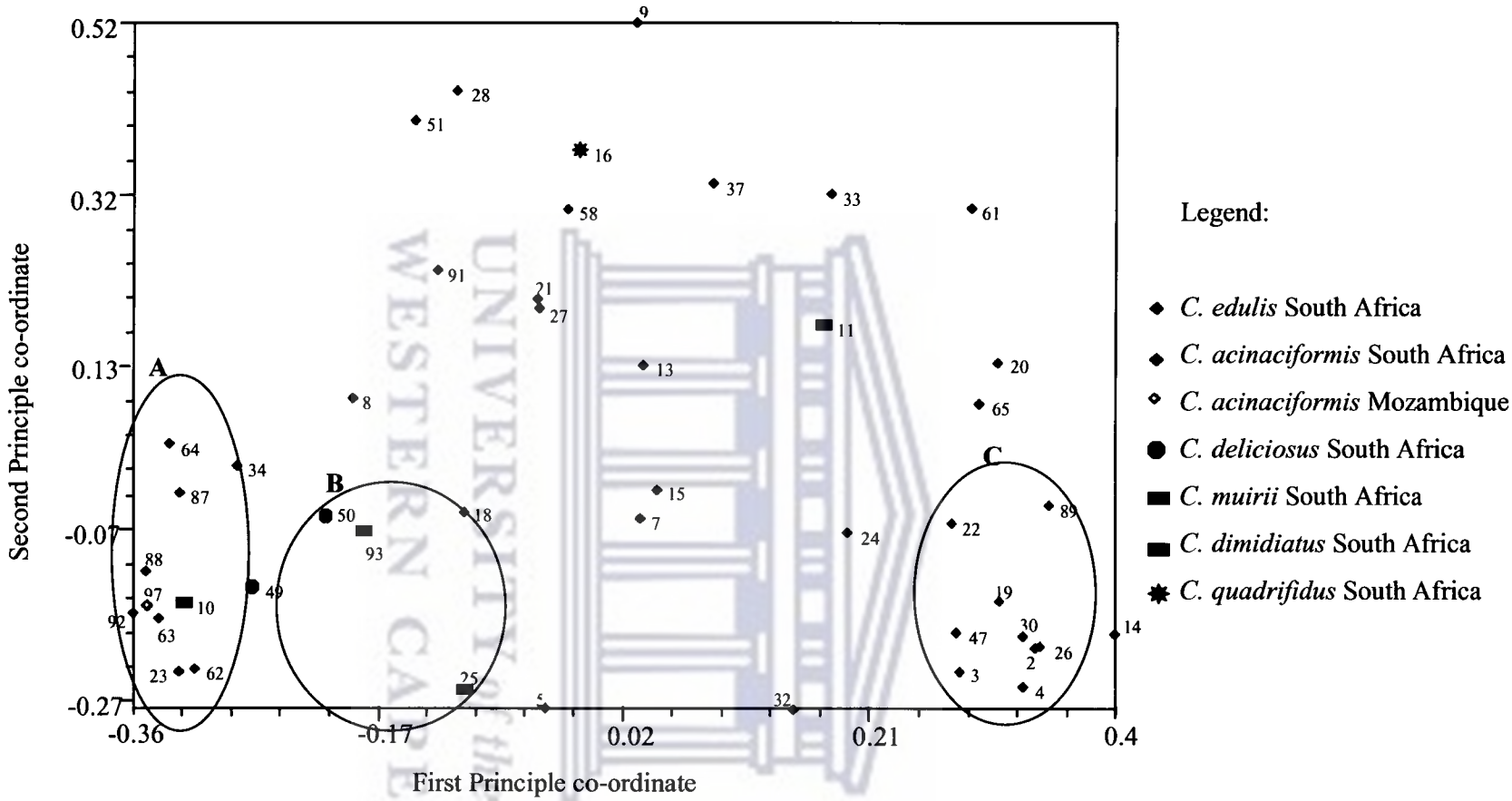


Figure 3.6 Principle co-ordinate analysis using Jaccard's co-efficient of similarity of African *Carpobrotus* taxa based upon 230 AFLP markers generated using primer combination G1 with a percentage variance of 22 for both axes. The numbers refer to the voucher information in Appendix 1. Three clusters are identified comprising of Group A, Group B and Group C.

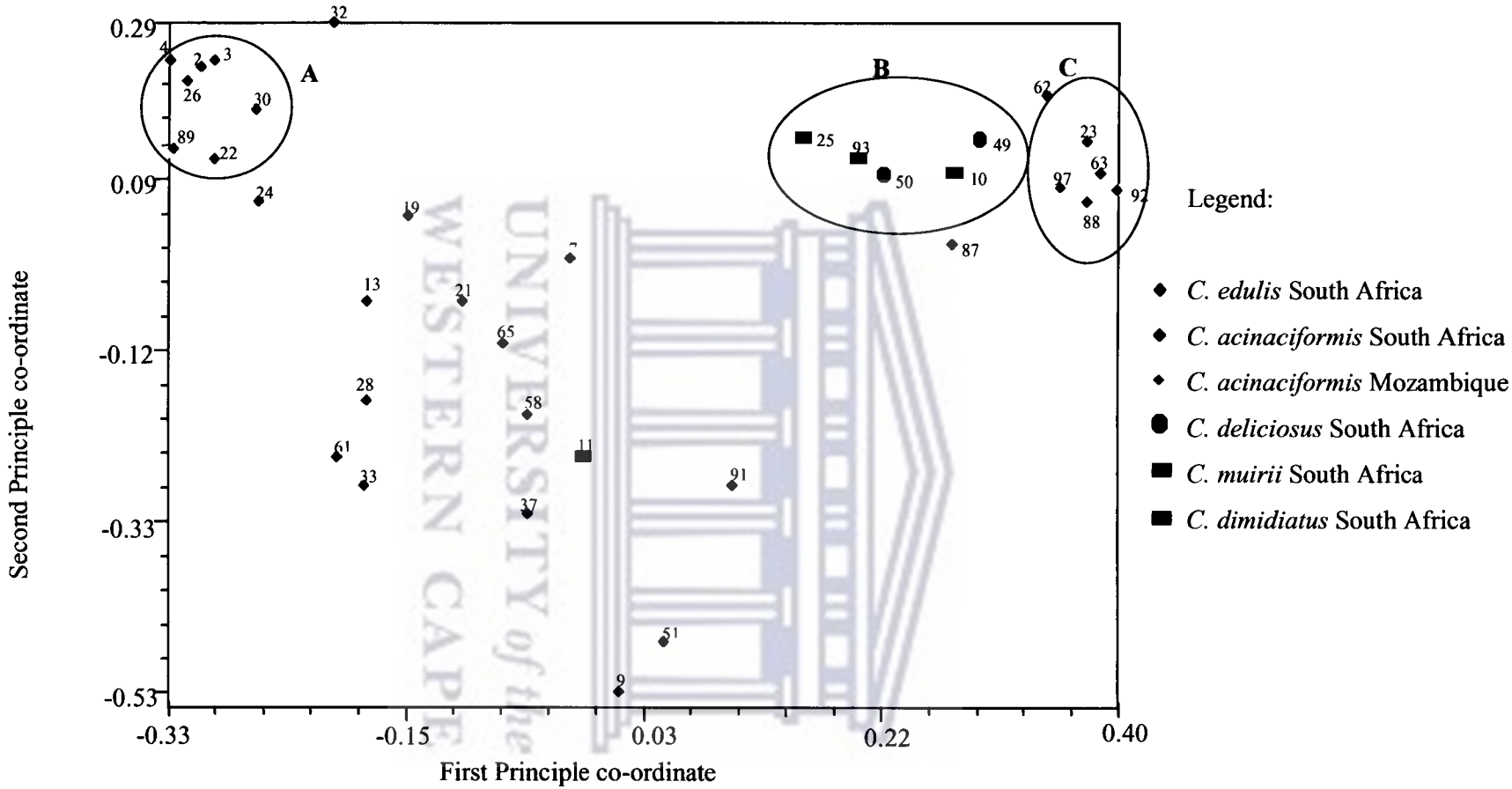


Figure 3.7 Principle co-ordinate analysis using Jaccard's co-efficient of similarity of African *Carpobrotus* taxa based upon 482 AFLP markers generated using primers both B1 and G1 with a percentage variance of 21.7 for both axes. The numbers refer to the voucher information in Appendix 1. Three clusters are identified comprising of Group A, Group B and Group C.

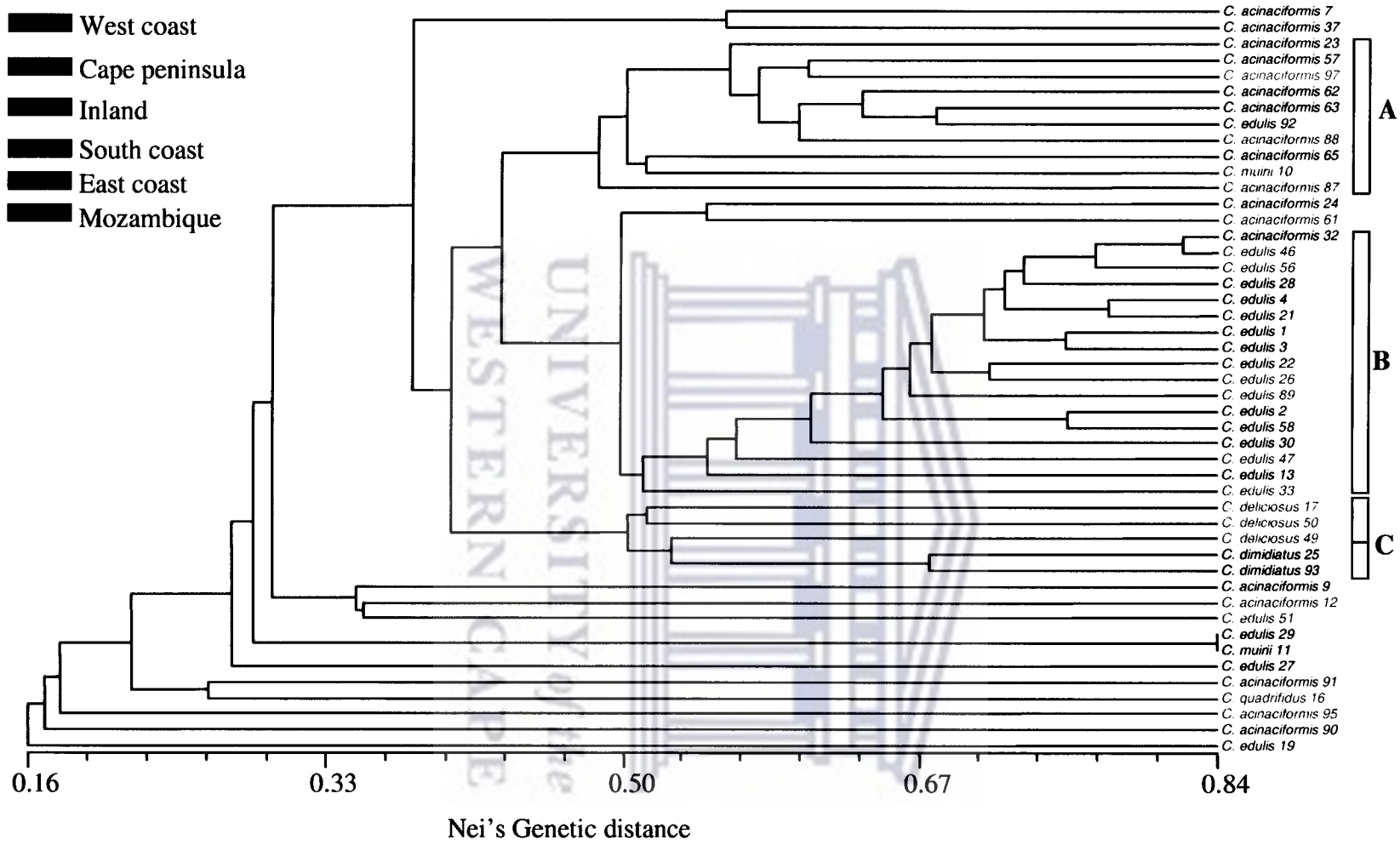


Figure 3.8 Mid-point rooted UPGMA tree using Nei's genetic distance of African *Carpobrotus* individuals based upon 254 AFLP markers generated using primer combination B1. The numbers refer to the voucher information in Appendix 1.

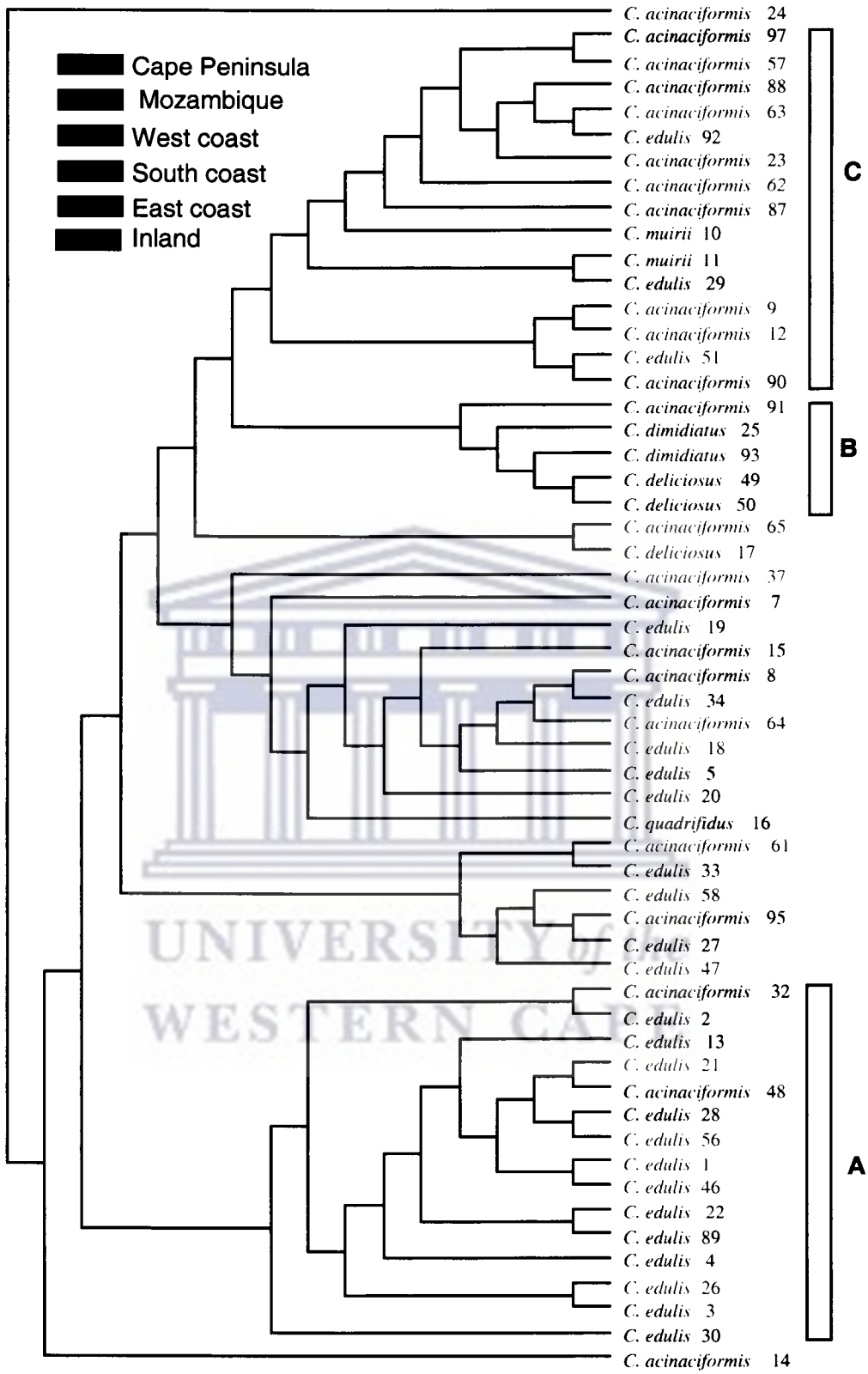


Figure 3.9 Neighbour-Joining tree of Africa *Carpobrotus* taxa based upon 254 AFLP markers generated using primer combination B1. The numbers refer to the voucher information in Appendix 1.

The main aims of this thesis were to elucidate phylogenetic relationships within the genus *Carpobrotus,* and furthermore to investigate levels of genetic variability in two species commonly exploited for their medicinal value (*C. acinaciformis* and *C. edulis*). From the outset, this study attempted to use DNA sequence information from the *psbA-trnH* intergenic spacer to recover species-level relationships within *Carpobrotus*. However, this did not prove to be possible, because sequences from this region were identical for the taxa sampled (with the exception of limited indel activity). *A priori* it is difficult to determine which regions will contain informative characters for a given group, but the *psbA-trnH* intergenic spacer has been shown to provide useful information in cases where other commonly exploited regions (e.g. *trn L-F* region of plastid DNA and ITS of nuclear DNA) have been insufficiently variable. For example Sang *et al.* (1997) found the *psbA-trnH* region to be variable in species-level phylogenetic reconstruction of *Paeonia*.

However, the inability to utilize DNA sequence data for phylogenetic reconstruction in *Carpobrotus* is perhaps not surprising since reconstruction of relationships even among genera of subfamily Ruschioideae (to which *Carpobrotus* belongs) by Klak *et al.* (2003) proved to be largely unmanageable due to a paucity of phylogenetically informative sites. In this study Klak *et al.* (2003) sampled four non-coding regions from the plastid genome (*trnL-F* region, *rps16* intron, *psbA-trnH* intergenic spacer and *atpB-rbcL* intergenic spacer) for 40 genera of the subfamily Ruschioideae but relationships were largely unresolved.

It is unlikely that any DNA regions, for which universal sequencing primers are available, currently exist that will assist in species-level phylogenetic reconstruction within *Carpobrotus*. This may be explained by the recent radiation of the majority of Ruschioideae taxa. Klak *et al.* (2004) have estimated that some 1500 taxa belonging to the tribe Ruschiae have radiated in the last 3.5-8.7 million years. This represents the fastest continental radiation in plants thus far documented. There are now many examples in the literature of taxa that have radiated recently in evolutionary time both from South Africa and elsewhere. These include the rapid and recent origin of species richness in *Phylica* (Richardson *et al.* 2001), and *Inga* (Richardson *et al.* 2001). In all

these cases variability within the DNA sequence data in question was limited making robust phylogenetic inferences difficult or impossible at the species-level.

For this reason, AFLPs were used as a source of phylogenetic information with which to build a species-level phylogenetic tree for *Carpobrotus*. AFLPs are not usually used as a source of characters in phylogeny reconstruction, but this approach is now being used more routinely to address species-level relationships. Due to the variability of AFLP markers and the increasing probability of detected fragments being homoplastic with increasing genetic distance between taxa, only comparisons among closely related taxa are appropriate (Mueller & Wolfenbarger 1999). Nevertheless, Richardson *et al.* (2003) concluded that AFLPs were an extremely useful tool for the purposes of species delimitation in oceanic island representatives of *Phylica*. Similarly, Reeves *et al.* (in press) and Albertson *et al.* (1999) found AFLPs to be extremely useful in reconstruction of species-level relationships in *Protea* and Cichlid fish respectively.

However, there are a number of limitations associated with AFLPs. In some cases the technique may not be informative at taxonomic levels higher than the population level, due to the restriction sites evolving at rates too fast to obtain a good phylogenetic signal (Ulrich *et al.* 1999). Another limitation is that the automated approach makes it difficult to determine whether weak bands are due to noise or represent true bands, and thus eliminating bands that are difficult to score can sometimes be subjective.

The first objective of this part of my study was to carry out an AFLP primer trial, owing to the fact that it was impossible to determine beforehand which primer combinations would provide suitable markers in *Carpobrotus*. In order to achieve this, a trial using 35 primer combinations at the selective amplification stage was performed. Subsequently two primer combinations were chosen to expand the study. As a result AFLP markers were generated for 70 *Carpobrotus*, *Disphyma* and *Sarcozona* taxa using one set of primers (referred to as B1), and 66 taxa using a second combination (referred to as G1).

This study was undertaken with the primary aim of resolving relationships between *Carpobrotus* species in South Africa, and determining species limits by assessing

their genetic distinctness. For conclusions to be drawn regarding species-level relationships, it is a prerequisite that multiple representatives of a species are more similar to one another than they are to their close relatives, i.e. these representatives are required to be monophyletic in a phylogenetic context. However, NJ and UPGMA analyses of the South African *Carpobrotus* species included in this study revealed that coherent groupings for taxa of the same species were not retrieved. Instead, only broad groupings could be identified, comprised of *C. edulis*, *C. acinaciformis*, *C. deliciosus* and *C. dimidiatus* taxa, but many individuals of these species were also placed as intermediate to these groups, or embedded in different species groups.

Despite the fact that these AFLP data have not allowed phylogenetic relationships within *Carpobrotus* to be addressed, some genetic affinities are worth mentioning. UPGMA, NJ and PCO analyses all group *C. deliciosus* and *C. dimidiatus* together. *Carpobrotus dimidiatus* has leaves that are consistently smaller than those of the other southern African members of the genus, a turbiniform receptacle and purplish flowers (50-60 mm in diameter). *C. deliciosus* has a subglobose receptacle and purple flowers (Glen & Wisura 1993). Thus based upon morphological features the only common characteristic that these two species share is flower colour. Although the distribution ranges of these two species do not overlap, they are virtually continuous. *Carpobrotus deliciosus* occurs in the Southern and Eastern Cape and *C. dimidiatus* extends along the coast from the Transkei to Mozambique. Although there is no mention in the literature to suggest that these two taxa may be sister species the genetic data presented here imply that this may be the case.

Carpobrotus muirii and *C. acinaciformis* have been considered to be closely related to each other based on morphological data. *Carpobrotus muirii* has a globose receptacle, narrow leaves and purple flowers, whereas *C. acinaciformis* has robust leaves, 15-25 mm thick, and 60-70 mm wide flowers (Glen & Wisura 1993). In all other respects, *C. muirii* and *C. acinaciformis* are alike. However, in the UPGMA analysis presented here only one of the two included individuals of *C. muirii* grouped nearby with individuals of *C. acinaciformis* (Chapter 3; Figures 3.8). The other individual was placed at the base of the tree with a representative of *C. edulis*. Owing to the fact that *C. muirii* is represented by only two specimens in this study, further sampling will be required to elucidate its affinities to *C. acinaciformis*. *Carpobrotus quadrifidus* was

only represented by a single individual in this study owing to difficulty in collecting this species from Namaqualand, and therefore conclusions regarding the genetic coherence of this species could not be addressed here. With additional time and resources, increased sampling of all the southern African *Carpobrotus* species (with the exception of *C. edulis* and *C. acinaciformis*) would be desirable to further shed light of the genetic affinities of these taxa.

The unclear phylogenetic pattern detected among South African species of *Carpobrotus* may be interpreted as the occurrence of relatively recent or still on-going genetic exchange among most or all of the individuals included. Presence of genetic structure should result from lack of gene flow, caused by either geographical isolation or the breeding system. However, these data do not indicate that gene flow is more frequent within individuals of the same species than among individuals of different species.

As depicted in the NJ and UPGMA figures, these data also show no significant separation of individuals with geographical distance. This lack of correlation between geographical isolation and genetic diversification may suggest that genetic exchange, both within and between species, is still ongoing over large distances. Because these taxa are not genotypically distinct, one possible conclusion may be that they are not appropriately recognized taxonomically. An alternative explanation could be that the intermediate individuals have resulted from hybridization.

Studies by Suehs *et al.* (2001, 2003, 2004) suggest that hybridization has contributed to the successful invasion of *C. edulis* and *C. acinaciformis* in the Mediterranean basin (Suehs *et al.* 2004). Interspecific hybridization can provide new genetic combinations potentially favorable in new environments, especially if hybrid vigor is involved (Abbott 1992). Multilocus isozymic variation and reproductive ecology results by Suehs *et al.* (2001) have strongly suggested that on one site on the island of Bagaud (south-eastern France) invasive *C. acinaciformis* individuals are of hybrid origin (these taxa are now referred to as *C. affine acinaciformis*). Once established it is also thought that these taxa have further generated different introgressed types from *C. edulis* genotypes.

In this study, PCO analysis of *Carpobrotus* taxa from South Africa, Australia and the Mediterranean Basin clearly placed individuals of *C. affine acinaciformis* (sampled from the island of Bagaud) between major groupings comprised of *C. edulis* and *C. acinaciformis* from South Africa (Chapter 3; Figure 3.2). In agreement with the isozyme studies of Suehs *et al.* (2001), these AFLP data also indicate that *C. affine acinaciformis* is of hybrid origin between *C. acinaciformis* and *C. edulis*. Taxa sampled from the Mediterranean Basin purported to be *C. edulis* were also included in this study. However, the pattern associated with the affinity of these taxa to South African *C. edulis* was much less clear. PCO analysis of AFLP markers generated using primer combination B1 (Chapter 3; Figure 3.2) placed only one of the four included representatives of *C. edulis* from the Mediterranean with *C. edulis* from South Africa. PCO analysis of AFLP markers generated using primer combination G1 (Chapter 3; Figure 3.3) did not place any of the five included representatives of *C. edulis* from the Mediterranean with *C. edulis* from South Africa. In the combined PCO analysis of markers from B1 and G1, two of the three included representatives of *C. edulis* from the Mediterranean were grouped with *C. edulis* from South Africa. However, in this latter analysis fewer individuals of *C. edulis* and *C. acinaciformis* were sampled and thus the overall affinities among these taxa were less clear. These AFLP results may suggest that there is also significant introgression within *C. edulis* in the Mediterranean Basin. Hybrid vigor in *C. edulis* may again account for the successful invasion of *Carpobrotus* outside of South Africa.

Natural hybrids have been reported between *C. acinaciformis* and *C. edulis* in South Africa (Wisura and Glen 1993). During my fieldwork, morphological intermediates often made it difficult to distinguish between *C. edulis* and *C. acinaciformis*, and although I did not collect plants from the wild whose identification was dubious, it seems clear from these results that some introgressed individuals of *C. edulis* and *C. acinaciformis* were sampled. Since the distribution ranges of *C. edulis* and *C. acinaciformis* overlap to a large extent it is perhaps not surprising that hybridization is common between the two species. PCO analysis also highlighted taxa that appeared to have been misidentified (Chapter 3, Figure 3.5). One individual of *C. edulis* (#92) was placed with *C. acinaciformis* (Figure 3.5; Group C), and two individuals of *C. acinaciformis* (#24 & #32) were placed with *C. edulis* (Figure 3.5; Group A). However, the herbarium samples corresponding to these individuals were checked,

and based upon morphological features, the identification of these plants appeared correct. In these instances it appears that genetic diversity is not reflected in phenotypic expression.

Suehs *et al.* (2001, 2004) have also reported that *C. affine acinaciformis*, along with all introgressed types from their study in the Mediterranean Basin, showed higher levels of genetic diversity than *C. edulis*. The measures of Shannon's diversity index and Nei's gene diversity for samples of *C. affine acinaciformis* and *C. edulis* from the Mediterranean in this study also show *C. affine acinaciformis* to harbor greater genetic diversity (Chapter 3; Table 2a). However, these measures were lower than those demonstrated for *C. acinaciformis* and *C. edulis* from South Africa. This may be due to the fact that the sampling of individuals from South Africa was much larger than from the Mediterranean, and thus a greater proportion of the available gene pool was sampled from South Africa. Nevertheless, genetic diversity measured for *C. affine acinaciformis* was close to that of *C. acinaciformis* from South Africa - despite there being only six individuals sampled (as compared to 18 from South Africa). This evidence further suggests that *C. affine acinaciformis* from the Mediterranean has gone through significant introgression by comparison with the 'true' *C. acinaciformis* from South Africa.

C. edulis is also found to hybridize with *C. chilensis* in California, and in Australia naturalized *C. edulis* and *C. chilensis* are found to hybridize with native species of *Carpobrotus* (D'Antonio 1998). In addition, intergeneric hybrids have also been observed between *Carpobrotus* and *Disphyma* – the latter is thought to be a close relative to *Carpobrotus* (Chinnock 1972).

Several taxa representative of *Disphyma*, *Sarcozona* and species of *Carpobrotus* native to Australia were included in this study. In the combined PCO analysis of AFLP markers generated using primer combinations B1 and G1, the Australian taxa formed a single group separate from the African and Mediterranean taxa. One surprising outcome therefore, was that the Australian species of *Carpobrotus* in this analysis did not group with the African species of *Carpobrotus*, but were more genetically similar to *Disphyma* and *Sarcozona* from Australia. In addition, no groupings within the Australian taxa could be identified, meaning that the three genera in question were not found to be genetically coherent. It seems clear that an

expanded study should include more representatives of these Australian species and closely allied genera. These preliminary results would suggest that not only are species within *Carpobrotus* genetically indistinct, but that members of this genus may still be exchanging genetic material with species of closely related genera. This may further complicate the quest to unravel species delimitations and hybridization in *Carpobrotus.*

It is not known how *Carpobrotus* arrived in Australia, but species of this genus have fleshy indehiscent friuts that are embedded in a mucilaginous substance - attractive to birds and mammals (D'Antonio 1998: Zedler & Scheid 1988). These fruits also have a high percentage of germination after passing through the digestive system of animals (D'Antonia *et al.* 1993). Additionally, human use of *Carpobrotus* as a food resource, and for medicinal and commercial purposes, may have aided dispersal of *Carpobrotus* to different countries, especially Australia. Subsequent geographical isolation may then have allowed the Australian species to diverge from members of the genus in other parts of the world.

The AFLP technique has been applied to a number of questions regarding hybridization. For example O'Hanlon *et al.* (1999) used AFLPs to compare genetic diversity in invasive forms of *Onopordum* (Compositae) from Australia with several know native European species. These species were introduced into Australia as ornamental plants and subsequently became major pasture weeds. Their analysis showed that a full range of genetic intermediates between *O. acanthium* and *O. illyricum* were present in Australia. They concluded that the current genetic patterns in Australia may be best explained by a combination of processes including introduction of hybrid material, and ongoing contact among hybridizing taxa.

In another study involving *Mangifera odorata* (Anacardiaceae; Teo *et al.* 2002) - a well-known fruit cultivated in Southeast Asia, AFLPs were applied to determine whether *M. odorata* was a hybrid between *M. indica* and *M. foetida.* Both *M. indica* and *M. foetida* were shown to possess unique AFLP bands, whereas *M. odorata* had additively inherited bands specific to *M. indica* and *M. foetida.* Teo *et al.* (2002) thus concluded that this evidence strongly suggests *M. odorata* to be of hybrid origin.

From this study, it is clear that the species that are commonly used in South Africa for medicinal purposes (namely *C. acinaciformis* and *C. edulis*) do hybridize freely. This is supported by observations based upon morphology and genetic identity, whereby the two species are often difficult to differentiate. In this respect it is possible that the medicinal qualities of different populations within the two species might differ to a great extent, depending on their geographic origin and degree of introgression. This may suggest that, in a bio-prospecting context, caution needs to be taken when sampling these plants in the wild. For example, Springfield *et al.* (2003), in their anti-microbial studies sampled an individual they thought to be *C. quadrifidus*. However, the sample was later identified as *C. acinaciformis*. In this case only morphological data was used to correctly identify the specimen in question but in future it may be possible to use genetic information to identify clones, or groups of similar individuals that have been shown to be medicinally useful

Appendix 1: *Carpobrotus* taxa used in this analysis, with voucher information and AFLP profiles obtained for each primer combination.

(-indicates that AFLP data were generated for this taxon). Primer combinations Mse1CAC + EcoR1TC (FAM) and Mse1CAC + EcoR1AA (JOE) are referred as B1 and G1 respectively.

Taxon	Country/ Region	Locality	Voucher information	Primer combination G1	Primer combination B1
Carpobrotus acinaciformis	South Africa/ West coast	Witklip	*Khunou AK7 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ West coast	Langebaan	*Khunou AK8 NBG*	√	-
Carpobrotus acinaciformis	South Africa/ West coast	Cape Flats Nature Reserve	*Khunou AK9 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ Inland	Montagu	*Khunou AK12 NBG*	-	√
Carpobrotus acinaciformis	South Africa/ West coast	Bloubergstrand	*Khunou AK14 NBG*	√	-
Carpobrotus acinaciformis	South Africa/ West coast	Mykonos	*Khunou AK15 NBG*	√	-
Carpobrotus acinaciformis	South Africa/ Cape Peninsula	Slangkop	*Khunou AK23 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ Cape Peninsula	Mistycliff	*Khunou AK24 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ West coast	Velddrif	*Khunou AK32 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ Cape Peninsula	Cape Point Nature Reserve	*Khunou AK37 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ South coast	Grootbrakrivier	*Khunou AK48 NBG*	-	-
Carpobrotus acinaciformis	South Africa/ Cape Peninsula	Boulders beach	*Khunou AK57 NBG*	-	√
Carpobrotus acinaciformis	South Africa/ Inland	Karbonkelberg	*Khunou AK61 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ West coast	Cape Flats Nature Reserve	*Khunou AK62 NBG*	√	√

Taxon	Country/ Region	Locality	Voucher information	Primer combination G1	Primer combination B1
Carpobrotus acinaciformis	South Africa/ West coast	Cape Flats Nature Reserve	*Khunou AK63 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ West coast	Cape Flats Nature Reserve	*Khunou AK64 NBG*	√	-
Carpobrotus acinaciformis	South Africa/ West coast	Cape Flats Nature Reserve	*Khunou AK65 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ South coast	Bredasdorp	*Khunou AK87 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ South coast	Stanford	*Khunou AK88 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ Cape Peninsula	Olifantsbos	*Khunou AK90 NBG*	-	√
Carpobrotus acinaciformis	South Africa/ Cape Peninsula	Scarborough	*Khunou AK91 NBG*	√	√
Carpobrotus acinaciformis	South Africa/ South coast	Potberg	*Khunou AK95 NBG*	-	√
Carpobrotus acinaciformis	Mozambique	Guinjata Bay	*Khunou AK97 NBG*	√	√
Carpobrotus affine acinaciformis	France/ Baguad	Unknown	*Carey Suehs 3; AK68 NBG*	√	√
Carpobrotus affine acinaciformis	France/ Baguad	Unknown	*Carey Suehs 5; AK72 NBG*	-	√
Carpobrotus affine acinaciformis	France/ Baguad	Unknown	*Carey Suehs 7; AK74 NBG*	-	√
Carpobrotus affine acinaciformis	France/ Baguad	Unknown	*Carey Suehs 9; AK 78 NBG*	-	√
Carpobrotus affine acinaciformis	France/ Baguad	Unknown	*Carey Suehs 10; AK80 NBG*	-	√
Carpobrotus affine acinaciformis	France/ Baguad	Unknown	*Carey Suehs 11; AK82 NBG*	-	√
Carpobrotus chilensis	Australia	Unknown	*Toelkom 9451;*	√	√

Taxon	Country/ Region	Locality	Voucher information	Primer combination G1	Primer combination B1
			AK103 NBG		
Carpobrotus chilensis x *Carpobrotus rossi*	Australia	Unknown	*Toelkom 9453; AK108 NBG*	√	√
Carpobrotus deliciosus	South Africa/ South coast	University of Port Elizabeth Nature Reserve	*Khunou AK17 NBG*	-	√
Carpobrotus deliciosus	South Africa/ South coast	Wilderness, George	*Khunou AK49 NBG*	√	√
Carpobrotus deliciosus	South Africa/ South coast	Wilderness, George	*Khunou AK50 NBG*	√	√
Carpobrotus dimidiatus	South Africa/ East coast	Umkobi beach	*Khunou AK25 NBG*	√	√
Carpobrotus dimidiatus	South Africa/ East coast	Umhlanga Nature Reserve	*Khunou AK93 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Cape Flats Nature Reserve	*Khunou AK1 NBG*	-	√
Carpobrotus edulis	South Africa/ West coast	Vredenburg	*Khunou AK2 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Sunset beach, Milnerton	*Khunou AK3 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Ganskraal	*Khunou AK4 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Langebaan	*Khunou AK5 NBG*	√	-
Carpobrotus edulis	South Africa/ Inland	Houwhoek	*Khunou AK6 NBG*	-	-
Carpobrotus edulis	South Africa/ West coast	Bloubergstrand	*Khunou AK13 NBG*	√	√
Carpobrotus edulis	South Africa/Cape Peninsula	Fish Hoek	*Khunou AK18 NBG*	√	-
Carpobrotus edulis	South Africa/ Cape Peninsula	Olifantsbos	*Khunou AK19 NBG*	√	√
Carpobrotus edulis	South Africa/ Cape	Olifantsbos	*Khunou AK20 NBG*	√	-

Taxon	Country/ Region	Locality	Voucher information	Primer combination G1	Primer combination B1
	Peninsula				
Carpobrotus edulis	South Africa/ Cape Peninsula	Teeberg	*Khunou AK21 NBG*	√	√
Carpobrotus edulis	South Africa/ Cape Peninsula	Silvermine	*Khunou AK22 NBG*	√	√
Carpobrotus edulis	South Africa/ Inland	Piekenierskloof Pass	*Khunou AK26 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Saldanha	*Khunou AK27 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Saldanha	*Khunou AK28 NBG*	√	√
Carpobrotus edulis	South Africa/ West coast	Mykonos	*Khunou AK29 NBG*	-	√
Carpobrotus edulis	South Africa/ West coast	Koeberg Nature Reserve	*Khunou AK30 NBG*	√	√
Carpobrotus edulis	South Africa/ South coast	Bredasdorp	*Khunou AK33 NBG*	√	√
Carpobrotus edulis	South Africa/ South coast	Bredasdorp	*Khunou AK34 NBG*	√	-
Carpobrotus edulis	South Africa/ Inland	Worcester	*Khunou AK46 NBG*	-	√
Carpobrotus edulis	South Africa/ Inland	Robertson	*Khunou AK47 NBG*	√	√
Carpobrotus edulis	South Africa/ South coast	Kleinbrak rivier	*Khunou AK51 NBG*	√	√
Carpobrotus edulis	South Africa/ Inland	Riversdale	*Khunou AK56 NBG*	-	√
Carpobrotus edulis	South Africa/ Cape Peninsula	Cape Point Nature Reserve	*Khunou AK58 NBG*	√	√
Carpobrotus edulis	South Africa/ South coast	Hermanus	*Khunou AK89 NBG*	√	√
Carpobrotus edulis	South Africa/ Cape Peninsula	Scarborough	*Khunou AK92 NBG*	√	√
Carpobrotus edulis	France/ Baguad	Unknown	*Carey Suehs 4; AK69 NBG*	√	-
Carpobrotus edulis	France/ Baguad	Unknown	*Carey Suehs 6; AK73 NBG*	√	√

Taxon	Country/ Region	Locality	Voucher information	Primer combination G1	Primer combination B1
Carpobrotus edulis	France/ Baguad	Unknown	*Carey Suehs 8; AK77 NBG*	-	√
Carpobrotus edulis	France/ Baguad	Unknown	*Carey Suehs 12; AK83 NBG*	√	√
Carpobrotus edulis	France/ Baguad	Unknown	*Carey Suehs1; AK66 NBG*	√	√
Carpobrotus edulis	France/ Baguad	Unknown	*CareySuehs 2; AK67 NBG*	√	-
Carpobrotus edulis	Australia	Unknown	*Toelkom 9449*; AK 99 *NBG*	√	-
Carpobrotus edulis	Australia	Unknown	*SDH8574; AK105 NBG*	-	-
Carpobrotus modestus	Australia	Unknown	*Toelkom 9445; AK100 NBG*	√	√
Carpobrotus modestus	Australia	Unknown	*R. J. Chinnock 9568;AK111 NBG*	√	√
Carpobrotus muirii	South Africa/ South coast	Bredasdrop	*Khunou AK10 NBG*	√	√
Carpobrotus muirii	South Africa/ South coast	Pauline Böhen Nature Reserve, Stilbaai	*Khunou AK11 NBG*	√	√
Carpobrotus quadrifidus	South Africa/ Inland	Bitterfontein	*Khunou AK16 NBG*	√	√
Carpobrotus rossii	Australia	Unknown	*Toelkom 9452; AK102 NBG*	√	-
Carpobrotus rossii	Australia	Unknown	*S. D. Hopper 8574; AK114 NBG*	√	√
Carpobrotus rossii	Australia	Unknown	*Toelkom 9446;AK101 NBG*	√	√
Disphym australe	Australia	Unknown	*R. J. Chinnock*	√	√

Taxon	Country/ Region	Locality	Voucher information	Primer combination G1	Primer combination B1
			9566;AK113 NBG		
Disphym australe	Australia	Unknown	*R. J Chinnock 9420;AK110 NBG*	√	√
Disphyma crassifolium	Australia	Unknown	*R. J. Chinnock 9564; AK109 NBG*	-	√
Disphyma papillatum	Australia	Unknown	*R. J. Chinnock 9567; AK104 NBG*	√	√
Sarcozona bicarinata	Australia	Unknown	*R. Taylor 854; AK112 NBG*	√	√
Sarcozona praecox	Australia	Unknown	*Augusta o'Leany 3348;AK 98 NBG*	√	√
Sarcozona praecox	Australia	Unknown	*D. E Symon 17030; AK115 NBG*	√	√

Appendix 2: Aligned *psbA-trnH* intergenic spacer sequences for sixteen *Carpobrotus* taxa

SA = South Africa, AUS = Australia and Med = Mediterranean Basin.

*Carpobrotus edulis*_SA TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus acinaciformis*_SA TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus muirii*_SA TCCCTCTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus deliciosus*_SA TTTCCATAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus dimidiatus*_SA TCTTCATAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carporotus quadrifidus*_SA ??????????????????????????TGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Sarcozina bicarinata*_AUS TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Sarcozona praecox*_AUS TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus modestus*_AUS TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobotus rossii*_AUS TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus edulis*_AUS ??TGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus chilensis*_AUS TCCCTTTAGACCTAGCTGCTATCGAAGCTCCATCTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus edulis1*_Med TCCCTCTAGACCTAGCTGCTATTGAAGCTCCATTTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus edulis2*_Med TCCCTCTAGACCTAGCTGCTATTGAAGCTCCATTTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus affine acinaciformis1*_Med TCCCTTTAGACCTAGCTGCTATTGAAGCTCCATTTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA
*Carpobrotus affine acinaciformis2*_Med TCCCTTTAGACCTAGCTGCTATTGAAGCTCCATTTACAAATGGATAAATTTTCGTTTTTTGTTTAGTTTAGTGTATACGAGTTA

Carpobrotus edulis_SA	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus acinaciformis*_SA	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus muirii*_SA	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus deliciosus*_SA	TTGAAAGTAAAGGGGGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTCTTC
*Carpobrotus dimidiatus*_SA	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carporotus quadrifidus*_SA	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Sarcozona bicarinata*_AUS	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Sarcozona praecox*_AUS	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus modestus*_AUS	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobotus rossii*_AUS	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus edulis*_AUS	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus chilensis*_AUS	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus edulis1*_Med	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus edulis2*_Med	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus affine acinaciformis1*_Med	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC
*Carpobrotus affine acinaciformis2*_Med	TTGAAAGTAAAGGGGCAGTACCGATTTCTTGAAAGAACAAGAAATTGGTAATTGCTCCTTTCCTTGTTTTGAATTTACTTC

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Summary

During the course of this thesis, AFLP technique was successfully applied to the Aizoaceae taxa. This study involved a substantial component of laboratory work including generating and analysing AFLP data, the operation of an ABI377 automated DNA sequencer, and use of GeneScan and Genotyper software (Applied Biosystems).

The initial motivation for this study was to reconstruct relationships between species of the genus *Carpobrotus*. However, it soon became clear that this would be impossible via the use of DNA sequence data. This was due to the inability to find DNA regions with sufficient variable characters for phylogenetic reconstruction.

Therefore applying the AFLP fingerprinting technique to the question of species-level relationships in *Carpobrotus* resulted in individuals of the same species not forming distinct groups. This has subsequently led to the conclusion that gene flow is common across species boundaries in *Carpobrotus* as a result of hybridisation. An alternative explanation for the pattern observed may be that current species delimitations in this group are not well defined. These results also concord well with isozyme studies carried out for *Carpobrotus* taxa in the Mediterranean Basin. Increased sampling of species other than *C. acinaciformis* and *C. edulis* would allow further investigation into the genetic delimitation and affinities of *Carpobrotus,* and shed light on the extent of gene flow between these other taxa.

In the biotechnological age the use of molecular techniques has the potential to greatly improve the bio-prospecting process. This has particular relevance to plants with medicinal properties, such as *Carpobrotus*, whereby targeted selection of genotypes with well-characterised medicinal properties may increase efficiency. Genetic and phylogenetic information can also help to identify closely related species and genera that may share similar metabolic pathways and properties. These issues are quickly coming to the forefront in the new South Africa, with the opening up of traditional knowledge systems, and subsequent integration and contact with the biotechnology community.