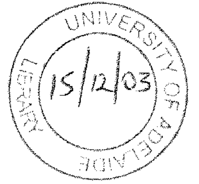

A study into the domestication of *Solanum centrale*, Australian bush tomato

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Summary

Solanum centrale L. the Australian bush tomato is a perennial, undershrub that is geographically restricted and a rare species. Mainly used as flavouring in value added products, this species is showing promise in both domestic and overseas markets. The supply of wild bush tomatoes is variable and hence unreliable. This has prompted the establishment of a number of commercial plantations in Australia to meet market demands.

The steps towards domestication covered by this study included improving methods of propagation, investigating the breeding system of the species and developing hybridisation techniques, investigating potential steroidal alkaloids in fruit, studying morphological and genetic diversity of *S. centrale* populations and identifying molecular markers for desirable traits. No serious domestication of *S. centrale* had taken place previously.

Vegetative methods of propagation were explored. Rooted cuttings with at least 90% survival rate were achieved with IBA at 3 000 and 8 000 ppm. A preliminary investigation of the reproductive biology of *S. centrale* with a view to artificial hybridisation was carried out. High levels of variability in the fertility of the plant were identified. This study indicated self-incompatibility and that this species can outcross under natural and artificial conditions. A controlled pollination method was developed.

The presence of steroidal alkaloids in the leaves and fruit was investigated. A number of methods of extraction and analysis were tested, confirming the presence of alkaloids in the immature fruit and leaves, but not in the mature fruit.

The pattern of morphological and genetic variability was investigated using plants grown from seeds collected from various wild populations found in natural habitats in Australia. Eight vegetative and floral characters were used with morphological data analysed using the hierarchical clustering method, unweighted pair group

arithmetic averaging (UPGMA) and the non-hierarchical ordination methods multidimensional scaling (MDS) and principal component analysis (PCA). The results for the population study of 100 individuals showed a high degree of morphological variation. For the study involving 10 isolated populations 10 clusters were produced each corresponding closely with the 10 different populations.

Diversity within the species was further investigated using RAPD-PCR, and analysed using hierarchical and non-hierarchical distance methods. Samples of DNA from individual plants were amplified with six different 10-mer primers to produce RAPD fragments. One hundred individual plants were selected and their DNA fingerprints compared. These were used to generate an UPGMA dendrogram based on similarity, an ordination derived by MDS and a minimum spanning tree (MST) to show the relative dissimilarities between the individuals tested.

The data subjected to MDS showed the presence of ten molecular clusters matching a dendrogram constructed using the simple matching coefficient with UPGMA clustering. The ten molecular clusters were significantly different. Each cluster consisted of the ten individuals from each of the populations investigated suggesting that there was a significant genetic differentiation between populations. The distribution of the clusters suggests that the gene flow and therefore pollination was localised within the populations.

For any crop species, the gains that can be made from selection depend to a large extent on the genetic variability of the population. The genetic similarities found between the 100 individuals from one population varied from 72% to 95%, confirming the existence of high genetic diversity in the gene pool. Two molecular clusters were identified, neither of which was significantly different indicating that random gene flow was a feature of this population.

A RAPD marker linked to non-prickliness was identified by bulked segregant analysis (BSA). To increase the utility of the RAPD marker for non-prickliness, it was converted to a sequence tagged site by developing primers specific for the sequence of the RAPD band. These primers were used to screen *S. centrale*

individuals for non-prickliness. This marker may facilitate the management of *S. centrale* breeding and selection for the bushfood industry, by providing an initial screening for non-prickliness.

This study contributed to the knowledge essential for further improvement of *Solanum centrale* as a commercial crop.

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Chapter 1 General Introduction

Australia's unique landscapes and climates, combined with its isolation, had large impacts on the development of the associated extant flora. While Aboriginals utilised the native flora as a food source for thousands of years, this so-called 'bushfood' is something of a novelty in modern Australia. Recently Australian bushfoods have increased in popularity, with more adventurous consumers seeking new flavours and textures, based on the concept of a truly Australian cuisine. Thus, there is the potential to develop a highly organized industry, based around commercial plantations. Currently the availability and price of wild food is highly variable as supply is often limited to several seasonal weeks, and is constrained by the size of the harvest from wild populations, supplemented by a limited amount of cultivated product. There is significant export potential for Australian bushfood, as demonstrated by sales of quandong flesh overseas of approximately $0.5 M dollars (Graham and Hart, 1998). Further research into Australian bushfoods is required to improve productivity and consistency of such crops (Latz, 1995).

One Australian endemic plant with potential as a commercial bushfood is *Solanum centrale* (Solanaceae), the Australian bush tomato. Mainly used as flavouring in value added products, this species is showing promise in both domestic and overseas markets. The supply of wild bush tomatoes is variable and hence unreliable. This has prompted the establishment of a number of commercial plantations in Australia to meet market demands. The establishment of these plantations has highlighted a number of domestication problems associated with commercial production.

These problems include:

- variability in prickliness, which results in handling difficulties,
- variability in yield and size of the fruit (D. Symon, pers. comm.),
- poor fruit set and development (A. Beal, pers. comm.),

- variability in fruit alkaloid content throughout the growing season. Alkaloids have been selected against in the cultivated potato *Solanum tuberosum* (Correll, 1962), such that levels are generally below the acceptable maximum of 200 mg/kg^{-1} fresh weight.

Seeds of bush tomato are collected from wild populations throughout Australia, providing a high level of genetic diversity and the potential for selecting superior plants for desirable characters. Knowledge of diversity within *S. centrale* populations is poor, and this project aims to identify quality characters of potential value in future breeding programs.

1.1 Australian Bushfood and its commercial potential

Bushfood is a term commonly used by Aboriginals and Europeans alike to describe foods obtained from native plants and animals. In central Australia, plants provided the bulk of the food for Aboriginals prior to European arrival. They utilised about 200 species for a great variety of purposes, including material for implements, medicines, narcotics and decoratigns. Only a few species formed a staple bulk food supply. Most bushfoods are currently considered to be an acquired taste by western cultures.

Aboriginals practised low-intensity agriculture using fire to promote the growth of many edible plants. The adaptation of these plants to fire has important implications for commercial cultivation. For example, bush tomato seeds germinate more readily when exposed to water saturated with wood smoke, compared to water alone (Griffin, 1985). Many bushfood plants grow in fire-prone habitats such as spinifex plains. They are usually most abundant in the first few years after fire, before other plants re-establish dominance. This suggests that many bushfood species prefer open, sunny, well-drained positions some even preferring disturbed situations. Other bushfood plants have evolved in habitats where fires seldom occur, such as rocky cliff faces and these plants can be cultivated in different situations than those required for fire-tolerant species (Griffin, 1985).

Bushfoods were introduced to restaurants in the mid-1980s and the range has been steadily expanding since. The broad range of endemic plants currently exploited for bushfood is shown in Table 1.1. Current species selection has been strongly influenced by culinary successes from restaurants specialising in Australian native cuisine. Further development of the bushfood industry may offer a commercial supplement to marginal agricultural enterprises, both irrigated and dryland. In the short term, the industry has potential to offer Landcare groups, Aboriginal communities, farmers, bush regenerators, and local councils alike, economic returns on revegetation programs with incentives to preserve wild populations. The limited existing bushfood industry is already making an impact on the survival of species in some areas. For this industry to prosper it must be established as a secure, long-term commercial activity (McCarthy, 1995).

Table 1.1 Australian plant species commonly used for food production (Graham and Hart, 1997).

Scientific Name	Common Name	Product	End Use
Acacia victoriae	Wattle Seed	cleaned seed	flour of varying quality
Santalum acuminatum	Quandong	fresh and dried fruit	sweet and savoury dishes
Syzygium australe	Lilly Pilly	fresh fruit	jams and sauces
Solanum centrale	Bush tomato	dried fruit	intense flavouring in savoury dishes
Podocarpus elatus	Illawarra Plum	fresh fruit	sweet and savoury dishes
Terminalia ferdinandiana	Kakadu Plum	fresh fruit	sweet dishes such as jams and sauces
Acronychia acidula	Lemon Aspen	fresh fruit	replacement for citrus/lemon flavour
Backhousia citriodora	Lemon Myrtle	fresh and dried leaf	teas, sorbets, asian dishes, meat dressings
Kunzea pomifera	Muntries, Munthari	fresh fruit	sweet dishes, similar flavour to apple
Prostanthera rotundifolia	Native Mint	fresh and dried leaf	Replacement for mint flavour
Tasmannia lanceolata	Native Mountain Pepper	dried leaf berry	intense flavour used as coarsely cracked pepper
Syzygium leuhmannii	Riberry	fresh fruit	sweet dishes and flavouring to meat dishes
Eremocitrus glauca	Wild Lime	fresh fruit	flavouring to sweet and savoury dishes
Hibiscus sabdariffa	Wild Rosella	fresh flower	petals make jelly and used as dessert garnish
Hibiscus heterophyllus	Native Rosella	fresh flower	same as wild rosella, tart flavour
Davidsonia pruriens	Davidson Plum	fresh fruit	sweet dishes
Tetragonia tetragonioides	Warrigal greens	fresh leaf	salad vegetable in place of baby spinach

Analysis of Australian bushfoods (Brand *et al.*, 1983) has shown that they are often richer in vitamins, trace elements and protein than similar 'traditional crops'. For example, many wild fruits have high protein, fat and carbohydrate levels, compared with apples, pears and stone fruits. The main plant-derived foods eaten by Aboriginals, were berries, grains, seeds, fruits of the native tomato and other *Solanum* species, wild fig, and quandong (Issacs, 1987).

Bushfood products are generally marketed through retail specialty food stores with distributors supplying the hospitality/catering sectors, tourist outlets in major department stores and airports, and larger end-users such as airlines and resorts. Products such as pastas, dressings, chutneys, salsas, jams, jellies, and spices have also been successfully marketed at international food fairs. The value of bushfood exports is set to increase with current markets existing in London, Zurich, and New York. The Australian Native Bushfood Industry Committee estimated the value of the industry at $10-12 M in 1997 (Graham and Hart, 1997).

Commercial production of native species is expanding, but raw produce is still predominantly harvested from the wild. Reliance on wild populations poses problems for the expansion and growth of the industry including the high cost of collecting produce, seasonal variability, and varying product quality. Thus, production methods need to be developed to provide improved consistency and quality and a reliable supply of product. Stringent food handling and safety procedures must also be adopted. Without these measures, the risk of a food safety incident or the inability to supply demand could jeopardise the industry (Read and Pepper, 1998). At present, the main steps towards sustainable and prosperous growth in the industry involve:

- establishing sustainable markets;
- improving communication networks and dissemination of information;
- establishing recognised food safety and quality standards;
- setting up sustainable and profitable production systems;
- improving education and training in all industry sectors;
- improving raw produce through genotype selection of quality characters (Graham and Hart, 1997; Phelps, 1997).

Table 1.2 illustrates bushfoods suitable for commercial production in the arid zone of far-western NSW where a 20 ha plantation would provide an internal rate of return of just fewer than 9%. It is estimated that, over a 5- year period, the operation would be financially viable and provide employment for a manager and four full time staff (Payne, 1998).

Table 1.2 Feasibility study on key regional species in the arid zone of far-western NSW (Payne, 1998).

Key Regional Species

Common Name	Scientific Name	Density	Estimated Yield	Yield	Farm Gate Value	Gross Return	Operating Costs	Net Return
		Plants/ha	kg/plant	kg/ha	$/kg	$/ha	$/ha	$/ha
Wattle Seed	*Acacia victoriae*	625	1.5	937	5.0	4 688	3 700	988
Wild Lime	*Eremocitrus glauca*	625	2.0	1 250	6.5	8 125	4 375	3 750
Bush Tomato	*Solanum centrale*	3 300	0.5	1 650	8.0	13 200	5 000	8 200
Native Thyme	*Ocimum tenuiflorum*	5 000	0.2	1 000	55.0	55 000	22 500	32 500
Kurrajong	*Brachychiton* spp.	275	2.0	550	15.0	8 250	3 325	4 925
Wild Orange	*Capparis* spp.	625	2.0	1 250	5.5	6 875	4 300	2 575
Quandong	*Santalum acuminatum*	833	1.0	833	20.0	16 660	3 700	12 960

Operating costs, excluding harvesting and sorting, but including amortised costs of plant and equipment, have been estimated at $2 500/ha. Harvesting costs have been estimated at $1 200/ha for Wattle Seed, $1 800/ha for Desert Lime, $2 500/ha for Bush Tomato, $200 000/ha for Native Thyme, $825/ha for Kurrajong, $1 800/ha for Wild Orange and $1 200/ha for Quandong. Land values and costs of irrigation have not been included, as they vary from one district to another.

1.2 The family Solanaceae

The Solanaceae family includes 90 genera and over 2600 species. The family is widely distributed in tropical and temperate regions, mostly in Central and South America, Africa, and Australia. Family members are terrestrial herbs, shrubs, or small trees that may be glaborous or pubescent, erect or straggling, and may have prickles. Leaves are either alternate or opposite, and range from simple to pinnate in form. Inflorescences can be terminal, lateral, axillary, leaf-opposed, cymose, appearing racemose, paniculate, or with solitary flowers. Flowers are actinomorphic or zygomorphic, and often bisexual with simple styles. Fruits are berries or capsules that are usually bilocular, which may be smooth, spiny or tuberculate, and often surrounded by an enlarged calyx. Seeds are numerous, often flattened, and the embryo may be curved, spiral or straight, with endosperm present (Purdie *et al.*, 1982; Haegi and Symon, 1986; Wheeler, 1987). In Australia, this family is represented by 23 genera containing about 200 species. Of these, 6 genera and 132 species are endemic, and 66 species naturalised.

Many species are economically important as food plants. These include, the potato (*Solanum tuberosum L.*), eggplant (*S. melongena L.*), tomato (*Lycopersicon esculentum*), and chilli/capsicum (*Capsicum* spp.). Others are cultivated as ornamentals and some (*Nicotiana* spp.) are sources of narcotics and medicinal drugs. Some species are toxic to mammals, such as blackberry nightshade (*S. nigrum*) (Purdie *et al.*, 1982; Haegi and Symon, 1986; Wheeler, 1987).

1.3 The genus *Solanum*

The genus *Solanum* includes about 1500 species that occur mainly in central and South America, Australia, and Africa. In Australia there are 117 species, of which 94 are native and 87 endemic. Most species contain steroidal alkaloids and are thus suspected of being toxic. Several are cultivated as a source of corticosteroid drugs and other steroid hormones. *Solanum* occurs in all areas except saline, alpine, and aquatic habitats. The highest number of species occurs in the tropics. Many also occur in arid or semi-arid areas are often widespread, with some having relict distributions. Many species are weedy and some are common after fire. Juvenile and mature plants often differ morphologically. Mature plants can exhibit great variation, especially in leaf morphology, distribution of prickles and, to a lesser extent, indumentum. Most species are diploid and have a chromosome number of $2n = 2x = 24$ or $2n = 2x = 46$, although tetraploids and hexaploids have been identified (Symon, 1979a, 1981; Wheeler, 1987; Randell and Symon, 1976).

Species may be annual or perennial herbs, shrubs or small trees, with trailing or climbing habits. Plants may be hairy with stellate, non-glandular or glandular hairs, and may have prickles. Leaves are usually alternate (sometimes paired), may be simple or pinnate, and be entire or lobed and petiolate. Roots are fibrous, tuberous or rhizomatous. Inflorescences are usually terminal becoming lateral through the growth of axillary buds. Flowers are mostly bisexual (rarely unisexual) and are generally actinomorphic. The calyx is campanulate, cup shaped, 5-lobed and sometimes enlarges to enclose the fruit. The corolla is star shaped and deeply incised to rotate, rarely campanulate, and ranges in colour from purple to blue, less often white or yellow. Flowers have five stamens, which are usually equal in length and exserted, while the anthers have opening pores or short slits. Styles are erect or

sigmoid. The fruit is a succulent, chartaceous or bony berry and is usually globular. Seeds are compressed and circular to almost reniform in outline (Symon, 1979a; Symon, 1981; Wheeler, 1987).

1.4 *Solanum centrale*

Family: Solanaceae
Subfamily: Solanoideae
Tribe: Solaneae
Subgenus: Leptostemonum
Section: Oliganthes
Genus: *Solanum*
Species: *S. centrale*

Solanum centrale is an evergreen, perennial herb or undershrub that grows to approximately 45 cm and often has a sprawling habit. However, some genotypes have an upright habit that is more amenable to cultivation and fruit harvesting. *Solanum centrale* has grey-green to green leaves, usually covered with fine silvery or rust coloured stellate hairs. Flowers are light-pink to dark-purple and may appear over long periods (Figure 1.1). The stems, petioles, flowers and fruits can be prickly (1-5 mm), but never the leaves or calyces. Leaves vary from ovate to oblong in shape and the lamina generally ranges from 1.5-6 cm long and 1-2 cm wide, although larger leaves can occur. The petiole ranges from 5-15 mm (sometimes 30 mm) long and can be entire to slightly undulate. The inflorescence consists of 1-6 flowers with a peduncle up to 10 mm long or absent, while the rachis ranges from 5-15 mm long, and pedicels from 6-10 mm long. The calyx is 4-6 mm long with triangular lobes 1.5-2.5 mm long. The stellate corolla is 15-25 mm in diameter and pale or deep purple in colour. Anthers are yellow and 4.5-6.5 mm long. Fruits are spherical, 10-20 mm in diameter, and turn from green to yellow to red during ripening. Fruits remain on the plant and dry to resemble a raisin (Figure 1.2). The number of seeds per fruit varies, and seeds are 2-4 mm long and pale-yellow or light-brown in colour (Black, 1934; Symon, 1979a; Symon, 1981). The chromosome number of this species is $2n = 2x = 48$ (Randell and Symon, 1976).

Solanum centrale is widespread in arid areas (150-300 mm rainfall per annum) of Western Australia, Northern Territory, and northern South Australia (Figure 1.3),

generally on red sandy soils. However, plants can be found on heavier textured soils in some locations and are common on roadsides and flood plains (Low, 1988, Latz, 1995). It is an important food plant for Aboriginal people who eat the fruit fresh or dried (Black, 1934; Symon, 1981). Fruits are also a source of food for some native fauna, such as emus (Noble, 1975).

Solanum centrale tends to grow in large numbers in confined and well-known areas maintained by Aboriginal people, not only by firing but also occasionally by damming watercourses after heavy rain so that any run-off services patches of fruiting vegetation (Issacs, 1987).

Figure 1.1 Flowering and fruiting branch and a fruit of *Solanum centrale* (Symon, 1981).

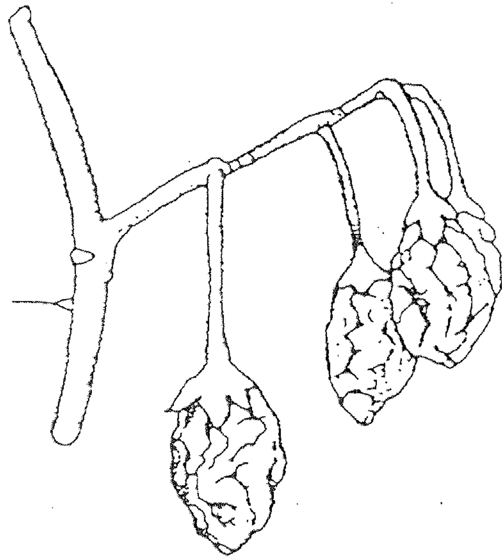


Figure 1.2 Dried fruit of *Solanum centrale* at harvesting stage (Black, 1934).

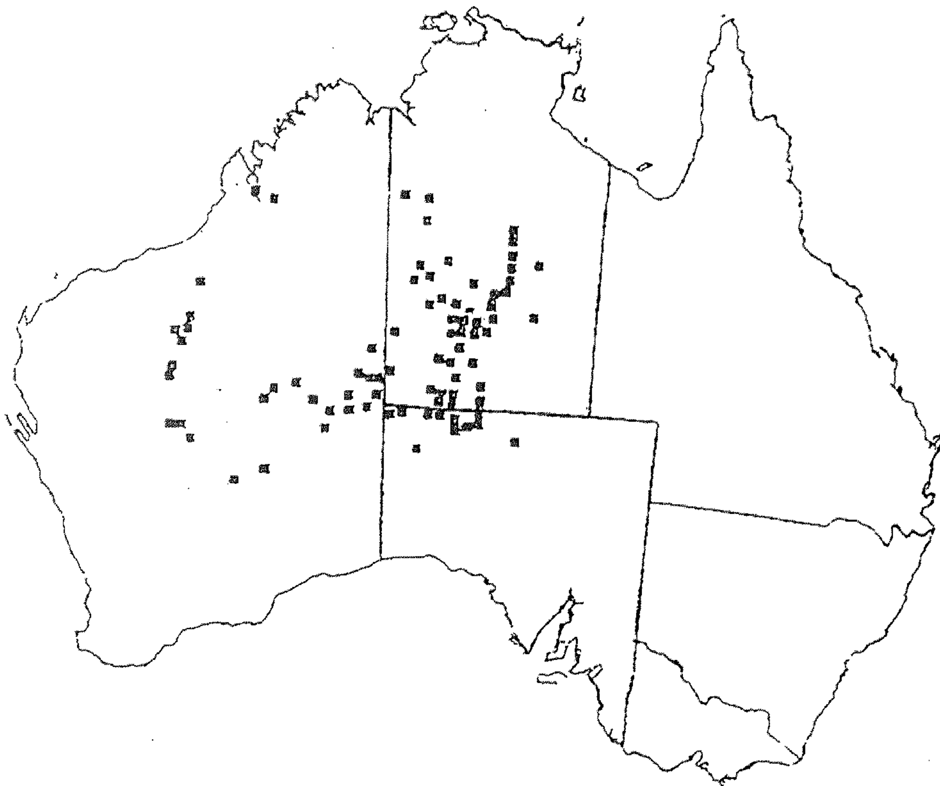


Figure 1.3 Distribution of *Solanum centrale* in Australia (Symon, 1981).

1.5 Project Aims

This research aims to investigate six areas pertaining to domestication and development of improved quality of the native bush tomato, *Solanum centrale*:

1. Investigation of propagation methods for commercial production of *S. centrale* (Chapter 4).
2. Confirmation of steroidal alkaloids in *S. centrale* leaves and fruit (Chapter 5).
3. Development of hybridisation techniques for *S. centrale* to increase breeding program efficiency (Chapter 6).
4. Morphological variability within and between populations of *S. centrale* (Chapter 7).
5. Genetic variability within and between populations of *S. centrale* (Chapter 8).
6. Identification of a molecular marker linked to the presence or absence of prickles (Chapter 9 and 10).

The ultimate aim is the selection and propagation of *S. centrale* genotypes that have low variability, are high yielding, have uniform maturity, and low alkaloid content, and are non-prickly. This research will provide the first steps toward achieving this aim.

Chapter 2 Literature Review

2.1 Bush Tomato Production

2.1.1 History and culinary uses of bush tomatoes

The fruits of many *Solanum* species are referred to as bush or desert tomatoes and eaten throughout arid areas of Australia (Peterson, 1979). They are also known colloquially as 'sultanas', 'tomatoes' or 'raisins' depending on species (Table 2.1) and whether they are eaten raw or dried. Aboriginal women gathered various Solanum fruits, especially when they travelled over large distances (Issacs, 1987). The main species of bush tomatoes eaten by Aboriginals, as well as the Aboriginal name for the species, and the appearance of the fruit when eaten, is shown in Table 2.1.

Table 2.1 Species of bush tomatoes eaten by Aboriginals (Issacs, 1987).

Appearance (Eating stage)	Aboriginal name	Scientific name (*Solanum* spp.)
Sultana (dried)	yakajiri	*S. ellipticum*
Raisin (dried)	kampurarpa	*S. centrale*
Green tomato (raw)	wanakidji	*S. chippendali*
Yellow fruit (raw)	albaraji	*S. cleistogamum*
Yellow fruit (raw)	yipirntiri	*S. esuriale*

Bush tomatoes were staple foods of desert Aboriginals, eaten fresh or dried on sticks. Fruits develop prolifically during cooler months, or following heavy rains, eventually drying on the shrub. Dried fruits were also mixed with water into a sweet-sour paste and moulded into cakes weighing a kilogram or more (Peterson, 1979; Low, 1988). Western Queensland Aboriginals buried a cache of seed cakes as emergency food for travellers. The dried fruits last for years, and when needed they were soaked or pounded with water into a sweet paste. *S. centrale* and *S. chippendali* were the most important of the bush tomatoes for this purpose, but many others were used (Low, 1988).

In western cooking, bush tomatoes are used either whole or coarsely ground into a granular form, and impart an intense, earthy, pungent, tomato flavour. Products are added sparingly as a spicy condiment to sauces, marinades, chutneys, soups, stews, casseroles, and salads (Cherikoff and Issacs, 1988). The high amount of pectin in the fruit allows ground bush tomatoes to act as a thickener in dishes such casseroles (Anon, 1998).

2.1.2 Cultivation, propagation and market considerations

Bush tomatoes are reliant on natural pollination for fruit production; fruit is harvested, dried and crushed whole for sale in value-added products, such as the "Red Ochre" native food range. The bush tomato grows best on light-textured soils and is a hardy, fast growing species that is very drought, frost and lime tolerant (A. Beal, pers. comm.). To survive frost conditions, aboveground parts of the plant die back to ground level, with vigorous new growth arising from the crown of the remaining plant at the commencement of a growing season. For fruit to ripen, the plant needs a high number of sunshine hours and warm ambient temperatures. In the Riverland region of South Australia, if planted in late October after frost risk, the plant continues to flower and set new fruit throughout spring and summer. The fruit does not abscise when ripe so the entire crop can be harvested at the end of the season. The crop is usually harvested between February and April and an average yield of 0.5 kg per bush is produced in the second year. Commercial plantations are established at approximately 8 000 plants per ha (A. Beal, pers. comm.).

Commercial production relies on supplies of seed collected from the wild to be cost-effective, a practice leading to wide genetic variability. However, among crop plants there are a number of problems associated with mass-propagation of the bush tomato. These include the need to remove flesh from the seed due to the presence of germination inhibitors, breaking seed dormancy, slow and unreliable germination, susceptibility to root rot pathogens (*Pythium* sp. and *Phytophthora* sp.) once germinated, and pathogenic fungal attack of leaves and stems in humid conditions (Graham and Hart, 1998).

Seed dormancy prevents germination in a wide range of Australian species. One way to enhance seed germination in species commonly found in fire-prone areas is to use smoke derived from the burning of plant material (de Lange and Boucher, 1990; Roche *et al.*, 1994; Dixon *et al.*, 1995). The mechanisms of smoke are not clearly understood but there have been preliminary investigations which exclude physical degradation of the seed coat or nutritive support as a role for smoke (Baldwin *et al.*, 1994). Smoke may trigger germination by chemically interacting with inhibitors or promoters in the seed coat, endosperm, embryo or combinations of these tissues (Baldwin *et al.*, 1994). In many species the mechanism for dormancy is not known but smoke and/or heat can promote germination (Dixon *et al.*, 1995).

Because market preference is for the powdered product, fruits must be 'chip dry' for hammer milling to be successful. Moisture content tests determine the maximum acceptable moisture level in fruit prior to harvesting. Most raw bush tomato product traded is harvested from the wild in the Alice Springs region. "Farm gate" prices currently paid to collectors and commercial producers of *Solanum centrale* range from $15.00 to $25.00 per kg plus freight. Wholesale prices are approximately $55.00 to $60.00 per kg of whole, dried fruit. Wholesale value-added bush tomato products include, bush tomato chutney ($19-24/kg), bush tomato sauce ($18-25/L), bush tomato chilli jam ($20-22/L), bush tomato dressing ($15-16/L), bush tomato salsa ($19-25/kg), and ground bush tomato powder ($55-58/kg). These prices are lucrative in the short-term for the limited number of collectors and producers, but are severely limiting market growth. Despite a high degree of acceptance of the product itself, few processors, marketers or consumers are willing to pay the current high prices (Graham and Hart, 1998; A. Beal, pers. comm.). Price may be reduced by efficient large-scale production.

2.1.3 Nutritional value

The bush tomato is high in energy, protein, carbohydrates, fibre, thiamine, potassium, iron and vitamin C (Table 2.2). However, the vitamin C content decreases when the fruit is dried or processed (Brand *et al.*, 1983; Issacs, 1987; Cherikoff and Issacs, 1988). Low (1988) measured the nutritional composition of

native bush tomatoes and cultivated tomatoes (*Lycopersicon esculentum)* (Table 2.3), with a higher energy value recorded for the bush tomato.

Unripe fruits may contain the toxic alkaloid solanine, which is also present in green potatoes. Upon ripening the fruit changes from green to yellow, at which stage it is assumed that the solanine is fully metabolised (A. Beal, pers. comm.).

Table 2.2 Composition of two species of bush tomato fruits per 100 g edible portion (Brand *et al.,* 1983; James *et al.*, 1986, Miller *et al.*, 1993).

Fresh Fruit	Edible portion of sample	Energy	Water	Protein	Fat	Carbo-hydrates	Fibre	Ash	Na	K	Mg	Ca	Fe	Zn	Cu	Vitam C
	(%)	(KJ)	(g)	(g)	(g)	(g)	(g)	(g)	(mg)	(mg)	(mg)	(mg)	(mg)	(mg)	(mg)	(mg
Solanum centrale	100	606	61.2	3.8	Trace	34.8	7.8	1.9	52	448	34	40	2.9	0.1	2.8	9.0
Solanum chippendali	100	294	78.2	1.8	0.6	15.1	3.1	1.1	21	503	23	38	1.5	0.5	0.5	48.9

Table 2.3 The nutritional composition of the bush tomato and the cultivated tomato (Low, 1988).

Nutritional Composition	Bush tomatoes	Cultivated tomatoes
Water (%)	78.7	93.7
Protein (%)	1.1	1.0
Fat/100g (%)	0.6	0.3
Energy (kJ/100g)	430.0	87.0
Thiamine (mg/100g)	243.0	60.0
Ascorbic Acid (mg/100g)	9.0	22.0

2.2 Domestication

Domestication of any food plant involves the selection of variants with useful characters. These characters can include improved yield, ease of germination, improved size, flavour, colour and resistance to disease, insects and drought. Plants such as the common tomato or potato have a long history of domestication. This is not the case for the bush tomato, and because it is highly heterozygous there is potential for improvement.

Fantozzi (1994) defines quality as the presence of a set of characters that make it different and superior to a similar food not having the same characters. The definition of quality for the bush tomato is related to prickliness of this plant, and the yield, flavour, colour, size, and preservability of this fruit. Potential toxicity from high solanine levels is also a matter of concern to the industry. Quality can be affected by climate, genetics, agronomy and their interaction (Pannelli *et al.*, 1994).

Horticultural plant breeding relies on the selection of superior individuals or populations, based on gross morphological or agronomic characters, from either wild or cultivated populations (Woodson, 1991; Von Hentig, 1995). Individuals exhibiting extremes of the desired characters are noted, with seed or vegetative material collected for further study. Such plants may be grown under a range of conditions to test their response to environmental factors. Selection at species level requires a range of genotypes to be grown and assessed for desired characters, with genotypes possessing superior attributes selected for further testing (Woodson, 1991).

Very little work has been done on the domestication of *S. centrale*. Aspects that need to be refined include improving methods in propagation, and investigating potential steroidal alkaloids in fruit, as alkaloids have been found in other members of the Solanaceae. Steroidal alkaloids have been found to have an influence on human health. Other steps towards domestication of the species are developing hybridisation techniques and the breeding system of the species, studying morphological and genetic diversity of *S. centrale* populations and identifying

molecular markers for desirable traits. No serious domestication of *S. centrale* has taken place previously and work described in each of these chapters is a step towards this.

2.2.1 Propagation

There are two main methods of plant propagation sexual from seed and asexual from cuttings, layering, budding, grafting and micropropagation. Both methods have advantages and disadvantages. The main advantage with sexual reproduction is the generally large number of plants that can be raised cheaply. Some disadvantages with this method of propagation are transfer of disease, transplant difficulties and variability amongst individuals (Plumridge, 1989). The main advantage in vegetative propagation is that plants propagated are true to the parent plant in habit of growth, fruits, flowers and foliage. With vegetative propagation it is difficult to obtain large numbers of plants and it can be labour intensive, making the process expensive (Plumridge, 1989). Some Australian native plants are known for their difficulties with seed and vegetative propagation (Vigilante *et al.*, 1998). For this reason the method of propagation of a domesticated species must be investigated if success is to be achieved in commercial situations by meeting market demands.

2.3 Alkaloids and steroidal alkaloids

2.3.1 Alkaloids

Alkaloids are heterocyclic, nitrogen containing compounds (Everist, 1974; Symon, 1994) that are generally insoluble in water but extractable in organic solvents or weak acid. In pure form, most are crystalline solids but in plants they usually occur as soluble salts of organic acids, sometimes as glycosides. Most of them are very bitter to taste. Most produce strong physiological reactions when introduced into animals and the effects are extremely varied, although some are apparently inactive. Many important medicinal compounds and toxins derived from plants are classified as alkaloids (Roddick, 1974). For example, narcotic alkaloids such as morphine, codeine, and cocaine are utilised for pain relief. Other commonly used alkaloids include caffeine, quinine, serotonin, strychnine, and nicotine (Anon, 1994).

2.3.2 Steroidal alkaloids in *Solanum*

Steroidal alkaloids occur within various species in the genus *Solanum* and closely related genera that include the tomato (*Lycopersicon* spp.) and weedy species. These alkaloids are often present as glycosides such as solanine. The solanine molecule contains a sterol group and therefore may be classified as a steroidal alkaloid with saponin-like properties (Kingsbury, 1964). Chemical structures of steroidal alkaloids commonly found in *Solanum* spp. are shown in Figure 2.1.

Steroidal alkaloids are found in all tissues of the potato plant, and it has been suggested that they are some of the most serious toxic components contaminating the human diet (Hall, 1992). The most predominant in cultivated potatoes are α-solanine and α-chaconine (Kuc, 1982), being derivatives of the aglycone solanidine. Solanidine is associated with undesirable flavour, mammalian toxicity and teratogenicity (Hellenäs *et al.*, 1995). Inhibition of cholinesterase may also be linked to toxicity of the alkaloids (Hellenäs *et al.*, 1995). The accumulation of steroidal alkaloids in plant tissues may be regulated by some environmental conditions, such as fungal infection and light. Mechanical injury during postharvest handling can also negatively affect quality and safety (McKee, 1955; Allen and Kuc, 1968). Alkaloids are present at high levels in green or immature potatoes (Jadhav and Salunkhe, 1975; Jadhav *et al.*, 1981). In the cultivars Kennebec and Tarago, normal levels range from between 8 to 12 mg/100 mg of tuber. However, in wild species such as *Solanum acaule,* values range from 35 to 126 mg/100 g fresh weight, which may create problems for breeding programs. The highest levels of alkaloids are found in leaves, flowers, sprouts, and within the tuber, especially in the outer tuber layers. Peeling potatoes may lower the levels consumed but this also reduces the nutritional value of the potato. The maximum acceptable alkaloid level in potatoes is 20 mg/100 g fresh weight (Gregory, 1984).

Steroidal alkaloids accumulate in tubers as a result of infection by various micro-organisms including the late blight pathogen, *Phytophthora infestans*. This response may play a role in disease resistance to some fungal pathogens often entering at sites created by micro-organisms (Allen and Kuc, 1968). Therefore,

reduction of alkaloids in potato foliage and tubers for health and flavour reasons may reduce the ability of these organs to resist disease (Kuc, 1984).

Potato leafhoppers are currently controlled by insecticides, which are expensive and often environmentally damaging. Steroidal alkaloids in potato leaves are toxic to leafhoppers and breeding new leafhopper-resistant potato varieties reduces insecticide use (Zhang and Michell, 1997). Tuber alkaloid levels are heritable and can vary considerably between different varieties, with α-solanine and α-chaconine accounting for more than 95% of the alkaloids found in potato tubers (Guseva and Paeshnichenko, 1957).

Current research involves optimising both the extraction procedure and condition for high performance liquid chromatography (HPLC) analysis for potato steroidal alkaloids from commercial cultivars and some hybrids. The main aim of this research is to determine the structure and biological activity of novel steroidal alkaloids (Kuronen and Väänänen, 1998).

High levels of steroidal alkaloids have been found in the Kangaroo Apple (*Solanum aviculare*). The chemical skeleton can be transformed into pregnanes, which are important intermediates in the industrial production of hormonal steroids. Solasodine, found in the Kangaroo Apple, has received special interest as it can be converted to a nitrogen analogue of an ecdysone and may be promising as an insect control agent. Exposure to ecdysones induces moulting in insects at abnormal times, disturbing their normal development and resulting in death. Ecdysones are active at low concentrations, are virtually inactive in other animals and appear harmless to vertebrates and man. Other alkaloids found in *S. aviculare* include tomatidenol, solanaviol, solaradixine, solashabinine, solaradinine, solarmargine and solasonine (Bradley *et. al.*, 1978; Schreiber, 1979).

Any species of *Solanum* should be considered potentially poisonous unless determined otherwise (Kingsbury, 1964). Toxicity of solanine-type alkaloids is not reduced by drying the plant tissue (Everist, 1974). This applies to leaves and immature fruits, although ripe fruits of many species appear to be safe. The

glycoalkaloid solanine, is present in *S. nigrum* (Blackberry Nightshade) in green berries, and diminishes as the berry ripens. Solanine is present in two forms, α-chaconine and α-solanine (Figure 2.1), which are both toxic by inhalation, in contact with skin, or if swallowed. The melting points for α-solanine and α-chaconine are 285°C and 286°C respectively. Both are pyridine-soluble and generally stable (Anon, 1988).

2.3.3 Common steroidal alkaloids

Solanine is a toxic substance and is usually present in all green parts of the *Solanum* plant. It tastes faintly bitter and acrid (Sanyal and Ghose, 1979). The intact molecule has the physical properties of a saponin because it forms a semi-permanent froth when shaken with water. Chemically it is considered to be a glycoside as it consists of a sugar (solanose) bound to an aglycone, solanidine (Kingsbury, 1964).

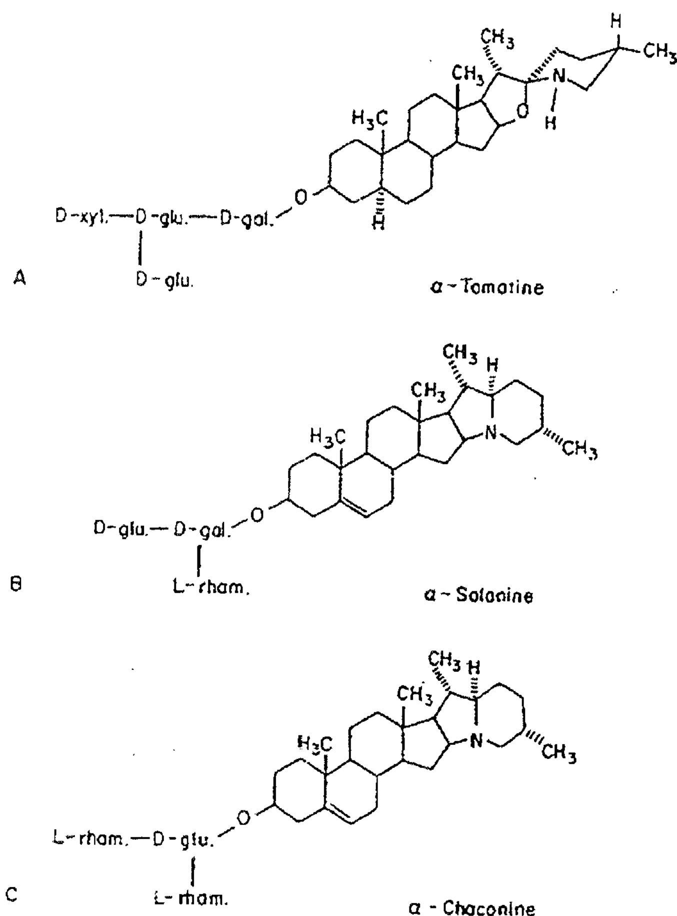


Figure 2.1 Steroidal alkaloids commonly found in *Solanum* spp. (Schreiber, 1979).

α-tomatine is a steroidal glycoalkaloid that is found in unripe fruit of some tomato species (Eltayeb and Roddick, 1984) and in some potato species. The compound consists of an alkaloid (aglycone) tomatidine and a sugar moiety of four monosaccharides (Keukens *et al.*, 1994). It is toxic to humans (Nishie *et al.*, 1975; Friedman *et al.*, 1992; Ripperger and Schreiber, 1981; Wilson *et al.*, 1961) and has been linked to host-plant disease resistance (Barbour and Kennedy, 1991; Dahlman and Hibbs, 1967; Roddick, 1974), antifungal activity (Jiratko, 1993), and growth inhibition of fruitworm, spiny bollworm larvae (Elliger *et al.*, 1981; Weissenberg *et al.*, 1986) and moth eggs (Lu and Chu, 1992).

There have been several different methods developed for measuring α-tomatine levels in tomatoes including bioassays (Fontaine *et al.*, 1948; Ali and Schloesser, 1977), colorimetric assays (Tukalo and Ivanchenko, 1976; Bajaj *et al.*, 1988; Ostrzycka, 1989; Sosic, 1971), gas chromatography (Juvik *et al.*, 1982), and fast bombardment mass spectroscopy (Price *et al.*, 1986). Most of these methods result in low recoveries and high variation. HPLC is proving the most successful (Friedman *et al.*, 1994; Keukens *et al.*, 1994).

2.3.4 Toxicity of steroidal alkaloids

2.3.4.1 Factors affecting steroidal alkaloid toxicity

Factors that affect alkaloid toxicity are plant, animal, and environment related. Some plants are poisonous at all growth stages, while others may be toxic for a single developmental phase only. In many cases all tissues of the plant are toxic, in others toxins may be concentrated in the leaves, stems, roots, flowers or seeds (Everist, 1974). Different animal species vary in their level of susceptibility to different steroidal alkaloids. The health of the animal and quality of other food sources can also affect the level of poisoning. An animal's previous experience with the plant can influence whether or not the plant is eaten again (Everist, 1974).

2.3.4.2 *Symptoms of steroidal alkaloid poisoning*

Solanine poisoning is characterised by both gastrointestinal and neural disturbances. Animals affected by solanine poisoning may die within a short time. Gastrointestinal effects include loss of appetite, nausea, abdominal pain, vomiting and either constipation or diarrhoea, sometimes containing blood. Neural problems include drowsiness and apathy, difficulty in breathing, salivation, trembling and progressive weakness leading to prostration, unconsciousness and sometimes death from paralysis. Pupils may be dilated, and in humans there may be a loss of reasoning ability and loss of nerve sensation. There is a possible risk of harm to developing foetus and overexposure may cause reproductive disorders in adults (Anon, 1988). Some animals exhibiting nervous symptoms may suffer for several days and then recover. In chronic cases animals become emaciated with a rough coat, loss of appetite, constipation and abdominal dropsy (Everist, 1974; Francis and Southcott, 1967; Van Gelder, 1991).

2.3.5 History of steroidal alkaloid extraction

Most steroidal alkaloids are only sparingly soluble in aqueous solutions at pH 7 or above. Extractions are therefore carried out under either non-aqueous conditions, at a low pH or both. Any combination of acid and heat is usually avoided, as this can cause hydrolysis, although some gas chromatography and colorimetric methods may require hydrolysis before analysis. Friedman and McDonald (1995) evaluated 10 solvent systems for extraction efficiency on dried, fresh, and processed potato tubers using the same extraction and clean-up procedures and then measuring α-solanine and α-chaconine by HPLC. These solvents included ethanol (Dabbs and Hilton, 1953), 2% acetic acid (Birner, 1969), 5% acetic acid (Speroni and Pell, 1980), 3% acetic acid in ethanol (Baker *et al.*, 1955), methanol-acetic acid-water (94:1:6) (Jonker *et al.*, 1992), 5% trichloroacetic acid (TCA) in 50% methanol (Bretzloff, 1971), 5% TCA in 75% methanol (Smittle, 1971), methanol-chloroform (2:1) (Wang *et al.*, 1972), chloroform-acetic acid-methanol (10:1:9) (Shih and Kuc, 1974), tetrahydrofuran-water-acetonitrile-acetic acid (5:3:2:0.1) (Bushway *et al.*, 1985), 0.5% sodium bisulphite in 2% acetic acid (Hellenäs, 1986) and 0.02 M 1-heptanesulfonic acid in 1% acetic acid (Carmen *et al.*, 1986). Solutions of acetic acid in alcohol were found to be poor performing for extracting α-solanine. The

solvent system utilised by Bushway *et al.* (1985) was less sensitive and did not give sufficient separation of α-solanine and α-chaconine when compared to the system used by Carmen *et al.* (1986).

Steroidal alkaloids are commonly purified in one of three ways:

(1) precipitation with ammonium hydroxide (Bushway *et al.*, 1979; Friedman and Dao, 1992).

(2) partitioning with either aqueous sodium sulphate (Wang *et al.*, 1972) or water-saturated *n*-butanol (Crabbe and Fryer, 1980; Friedman *et al.*, 1994).

(3) passing through a C_{18} ion-pair chromatography cartridge (Carmen *et al.*, 1986; Houben and Brunt, 1994; Kobayashi *et al.*, 1989; Morris and Petermann, 1985; Saito *et al.*, 1990).

A disadvantage of the ammonia precipitation method is that while α-solanine is insoluble in basic solutions, α-chaconine is partially soluble. With small-scale samples this could be significant causing an underestimation of α-chaconine levels. Using water-saturated *n*-butanol can avoid this problem. The most effective method for clean up is solid-phase extraction (SPE), this uses commercial C_{18} cartridges prior to estimating potato alkaloid levels by HPLC. The choice of sample clean-up method depends to a large extent on the extraction solvent and method of analysis.

2.3.6 Analysis of steroidal alkaloids

Solanine was initially determined gravimetrically following alkaline precipitation (Bömer and Mattis, 1924). Precipitation is now used as only part of modern-day clean-up procedures. Liquid chromatography (LC) and paper chromatography (PC) are outdated methods that were used for qualitative and quantitative separation (Paseshnichenko and Guseva, 1956). Current methodologies for the analysis of potato alkaloids and related compounds include colourimetry, thin layer chromatography (TLC), gas chromatography (GC), HPLC, and immunoassays (ELISA).

The most effective and popular method for alkaloid determination is HPLC, which has been continuously improved with respect to sample preparation and clean-up methods, column selection, and peak detection. Bushway *et al.* (1979) were the first

to describe the separation of α-solanine and α-chaconine by HPLC and subsequent improvements have been described (Bushway, 1982 a, b, c; Bushway *et al.*, 1980, 1986; Carmen *et al.*, 1986). HPLC is increasingly used because individual alkaloids and aglycone forms can be distinguish directly, it is performed at room temperature and can give an almost complete picture of the pattern of individual glycosides in one determination. The main disadvantages are the expense of equipment, the need for extensive clean up of samples, and the use of potentially toxic organic solvents that are expensive and require specialised handling and disposal. Almost all HPLC-based methods use UV detection between 200 and 215 nm.

2.4 Floral Biology

Detailed observation of flowers can lead to accurate assessment of the breeding system of a flowering plant (Sedgley and Griffin, 1989). Pollination is an essential stage in the reproduction of flowering plants. It involves the transfer of pollen from the male to the female part of a flower, and is carried out by an external agent in many crop plants. The most common pollinating agents are wind and insects (Crane and Walker, 1984).

Flowers are adapted for either self-pollination (autogamy) or cross-pollination (allogamy), although varying frequencies of both mechanisms may occur in the same species (Sedgley and Griffin, 1989). When pollen is transferred from the anther to the stigma of either the same flower or another flower on the same plant it is referred to as geitonogamy. For hermaphrodite flowers where both the male and female organs are produced in the same flower, self-pollination is favoured. Cross-pollination involves the transfer of pollen from one plant to the stigma of another plant by a pollinating agent. Factors that can influence whether cross- or self-pollination are favoured include: spatial relationships between the anther and the stigma, maturation of female and male organs and functional failure (Sedgley and Griffin, 1989).

The pollination mechanism can vary within a genus and even within a species. An example is in *Prunus* where the highly self-incompatible almond requires cross-pollination and the peach a mainly self-pollinating crop. Self-pollinating crops are

higher yielding than those that are cross-pollinated and selection pressure during domestication and breeding favours these individuals (Sedgley and Griffin, 1989).

2.4.1 Self-incompatibility

Mechanisms that prevent self-pollination are of crucial importance for maintaining genetic diversity within flowering plant (angiosperm) populations. Some flowers have developed barriers for their own pollen to prevent them from reaching the female organ (pistil) in the same flower or plant. Some plants have timing differences between their male and female flowerings involving different stages of maturity. The self-incompatibility systems creating a topological barrier (due to the morphologies of their flowers) are called heteromorphic self-incompatibility systems (deNettancourt, 1977; Ebert *et al.*, 1989; Kao and McCubbin, 1996).

The homomorphic self-incompatibility (SI) involves the rejection of self-pollen and was first recognized by Darwin. The homomorphic type is further classified into gametophytic and sporophytic types. In gametophytic type the pollen's own SI type is perceived by the stigma and should not match either of the plants SI alleles for successful fertilisation. In the sporophytic type, the two alleles of pollen's parent are recognised by the stigma and there should be no matching combination between the two alleles of the stigma and two alleles of the plant from which the pollen has derived to avoid self-rejection (Kao and McCubbin, 1996). The gametophytic type has been studied in a number of families including Solanaceae (including tobacco, potato and tomato) (Mau *et al.*, 1991; Matton *et al.*, 1992). The plant self-incompatibility system provides an example of balancing selection in the maintenance of their alleles (Clark and Kao, 1991; Richman *et al.*, 1996; Wu *et al.*, 1998).

2.4.2 Pollination in Solanaceae and *Solanum*

With more than 2 000 species, *Solanum* (Solanaceae) is the second largest genus of flowering plants. Flowers of the genus *Solanum* often have large conspicuous flowers consisting of a white or blue perianth, contrasting with brilliant yellow anthers. Although these flowers are conspicuous they offer no nectar, although

'pseudonectaries' or extra-floral nectaries may be present. Pollen is the sole reward for bees visiting *Solanum* flowers (Buchmann and Cane, 1989).

The flowers are generally hermaphrodite (Harris, 1903), but in *Solanum,* andromonoecious and androdioecious forms also occur. Andromonoecious refers to hermaphrodite and male flowers present on one plant, which is common in *Solanum*, while androdioecious refers to hermaphrodite and male flowers present on separate plants, and is rare in *Solanum* (Symon, 1979b).

For *Solanum*, pollen is harvested only by bees, which sonicate the anthers. The bees transmit strong vibrations to the flower androecium by using rapid contractions of their pterothoracic flight muscles. These vibrations propel the small light pollen out of small pores in distinct clouds of grains, which attach to the sonicating bee body (Buchmann *et al.*, 1977; Buchmann, 1983, 1986). Both solitary and social bees in over 50 genera and at least 7 families use floral sonication to harvest pollen (Buchmann *et al.*, 1977).

2.5 Genetic markers in plants

The process of designing and producing hybrids, which represent a compilation of desirable traits, is known as plant breeding (Allen, 1994). Traditionally, phenotypic characters have been used to select superior individuals. Such an approach is unreliable, as expression of many characters is sensitive to environmental conditions (Rafalski *et al.*, 1994; Torres *et al.*, 1993). This method is also time consuming as selection is often delayed, to allow complete expression of the trait, or delayed until the next generation to allow recessive traits to be assayed (Allen, 1994).

Molecular genetic markers provide a diagnostic tool that permits direct identification of a trait at any developmental stage, in an environment-independent manner (Rafalski *et al.*, 1994). Markers thus enhance selection and can also be applied to establish unique genetic fingerprints of individual genotypes, evaluate genetic relationships among individuals, and study genetic variation within and among populations (Allen, 1994). Indirect selection methods utilise markers for

traits of interest, and may prove more effective than direct selection. Indirect markers may be biochemical, morphological or molecular. The range of molecular techniques available for detecting genetic markers in plants provides flexibility of choice in most situations. Advances in DNA sequencing, data analysis, and the polymerase chain reaction have resulted in powerful tools that can be applied to the characterisation and evaluation of germplasm and other such genetic resources, and the identification of markers useful in breeding programmes (Jones *et al.*, 1997).

2.6 Genetic diversity

2.6.1 Molecular identification

The unique morphological expression found in all plants and animals depends on sequences found in the genomic DNA of their somatic cells. Since 'normal' environmental conditions do not affect this sequence, which is consistent in all somatic cells of an individual, the DNA fingerprint of an individual provides a unique identifier (Torres *et al.*, 1993). Molecular techniques can be used to identify markers linked to any specific gene or genomic region. Characterising genomes always consists of at least four phases: DNA isolation, DNA amplification, separation and visualisation of amplified products, and data collection and analysis (Trigiano and Caetano-Anolles, 1998).

DNA-based molecular markers have proven a powerful tool for estimating genetic diversity (Lu *et al.*, 1996; Sun *et al.*, 2001) by providing accuracy, speed, reliability, and cost-effectiveness that are essential for identification of quality characters in plant research and agriculture (Morell *et al.*, 1995). Of the current DNA fingerprinting techniques, RAPD and AFLP are most widely used.

2.6.1.1 Isozymes

Isozymes are different molecular forms of an enzyme (Markert and Møller, 1959). When plant protein extracts are separated by electrophoresis, isozymes form patterns called zymograms. Plant genotypes generally possess characteristic patterns of isozymes (Hartmann *et al.*, 1997). These have proven useful for varietal identification of olive (Pontikis *et al.*, 1980), and mandarin (Elisiário *et al.*, 1999) and for following gene flow in almond (Jackson and Clark, 1991), and cherry

orchards (Brant *et al.*, 1999), as well as characterisation of genetic resources in the genus *Chaenomeles* (Garkava *et al.*, 2000).

Disadvantages of using isozymes for molecular identification is the low level of polymorphism at the level of the cultivar, and the possibility that isozyme expression may be influenced by environmental conditions and/or management practices (Fabbri *et al.*, 1995). Further, only sexually mature individuals can be utilised when pollen DNA is required for analysis and pollen is only available during a short period.

Table 2.4 shows alternative DNA fingerprinting methods that may overcome limitations associated with using isozymes and morphological traits for identifying varietal polymorphisms and specific markers of interest.

Table 2.4 Characteristics of molecular systems for generating genetic markers.

Characteristics	RFLP	RAPD	Microsatellite	AFLP
Principles	Endonuclease restriction, Southern hybridisation	DNA amplification with random primers	Amplification of simple sequence repeats within DNA	Endonuclease restriction, DNA amplification with labelled primer
Type of polymorphism	Single base changes (Insertion, Deletion)	Single base changes (Insertion, Deletion)	Changes in length of repetitive sequences	Single base changes (Insertion, Deletion)
Genomic Abundance	High	Very High	Medium	Very High
Level of Polymorphism	Medium	Medium	High	Very High
Dominance	Codominant	Dominant	Codominant	Dominant
Amount of DNA required	2-10 µg	10-25 ng	50-100 ng	0.5-2 µg
Sequence information required	No	No	Yes	No
Radioactive detection required	Yes/No	No	Yes	Yes/No
Development Costs	Medium	Low	High	High
Start-up cost	Medium/High	Low	High	High

Adapted from Rafalski and Tingey (1993) and Hill *et al.* (1996).

2.6.1.2 DNA fingerprinting

DNA fingerprinting is a molecular sampling procedure exploiting complex genetic information contained in DNA. The DNA of an individual is identical whether extracted from leaves, stems, or fruit, and this identical DNA structure within all tissues of the same organism provides the basis for DNA profiling. However, differences in DNA methylation between tissues of the same plant may give different DNA fingerprints (Donini *et al.*, 1997).

Factors favouring DNA analysis are small sample requirements, the ability to rapidly replicate a sequence a million-fold or more, and the relative stability of DNA (Kirby, 1992).

A DNA fingerprint (or profile) is a series of bands, which represent DNA fragments that have been amplified from selected portions of the genome. Comparing DNA profiles from different but closely related organisms, such as plant cultivars, can reveal different nucleotide sequences (polymorphisms) that uniquely identify individuals (Trigiano and Caetano-Anolles, 1998).

DNA polymorphisms can be utilised as genetic markers to distinguish plant species, cultivars, and in some cases, individual plants (Brettschneider, 1998; Sale, 1995). If modifications of a gene exist at a specific locus in a population, the locus is said to be polymorphic. For a locus to be considered polymorphic, the most common allele must occur at a frequency of less than 99 percent at the locus (Kirby, 1992). Polymorphisms may range from a single nucleotide change, to variance in the number of tandem repeats in a repetitive DNA sequence (Kirby, 1992).

2.6.1.2 RFLPs

DNA-based molecular markers have proven a powerful tool for estimating genetic diversity (Monckton and Jeffreys, 1993). RFLP was the first molecular method used in estimation of genetic diversity of eukaryotic species (Lu *et al.*, 1996). Detection of RFLP markers involves the preparation of radioactively or fluorescently labelled DNA probes, digestion of genomic DNA with restriction enzymes, gel electrophoresis, and southern hybridisation. The RFLP patterns

depend on variations in restriction fragment length between individuals due to altered restriction sites resulting from the insertion or deletion of one or more nucleotides in their genomic DNA.

RFLP analysis of PCR (polymerase chain reaction)-amplified chloroplast DNA (cpDNA) has been developed as a novel method for detecting cpDNA variations and has been utilised for identifying seed parents in iris (Arnold *et al.*, 1991) and assessing taxonomic relationships in several genera including *Astragalus* (Liston, 1992). In eggplant and related *Solanum* species, cpDNA has been analysed for the purpose of taxonomic studies (Sakata *et al.*, 1991; Sakata and Lester, 1994).

RFLPs have also been applied to studying heritable traits, examining variation between and within populations, and constructing genetic linkage maps for a variety of organisms (Sale, 1995). Examples of horticultural crops for which RFLP analysis has been utilised include potato (Gebhardt *et al.*, 1989), maize and tomato (Helentjaris *et al.*, 1986; Young *et al.*, 1988; Ahn and Tanksley, 1993), rice (Sun *et al.*, 2001), pineapple (Duval *et al.*, 2001), and lettuce (Landry *et al.*, 1987).

RFLP analysis is of limited use in some horticultural crops, such as citrus (Morell *et al.*, 1995) due to the small number of probes available. Another limitation is that the number of RFLP polymorphisms identified per probe is often too low to allow development of detailed linkage maps (Apuya *et al.*, 1988; Keim *et al.*, 1990; Lin *et al.*, 1996). RFLP is also labour intensive, requires large amounts of genomic DNA (5-10 μg per plant), and usually involves the use of radioactive isotopes (Lin *et al.*, 1996).

2.6.1.3 PCR

PCR-based techniques are used routinely in the field of molecular biology (Clarke *et al.*, 1992) and have been used to detect polymorphisms in plants (Jones *et al.*, 1997). Using specially designed oligonucleotide primers, PCR is a method for amplifying regions of DNA flanked by these primers. Amplification of such a region is achieved via repeated cycles of denaturation, oligonucleotide annealing, and heat-stable DNA polymerase extension of the primers (Gibbs, 1990). The

extension products of one cycle serve as templates for following cycles, which theoretically results in a doubling of the number of target copies with each cycle. Fragments resulting from the amplification of DNA display genetic differences as length or sequence polymorphisms if primer-binding sites differ in separation (Aert *et al.*, 1998; Gibbs, 1990). Nucleic acid sequences can be generated in great abundance using PCR (Gibbs, 1990) allowing rapid construction of sequence-based phylogenies (Welsh and McClelland, 1990).

2.6.1.4 AFLP

Amplified fragment length polymorphism (AFLP) is a PCR-based technique allowing selective amplification of desired fragments from a complex mixture of DNA fragments resulting from restriction of genomic DNA (Matthes *et al.*, 1998; Vos *et al.*, 1995). Adaptors are ligated to the ends of the restricted fragments, and either a pre-selection step is performed using magnetic beads followed by a round of selective PCR, or two selective rounds of PCR are applied (Zabeau and Vos, 1993; Vos *et al.*, 1995). Selective restriction amplification is performed with a radioactive, chain luminescent, or fluorescently labelled primer on one end. AFLP markers are dominant because they are usually detected via presence or absence of amplified fragments, and do not require sequence information (Meksem *et al.*, 1995).

Many reports suggest that AFLP analysis has several advantages for the analysis of genetic diversity (Hill *et al.*, 1996) compared to other methods of analysis such as RFLP. One major advantage of AFLP is the short time required to assay large numbers of DNA loci (Lin *et al.*, 1996). In identifying soybean varieties, Vogel *et al.* (1994) reported that AFLP analysis detected about 12 times the number of polymorphic loci per assay as RAPD. Thus, AFLP results in an almost 50 fold decrease in time required to generate data compared to RFLP markers. Hill *et al.*, (1996) and Lin *et al.*, (1996) argued that AFLP analysis is an important technique for identifying polymorphisms and for determining genetic linkages by analysing individuals from segregating populations.

The technique has also been used to study the polymorphism, distribution, and inheritance of markers in rice populations (Maheswaran *et al.*, 1997) and genetic diversity of cultivated and wild olives (Angiolillo *et al.*, 1999). Maughan *et al.* (1995, 1996) studied the degree of AFLP variation both within and between wild and cultivated soybean and compared this to RFLP probes and microsatellites. Using AFLP, a number of polymorphic fragments were observed in soybeans, while only one polymorphic locus was detected using RFLP probes and microsatellites. AFLP marker analysis is more efficient than RFLPs and microsatellites (Becker *et al.*, 1995) but not as easy to perform as RAPD analysis.

The major difference between AFLPs and RFLPs is that PCR amplification is utilised instead of Southern hybridisation, for detection of restriction fragments (Vos *et al.*, 1995). Although difficult to perform initially, AFLPs were found to be highly reproducible, suggesting that they are as reproducible RFLPs (Jones *et al.*, 1997). However, the quality of the extracted DNA and the method of extraction may affect the profiles obtained, as AFLPs are based upon restriction digestion of DNA (Jones *et al.*, 1997). Attributed to probable differences in DNA methylation, differing AFLP patterns have been observed between different organs from the same wheat accession (Donini *et al.*, 1997). Current evidence suggests that AFLPs are as reproducible as RFLPs.

2.6.1.5 Microsatellites

DNA sequences composed of tandem repeats of short sequences are known as microsatellites (Jones *et al.*, 1997; Rafalski *et al.*, 1994), simple sequence repeats (SSRs) (Cregan, 1992), simple sequence length polymorphisms (SSLPs) (Tautz, 1989), or sequence tagged microsatellites (STMs) (Beckman and Soller, 1990). Microsatellites are highly polymorphic due to variability in the number of repeat units and are widely dispersed throughout the genomes of many organisms (Tautz, 1989; Morgante and Olivieri, 1993; Ciofi *et al.*, 1998; Jones *et al.*, 1997; Rallo *et al.*, 2000; Bertin *et al.*, 2001).

In plants the most frequently occurring simple repetitive sequence is AT (Lagercrantz *et al.*, 1993; Wang *et al.*, 1994), followed by AG and then AC

(Morgante *et al.*, 1998). SSRs are analysed by PCR amplification of a short genomic region containing the repeated sequence and subsequent size estimation of the repeat length by gel electrophoresis. As the flanking sequences at each of these sites may be unique, flanking primers are designed using sequence data of SSR loci. The resultant sequence tagged microsatellite usually identifies a single locus, which because of the high mutation rate of SSRs is often multi-allelic. SSRs are visualised on sequencing gels allowing resolution of single repeat differences and thus, detection of all possible alleles. SSRs provide highly informative markers because they are co-dominant (unlike RAPDs and AFLPs) and generally highly polymorphic (Tautz, 1989; Morgante and Olivieri, 1993; Koreth *et al.*, 1996).

The drawback to identifying DNA sequences flanking microsatellite loci is the expense and labour required (Rallo *et al.*, 2000). As knowledge of an organisms DNA sequences is required, widespread application of SSR-PCR in plants has been limited because of the relatively few DNA sequences known (Goodfellow, 1993).

2.6.1.6 RAPDs

Random amplified polymorphic DNA (RAPD) markers have been independently described (Williams *et al.*, 1990; Welsh and McClelland, 1990) as the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. Polymorphisms result from sequence differences in one or both of the primer binding sites, and are detected as the presence or absence of a particular RAPD band. The resulting polymorphic RAPD fragments are useful DNA markers in general fingerprinting or mapping applications, and behave as dominant genetic markers (Hu and Quiros, 1991; Rafalski and Tingey, 1993; Fabbri *et al.*, 1995; Trigiano and Caetano-Anolles, 1998; Woolley *et al.*, 2000).

RAPD involves the use of a single 'arbitrary' primer in a PCR reaction, resulting in the amplification of several discrete DNA products. Each product is derived from a region of the genome containing two short segments in inverted orientation, on opposite strands that are complementary to the primer and sufficiently close together for amplification to be successful. In RAPDs, amplification products are separated by gel electrophoresis stained with ethidium bromide and visualised

under ultra-violet light (Williams *et al.*, 1990; Welsh and McClelland, 1990), or visualised via silver stain (Caetano-Anolles *et al.*, 1991). It is now widely recognised that to obtain reproducible band profiles on gels, it is essential to maintain consistent reaction conditions (Jones *et al.*, 1997).

Williams *et al.* (1990) stated that RAPD polymorphisms are DNA segments that amplify from one individual but not another and are inherited in a Mendelian fashion. An advantage of these markers is that in the absence of specific nucleotide sequence information these primers detect polymorphisms that function as genetic markers and can be used to construct genetic maps for a variety of species (Williams *et al.*, 1990).

When compared to other PCR-based techniques, RAPD is simple, quick, and relatively inexpensive and can be used on minute quantities of DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990), thus increasing the likelihood of correct species identification (Williams *et al.*, 1990, 1993). Using recently developed molecular methods, in addition to traditional methods, can therefore refine taxonomic identification. Other benefits include the generation of a high degree of polymorphism, the use of a universal set of primers for a wide variety of species, and the avoidance of radioactive isotopes (Williams *et al.*, 1990, 1993; Torres *et al.*, 1993; Weerden *et al.*, 1992). RAPD analysis of a species for which there is little genetic information is a useful DNA fingerprinting technique (Williams *et al.*, 1990; Caetano-Anolles *et al.*, 1991; Caetano-Anolles and Gresshoff, 1994). RAPD markers do not require prior sequence information and can be viewed directly by agarose gel electrophoresis without the need for specific probe libraries (Grattapaglia and Sederoff, 1994; Paul *et al.*, 1997). Also, the number of RAPD polymorphisms identified per assay is higher than for RFLP (Lin *et al.*, 1996). However, Jones *et al.* (1997) stated that data derived from this technique could be difficult to emulate between laboratories due to reproducibility and band dominance limitations.

The technique usually employs short oligodeoxyribonucleotides to act as PCR primers. At low annealing temperatures a given primer binds to many sites

distributed in the genomic DNA template and DNA synthesis is initiated from the 3' terminal of these binding sites. An extension temperature of approximately 72°C results in strand elongation, and a denaturation temperature of 95°C is used to separate template DNA and the newly synthesised strand. Separated strands subsequently serve as template DNA when reaction temperature is decreased to a point where primer annealing is permitted again (usually 36°C for RAPDs). The outcome is that primer-template duplexes that are stable are transformed into accumulating amplification products (Williams *et al.*, 1990, 1993).

RAPD techniques have been used to detect polymorphisms in many perennials including grapevines (Collins and Symons, 1993), olive (Bogani *et al.*, 1994; Fabbri *et al.*, 1995; Vergari *et al.*, 1996; Mekuria *et al.*, 1999; Gemas *et al.*, 2000), *Banksia* (Maguire *et al.*, 1994), almond (Woolley *et al.*, 2000), garlic (Bradley *et al.*, 1996), and *Eucalyptus* (Keil and Griffin, 1994; Nesbitt *et al.*, 1995, 1997; Verhaegen and Plomion, 1996). In addition, RAPD techniques have proved simple and highly efficient for generating reliable markers identifying natural hybridisation within eucalypts (Sale *et al.*, 1996; Rossetto *et al.*, 1997; Neaylon *et al.*, 2001). RAPD markers were assessed for their usefulness at various taxonomic levels within the genus *Banksia* (Maguire and Sedgley, 1997). It was found that RAPDs are informative for closely related species, but not for more distant relatives, such as between distantly related series, sections and subgenera (Maguire and Sedgley, 1997). RAPD markers are routinely applied to constructing linkage maps (Yu and Pauls, 1993), and to determine genetic diversity and systematic relationships (Jarret and Austin, 1994, Wachira *et al.*, 1995), in a wide array of species.

Buffone *et al.* (1996) found that it is important to avoid the use of plant material infested with pathogens. During a study where RAPDs were applied to DNA isolated from sweet-potato plants infected with *Fusarium lateritium* and plants that were healthy, it was found that *F. lateritium* DNA successfully competes with sweet-potato DNA for primer binding.

2.6.1.8 *Marker Analysis*

Molecular methods are used to identify markers linked to specific genes or genomic regions. Several methods have been suggested for identifying genomic regions of interest with molecular markers. These include, bulked segregant analysis (BSA) (Michelmore *et al.*, 1991), the use of sequence-tagged sites (STS) (Paran and Michelmore, 1993), and pre-selection using nearly isogenic lines (NIL) (Olson *et al.*, 1989).

Using the RAPD method (Williams *et al.*, 1990) and BSA (Michelmore *et al.*, 1991) RAPD markers can be identified and linked to a dominant gene responsible for certain traits present in a plant. Two bulk DNA samples created by adding equal amounts of DNA from individuals from each population, differing in the trait of interest, are used to create DNA fingerprints that should differ significantly in the region linked to the trait. The benefits of using BSA with the RAPD-PCR technique are that it requires no prior knowledge of the genomic DNA being studied. The only requirement is that the DNA comes from a segregating population for the desired trait and the success of this depends on genetic divergence between the parents in the target regions. Bulked segregants can be made for any locus or genomic region once the segregating population has been constructed. BSA is a relatively rapid and simple protocol to perform (Michelmore *et al.*, 1991).

BSA has been used successfully to improve characters in several crops: e.g. freestone in peach (Warburton *et al.*, 1996), downy mildew resistance in lettuce (Michelmore *et al.*, 1991), anthracnose resistance in sorghum (Boora *et al.*, 1998), glaucousness of leaves in *Eucalyptus gunnii* (Wirthensohn *et al.*, 1999), rust resistance in *Phaseolus vulgaris* (common bean) (Haley *et al.*, 1993), fusarium wilt resistance in musk melon (Wechter *et al.*, 1995), rust resistance in poplars (Villar *et al.*, 1996), seed colour in *Brassica juncea* (Negi *et al.*, 2000), hypersensitive reaction of potato (*Solanum tuberosum* L.) to potato virus S (PVS) infection (Marczewski *et al.*, 1998), and olive leaf spot disease in olive (Mekuria *et al.*, 2001).

2.7 Conclusion

This project adopts a broad approach to establish the initial steps in domestication of *Solanum centrale*. By investigating the areas of propagation, steroidal alkaloids, hybridisation techniques and morphology the first steps toward plant improvement are developed. This work concentrates on genetic diversity within and between natural populations of *S. centrale* and attempts to identify a molecular marker linked to a desirable production trait.

Chapter 3 Plant Material

3.1 Sources of plant material

Plant material used in this project was sourced from ten locations known to contain natural populations of *S. centrale* (Table 3.1). Of the ten locations, eight were in the Northern Territory, and two in Western Australia (Figure 3.1).

Table 3.1 Codes for the ten different locations sourced for plant material.

Location Number	Description	Location Code
1	Utopia Station, Alice Springs region, Northern Territory	UT
2	Stirling Swamp, Alice Springs region, Northern Territory	ST
3	Laramba, Alice Springs region, Northern Territory	LA
4	Tanami Road, Alice Springs region, Northern Territory	TA
5	Gas Pipe, Alice Springs region, Northern Territory	GP
6	Stuart Highway, Alice Springs region, Northern Territory	SH
7	James Range, Alice Springs region, Northern Territory	JR
8	Kings Canyon Turnoff, Alice Springs region, Northern Territory	KC
9	Newman, Western Australia	NE
10	Ethel Creek, Western Australia	EC

3.1.1 Northern Territory

3.1.1.1 Utopia Station

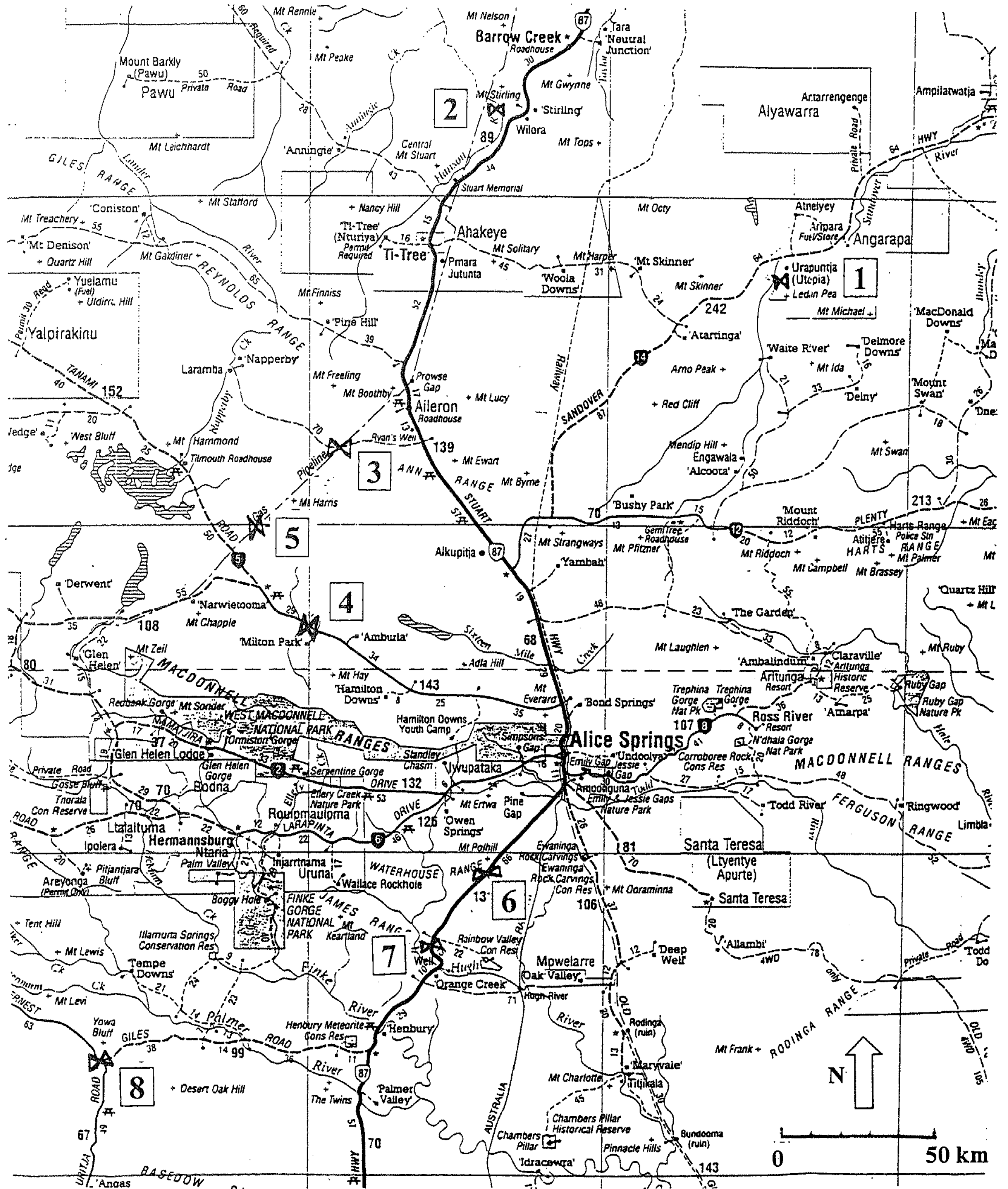
Location **UT** was Utopia Station 180 km north-west of Alice Springs, Northern Territory (latitude 22°25' and longitude 134°55') (Figure 3.2). The seed was collected by Aboriginal communities from Utopia Station and germinated by Australian Native Produce Industries (ANPI) nursery, South Australia, at the end of July 1998. Seedlings were pricked out in the middle of August 1998 and grown to an age of 3-4 months. One thousand of these seedlings (Figure 3.3) were purchased at the beginning of November 1998 for use in this study. This was the largest sampled area approximately 10km^2.

Figure 3.1 Map showing (A) Northern Territory and (B) Western Australian sampling locations of *Solanum centrale* in Australia.

B
A
N

Figure 3.2 Map showing eight sampled populations of naturally occurring *S. centrale* in the Northern Territory, Australia.

(1=**UT**, 2=**ST**, 3=**LA**, 4=**TA**, 5=**GP**, 6=**SH**, 7=**JR**, 8=**KC**)



Barrow Creek
Roadhouse
Tara
'Neutral Junction'
Mt Nelson
Mt Rennie
Mt Peake
Mount Barkly
(Pawu)
Pawu
Private Road
Mt Gwynne
Mt Stirling
'Stirling'
Wilora
Mt Tops
Artarrengenge
Alyawarra
Ampilatwatja
HWY
River
Mt Leichhardt
'Anningie'
Central Mt Stuart
Stuart Memorial
GILES RANGE
+ Mt Stafford
'Coniston'
Mt Treachery
'Mt Denison'
+ Quartz Hill
Mt Gardiner
REYNOLDS RANGE
+ Nancy Hill
'Ti-Tree' (Nturiya)
Permit Required
Ti-Tree
Ahakeye
Mt Solitary
Pmara Jutunta
Mt Harper
Mt Octy
'Mt Skinner'
Mt Skinner
'Woola Downs'
Atnelyey
Arlpara
Fuel/Store
Angarapa
Urapuntja (Utopia)
Ledan Pea
Mt Michael
'MacDonald Downs'
Yuelamu
(Fuel)
+ Uldirra Hill
Yalpirakinu
Mt Finniss
'Pine Hill'
242
'Atartinga'
'Waite River'
'Delmore Downs'
+ Mt Ida
'Delny'
'Mount Swan'
Laramba
'Napperby'
TANAMI
152
Mt Freeling
Mt Boothby
Prowse Gap
Aileron
Roadhouse
+ Mt Lucy
Arno Peak +
SANDOVER
+ Red Cliff
'Jedge'
West Bluff
Mt Hammond
Tilmouth Roadhouse
Pipeline
Ryan's Well
139
+ Mt Ewart
ANN RANGE
Mt Byrne
Mendip Hill +
Engawala
'Alcoota'
Mt Swan
+ Mt Harris
ROAD
Railway
'Bushy Park'
'Mount Riddoch'
PLENTY
Harts Range
Police Stn
HARTS RANGE
+ Mt Palmer
Atitjere
213
STUART
Alkupitja
Mt Strangways
Gemtree Roadhouse
Mt Pfitzner
'Yambah'
Mt Riddoch
Mt Campbell
Mt Brassey
'Quartz Hill'
'Derwent'
'Narwietooma'
Mt Chapple
108
'Milton Park'
'Amburla'
Sixteen Mile Creek
68
HWY
'The Garden'
Mt Laughlen +
Mt Ruby
'Glen Helen'
Mt Zeil
80
MACDONNELL
+ Adla Hill
'Ambalindum'
'Claraville'
Arltunga Historic Reserve
Arltunga Resort
Ruby Gap
Ruby Gap Nature Pk
+ Mt Hay
'Hamilton Downs'
143
Mt Everard
'Bond Springs'
Trephina Gorge Nat Pk
Trephina Gorge
Ross River
Resort
'Amarpa'
Redbank Gorge
Mt Sonder
WEST MACDONNELL NATIONAL PARK
RANGES
Hamilton Downs Youth Camp
Simpsons Gap
Alice Springs
107
NAMATJIRA
37
Ormiston Gorge
Glen Helen Lodge
Glen Helen Gorge
Standley Chasm
Serpentine Gorge
Iwupataka
Emily Gap
'Undoolya'
Jessie Gap
Corroboree Rock Cons Res
N'dhala Gorge Nat Park
MACDONNELL RANGES
Private Road
Gosse Bluff
Tnorala Con Reserve
70
Bodna
DRIVE
132
Ellery Creek Nature Park
Mt Ertwa
Pine Gap
Amoonguna
Emily & Jessie Gaps Nature Park
'Todd River'
FERGUSON RANGE
'Ringwood'
Limbla
Ltalaltuma
Roulpmaulpma
LARAPINTA
'Owen Springs'
126
Ipolera
Hermannsburg
Ntaria
Palm Valley
Injartnama
Uruna
Mt Polhill
Ewaninga
81
Santa Teresa
(Ltyentye Apurte)
Pitjantjara Bluff
Areyonga
(Permit Only)
WATERHOUSE RANGE
Wallace Rockhole
Ewaninga Rock Carvings Con Res
+ Mt Ooraminna
Santa Teresa
Boggy Hole
FINKE GORGE NATIONAL PARK
JAMES RANGE
Mt Keartland
106
+ Tent Hill
Illamurta Springs Conservation Res
+ Mt Lewis
Rainbow Valley Con Res
'Tempe Downs'
Mpwelarre
Oak Valley
'Deep Well'
'Allambi'
4WD
Private Road
Todd
Well
Hugh
Orange Creek
Hugh River
+ Mt Levi
ERNEST GILES ROAD
Palmer
Finke
River
Yowa Bluff
99
Henbury Meteorite Cons Res
'Henbury'
River
OLD
Rodinga (ruin)
RODINGA RANGE
Mt Frank +
'Maryvale'
N
'Palmer Valley'
The Twins
Mt Charlotte
Titjikala
+ Desert Oak Hill
AUSTRALIA
Chambers Pillar Historical Reserve
Chambers Pillar
Bundooma (ruin)
Pinnacle Hills
67
70
'Idracowra'
143
0
50 km
BASEDOW
'Angas
URITJA ROAD
1
2
3
4
5
6
7
8

Figure 3.3 Three- and four-month old *S. centrale* seedlings purchased from Australian Native Produce Industries Nursery, Paringa, South Australia at the beginning of November, 1998.

3.1.1.2 Alice Springs

The following populations were sourced during a trip to Alice Springs in March 2000 with the help of Mr. David Albrecht from the Herbarium located in the Alice Springs Desert Park, Parks and Wildlife Commission of the Northern Territory.

3.1.1.2.1 Fresh material

Fresh material from the first five populations described below was collected during the trip to Alice Springs. All seeds collected were germinated at the Waite campus (Chapter 4). From each population ten individuals were sampled for leaves and fruit (for seed) for genetic and morphological analysis in this project. Individuals were randomly selected over a $50m^2$ grid at about 10m intervals for each isolated population. Samples were also taken and used by the South Australian Herbarium as voucher specimens. Most population sites selected were distinct from each other based on the morphology and were found on red, sandy, free-draining soils (Figures 3.4-3.5).

- Location **TA**. Alice Springs Region, Tanami Rd (128 km from Alice Springs), latitude 23°42' S, longitude 133°00' E (Figure 3.2), collected 6/3/00.
- Location **GP**. Alice Springs Region, near gas pipe, latitude 23°00' S, longitude 132°80' E (Figure 3.2) collected 7/3/00.
- Location **SH**. Alice Springs Region, Stuart Highway, roadside adjacent red earth plain (440 m stretch of plants), latitude 23°13' S, longitude 133°66' E (Figure 3.2), collected 7/3/00.
- Location **JR**. Alice Springs Region, Stuart Highway, near James range, 24°44' S, longitude 133°47' E (Figure 3.2), collected 7/3/00.
- Location **KC**. Alice Springs Region, Ernest Giles Rd, Kings Canyon turn off, 99 km from the Stuart Highway, latitude 24°64' S, longitude 132°31' E (Figure 3.2), collected 7/3/00.

3.1.1.2.2 Herbarium seed store material

The last two locations were sourced by Mr. Albrecht in previous years and stored as voucher specimens in the herbarium located in the Alice Springs Desert Park, Parks and Wildlife Commission of the Northern Territory.

- Location **ST**. Alice Springs Region, Stirling Swamp, latitude 21°70' S, longitude 133°63' E (Figure 3.2), collected 12/8/96.
- Location **LA**. Alice Springs Region, 30 km southeast of Laramba, latitude 22°72', longitude 133°00' (Figure 3.2) collected 15/5/97.

Figure 3.4 ***S. centrale*** **plants sampled during the Alice Springs collection trip March 2000.**

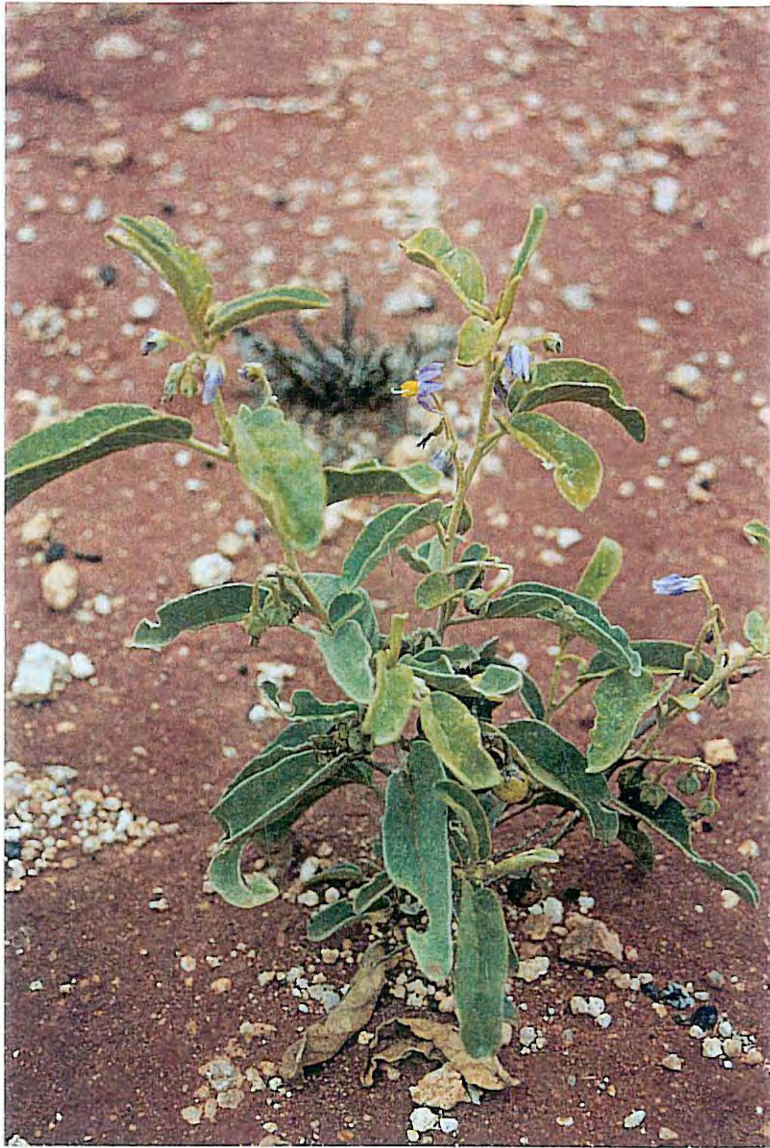
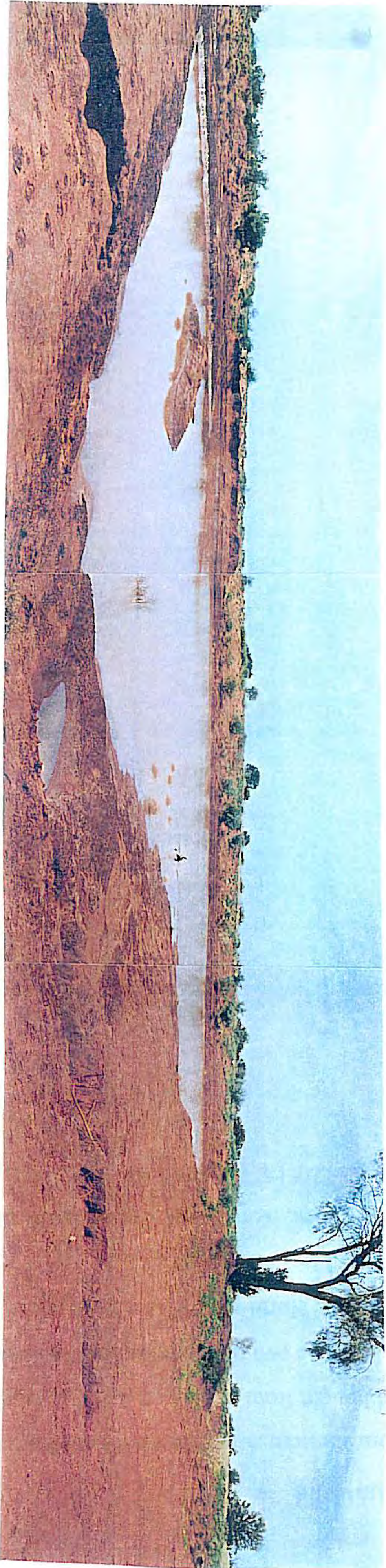


Figure 3.5 Sampling for location five, Alice Springs, Australia showing the distinctive red, sandy soil, which was characteristic of all collection sites.



3.1.2 Western Australia

Two populations from Western Australia were sampled for use in this study. Both originated from seed bought from a Western Australian seed company, KIMSEED Environmental Pty. Ltd. The two populations are described below:

- Location **NE**. Newman, North-west Western Australia, Pilbara Region, latitude 23°32' and longitude 119°75' (Figure 3.6).
- Location **EC**. Ethel Creek, North-west Western Australia, Pilbara Region, latitude 22°91' and longitude 120°15' (Figure 3.6).

Figure 3.6 Map showing two sampled locations of *S. centrale* in Western Australia.

(9=**NE**, 10=**EC**)

'Wallal Downs'
'Mandora'
Eighty Mile
Sandfire Roadhouse
Cape Keraudren
Poissonnier Point
Larrey Pt
Spit Pt
GREAT 291
Pardoo Roadhouse
'Pardoo'
'De Grey'
Port Hedland
Cape Thouin
'Boodarie'
South Hedland
'Goldsworthy'
Shay Gap
Callawa
'Callawa'
Cape Cossigny
Sherlock Bay
Depuch Is
'Mundabullangana'
'Pippingarra'
Pippingarra
'Strelley'
Strelley
'Carlindie'
Carlindie
Coongan
'Coongan'
202
Black Hill
De Grey
'Muccan'
'Yarrie'
201
HWY
'Sherlock'
Whim Creek
'Mallina'
'Indee'
'Wallareenya'
Lalla
Tabba Tabba
Lalla Rookh
Rookh
'Eginbah'
Bamboo Ck
'Warrawagine'
ISABELLA RANGE
'Pyramid'
'Kangan'
Kangan
Five Mile Hill
Marble Bar
Mt Edgar
'Mt Edgar'
MARBLE
GREGORY RANGE
'Yandeyarra'
'Abydos'
NORTHERN
261
YANDEYARRA
'Corunna Downs'
Carawine Gorge
'Woodie Woodie'
MILLSTREAM CHICHESTER NATIONAL PARK
Mt Richthofen
288
MUNGAROONA RANGE NATURE RESERVE
'Woodstock'
'Hillside'
'Coolawanyah'
'Mt Florance'
'White Springs'
'Hooley'
Nullagine
BAR
4WD 144
Mt Margaret
'Mulga Downs'
Wittenoom
42
Auski Roadhouse
RANGE
'Bonney Downs'
307
ROAD
Mt Cooke
Bold Cliff
Mt King
'Hamersley'
Hamersley Gorge
Weano Gorge
Knox Gorge
Kalamina Gorge
Mt George
Joffre Gorge
Mt Frederick
Fortescue River
Mt Marsh
'Noreena Downs'
Mt McLarty
Mt Divide
Munjina East Gorge Lookout
Fortescue Falls
Mt Bruce
'Marandoo'
141
'Marillana'
Roy Hill
Mt Lewin
Tom Price
Mt Nameless
KARIJINI NATIONAL PARK
'Juna Downs'
10
'Balfour Downs'
SALTBUSH
'Rocklea'
Mt Bennett
Mt Meharry 1249m
Mt Trevarton
'Ethel Creek'
'Billinooka'
Walagunya
ROBERTSON RANGE
197
126
Paraburdoo
OPHTHALMIA RANGE
Mt Newman
Newman
Shovelanna Hill
'Walgun'
Jigalong
Capricorn Roadhouse
9
JIGALONG
'Robertson Range'
'Mininer'
Angelo River
Turee Creek
'Prairie Downs'
'Sylvania'
Mt Boggola
Mt Bresnahan
Mundiwindi
'Bulloo Downs'
165
'Weelarrana'

N
LIT
Perry River
Ashburton
Mt Vernon
'Mt Vernon'
4WD
LOFTY RANGE
0 50 km
Lake Wilderness

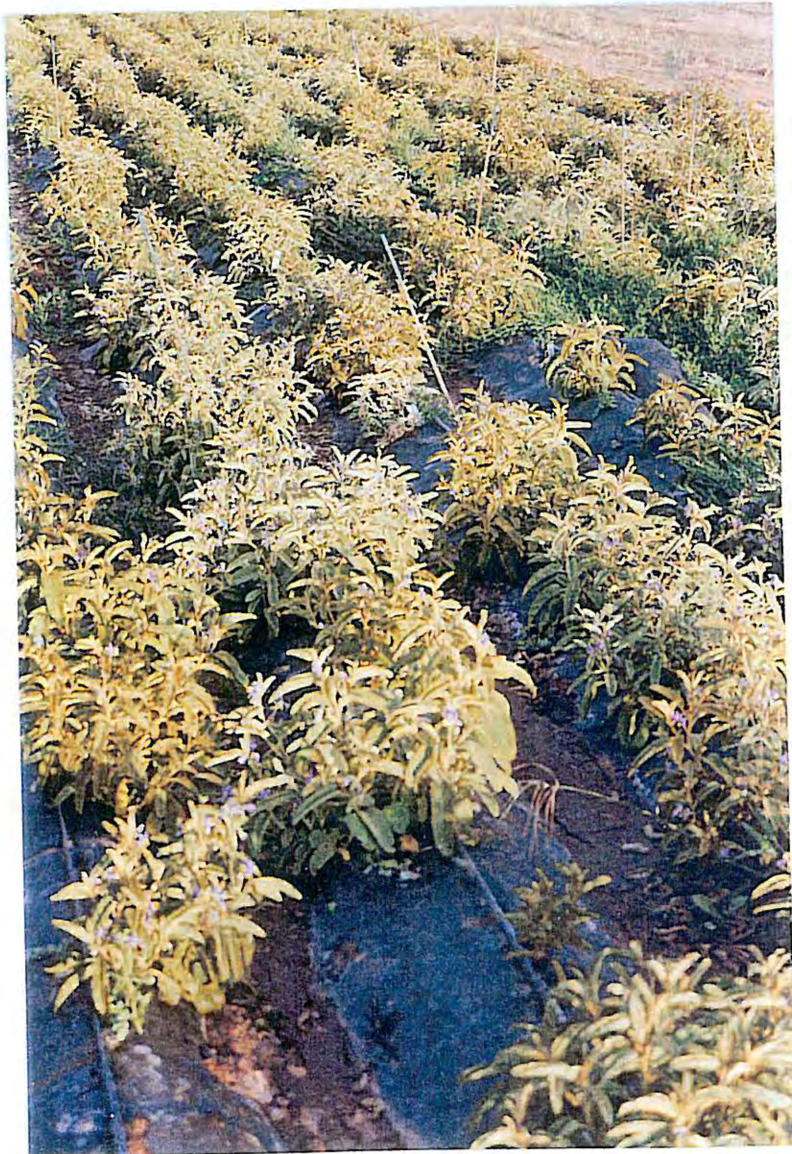
3.2 Waite Campus plantation

A plantation of 700 bush tomato (*S. centrale*) plants from the Utopia Station population was established at the Waite Campus, University of Adelaide, South Australia (latitude 34°58' S, longitude 138°38' E, altitude 100 m) on the western side of Waite road (Figures 3.7-3.9). Planting was in early November 1998 when the soil temperature was above 15°C and there was no risk of a severe frost. Average spacing of plants was 0.6 m x 0.9 m and irrigation was provided to simulate current commercial practice in native food plantations This comprised drip irrigation (2 L/hour drippers) two to three times a week for four hours depending on weather conditions during hotter months where temperatures were on average above 28°C. The plantation was set up in 10 rows approximately 45 m long, raised up on mounds for improved drainage, and gypsum was added before planting to further enhance drainage. The mounds were covered with weed mat (Matrix weed control mat, supplied by J.W. Cranston & Co. Ltd., Woodville, South Australia) to inhibit weed growth. Six metre headlands were left around the perimeter of the plantation to allow for the tractor to manoeuvre around the plantation. No fertiliser was added to the plantation.

Figure 3.7 Establishment of plantation site at the Waite Campus, University of Adelaide (September 1998). The site was watered by drip irrigation, placing weed mat in between rows inhibited weed growth, and gypsum was added to the soil to improve drainage.

Figure 3.8 Completed plantation site at the Waite Campus, University of Adelaide (November 1998).

Figure 3.9 Plantation at the Waite Campus, University of Adelaide three months after establishment (February 1999).



3.3 Potted plants

The remaining 300 plants from Utopia Station, Northern Territory were initially potted in 1.5 L pots and then potted on into 2 L pots (Figures 3.10 - 3.11). The following media was used.

Bulk Ingredients	**Quantity**
Composted pine bark	440 L
Coarse white sand	220 L
Total	660 L

Fertilisers	**Quantity**
Ferrous sulphate ($FeSO_4$)	528 g
Dolomite lime	660 g
Agricultural lime	330 g
Gypsum	330 g
Osmocote (N/P/K = 17/1.6/9)	1980 g

The soil pH was approximately 5.5-6 and was measured using a standard soil pH kit.

This media was chosen because it had been successfully used for other native plants in research carried out at University of Adelaide and it had properties similar to soils found in the natural habitat of the species such as free draining soil. Plants were watered every 1-2 days depending on weather conditions. Additional Osmocote for Australian natives was added for slow release fertilisation.

Individuals were randomly selected from both the plantation and potted plants for use in the morphological, molecular and steroidal alkaloid studies of this project. Plants derived from seeds collected in Western Australia and the Northern Territory mentioned previously were used in the morphological and molecular diversity studies.

Figure 3.10 *S. centrale* from Utopia Station seed planted in pots at the Waite Campus, University of Adelaide (November 1998).

Figure 3.11 Potted plants of *S. centrale* at the Waite Campus, University of Adelaide (January 1999).

3.4 Pests and diseases of *Solanum centrale*

The following diseases and pests of natural and plantation populations of *S. centrale* were identified.

Insect pests

- Aphids, *Macrosiphum* sp. (Hemiptera: APHIDIDAE)

Cluster on stems and the undersides of the leaves of the plant, sucking the sap. To control aphids insecticidal soaps were used. Potential problem is transfer of viruses.

- Mealy bugs, *Pseudococcus* sp. (Hemiptera: PSEUDOCOCCIDAE) (Figure 3.12)

This was a major problem with plants in the glasshouse. A number of sprays were used, including Lebaycid® (Bayer, Active constituent 550 g/L Fenthion (anticholinesterase compound)) and Confidor® (Bayer, Active constituent 200 g/L Imidacloprid). However, control was difficult to achieve and plant deaths were noted.

- Crusader bugs, *Mictis profana* (Hemiptera: COREIDAE) (Figure 3.13)

The three hemipteran species mentioned above are likely to be key pests of *S. centrale* because they:

(1) are vector pathogens

(2) feed on the secreted sugar of the species that can then cause sooty mould by disease transfer. Ants also feed on these secretions but at the same time protect the plant from predators and parasites.

(3) reduce plant vigour by feeding from vascular system

- *Alloeocysta* sp. (Lepidoptera: TINGIDAE)

Probably a new native species of moth (G. Cassis, Pers. comm.) lives in and feeds on juvenile fruit.

- Caterpillars (Lepidoptera: SPHINGIDAE) (Figure 3.14)

Eat foliage of *S. centrale*.

- Weevils (Coleoptera: CURCULIONIDAE) larval eat fruit in storage; treat with Erinol.

Disease pests

- Root rot pathogens, *Pythium* sp. and *Phytophthora* spp.

While these were not a problem for any plants grown during this study it has been found to be a problem for many *S. centrale* growers. In the case of *Phytophthora*, motile spores accumulate in the soil and attack the plant roots. Disease usually occurs when the soil is wet and waterlogged as this aids in spore dispersal. Avoiding heavy watering and improving soil drainage using products such as gypsum appeared to control disease. Both *Pythium* and *Phytophthora* can affect the seedlings of *S. centrale* by producing typical damping-off symptoms or in some cases pre-emergence decay. Affected seedlings collapse and die. To reduce this risk, all pots used for seed germination were cleaned with boiling water before use.

- Black Sooty mould (Figure 3.15)

Black mould was present on the fruits of the species when the weather was humid and towards the end of the growing season.

Other pests

- Weeds

Weeds found in the plantation and pots sometimes reached large numbers and competed with the *S. centrale* plants, resulting in stunting and some plants dying. Hand weeding was used to control this problem.

- Rodents

Both rats and mice were found in the plantation, eating the fruit as it began to ripen. No control was applied, as the damage was not significant.

Figure 3.12 Mealy bugs (*Pseudococcus* sp.) present on *S. centrale*. Note feeding from central leaf vein.

Figure 3.13 Crusader bug (*Mictis profana* (Hemiptera: COREIDAE)) a pest of *S. centrale*. Alice Springs collection trip, March 2000.

Figure 3.14 ***Alloeocysta*** **sp. (Hemiptera: TINGIDAE), a pest of** ***S. centrale.*** **Alice Springs collection trip, March 2000. Note feeding damage on leaf edges.**

Figure 3.15 Black sooty mould present on the fruit and stems of ***S. centrale.***

Chapter 4 Propagation

4.1 Introduction

Some methods of plant propagation allow multiplication of plants whilst preserving their essential genetic characteristics. Such methods utilise propagules representing a specific genotype. Propagules include seeds and cuttings, which were utilised in this project. To maintain a consistent genotype in the plant population being propagated, it is important to control the selection of the seed parents or the vegetative source of the propagule (Hartmann *et al.*, 1997).

4.2 Seed germination

A number of different techniques were tested to germinate *S. centrale* seed. These included: scarification (mechanical, acid, and hot water scarification), and stratification (moist chilling and refrigerated stratification). These techniques were not successful, and resulted in little or no seed germination. Mini-greenhouse propagation units (Yates) were also used in an attempt to germinate seeds. These units have a clear plastic cover, which lets light in and protects fragile seeds during germination. Punnets were filled with seed raising mix and soaked before seeds were placed in each capsule, covered over with seed raising mix and watered with a spray bottle. The propagation units were watered daily or as required.

Due to the difficulty of germinating several Australian native species (Vigilante *et al.*, 1998), other novel methods of seed germination have been trialed. Following initial research carried out in South Africa (deLange and Boucher, 1990), further studies at Kings Park and Botanic Garden in Western Australia (Dixon *et al.*, 1995) found that smoke increases germination for many Australian native plants. The use of smoke-derived materials can promote germination of species that are difficult to germinate by conventional means, aid earlier and uniform germination, and produce seedlings that are more robust. The exact mechanism by which smoke breaks dormancy is unclear (Roche *et al.*, 1994; Vigilante *et al.*, 1998). It may interact chemically with the seed coat, endosperm or embryo to enhance seed germination. The active ingredients are

expected to be smoke-specific signal molecules, possibly promotive hormones (Baldwin *et al.,* 1994).

Smoke can be applied in three ways:

- aerosol smoke applied to seed trays;
- direct application of aerosol smoke to seed;
- smoke water applied to seed trays and smoke water imbition of seed.

S. centrale can be germinated in this way because it is a species adapted to bushfire conditions, responds well to smoke and can be difficult to germinate by conventional means (Roche *et al.*, 1994; Vigilante *et al.*, 1998). All three methods were tested on freshly collected mature *S. centrale* seed.

4.2.1 Materials and methods

For each method discussed below 100 *solanum centrale* seeds were sown. This was repeated three times for each of the three methods applied.

- **Method 1 - Aerosol smoke applied to seed trays**

Using a protocol developed in conjunction with Ray and Pat Rogers from 'Tanamara' bushfoods, *S. centrale* seeds were smoked in the following way. To smoke the seed a barbecue was used. Seeds were sprinkled into trays (20 x 30 cm) and then covered with perlite. The material used to make smoke in the barbecue consisted of ground up bush tomatoes and was placed in a metal container in the bottom of the barbecue (pie holder was used). This technique is very similar to fish smoking. Burning methylated spirits, using a gas lighter from a fish smoker, produced a flame. Smoking was for approximately 20-30 minutes at approximately 50°C after which trays were left to cool before being removed and placed on a heat mat set at 40°C. Trays were then covered with the tops of mini-greenhouses (Yates) to create a more humid environment. Hydroponic solution (Green Air®, USA) containing 100 L of water, 60 g hydroponic nutrient and 40g calcium nitrate was used to water the developing seedlings after germination. The hydroponic nutrient was made up of the following:

Total Nitrogen	3%
Nitrate Nitrogen	3%
Available Phosphoric Acid (P2O5)	10%
Soluble Potash (K2O)	19%

Total Magnesium (Mg)	3%
Water Soluble Magnesium (Mg)	3%
Sulphur (S) (Combined)	4%
Boron (B)	0.03%
Copper (Cu) (Chelated)	0.02%
Iron (Fe) (Chelated)	0.2%
Manganese (Mn) (Chelated)	0.05%
Molybdenum (Mo)	0.003%
Zinc (Zn) (Chelated)	0.05%
Chlorine (CL) not more than	0.12%

- **Method 2 - Direct application of aerosol smoke to seed**

Seed was collected, cleaned and placed in the barbecue as mentioned previously. Seeds were then planted in trays containing perlite and treated as in the first method.

- **Method 3 - Smoke water applied to seed trays + smoke water imbition of seed**

Seeds were soaked overnight (12h) in commercially available smoked water (Murray Plains Wildflower Farm, Pinjarra, Australia) according to manufacturing instructions and then planting into trays containing perlite or vermiculite. Seeds were watered every day with the smoke water until germination (emergence of the shoot) had occurred. Mini-greenhouses were also used with this method in order to create a humid atmosphere.

4.2.2 Results

As Table 4.1 shows, aerosol smoking of seed in trays was the method that produced the highest percentage of germination for *S. centrale*. The use of a mini-greenhouse appeared to improve germination of the seeds (Figure 4.1 and 4.2). Other methods produced approximately the same percentage of germination. Seed germination was carried out using the aerosol smoking method on collections made in Alice Springs and on seed purchased from Western Australia.

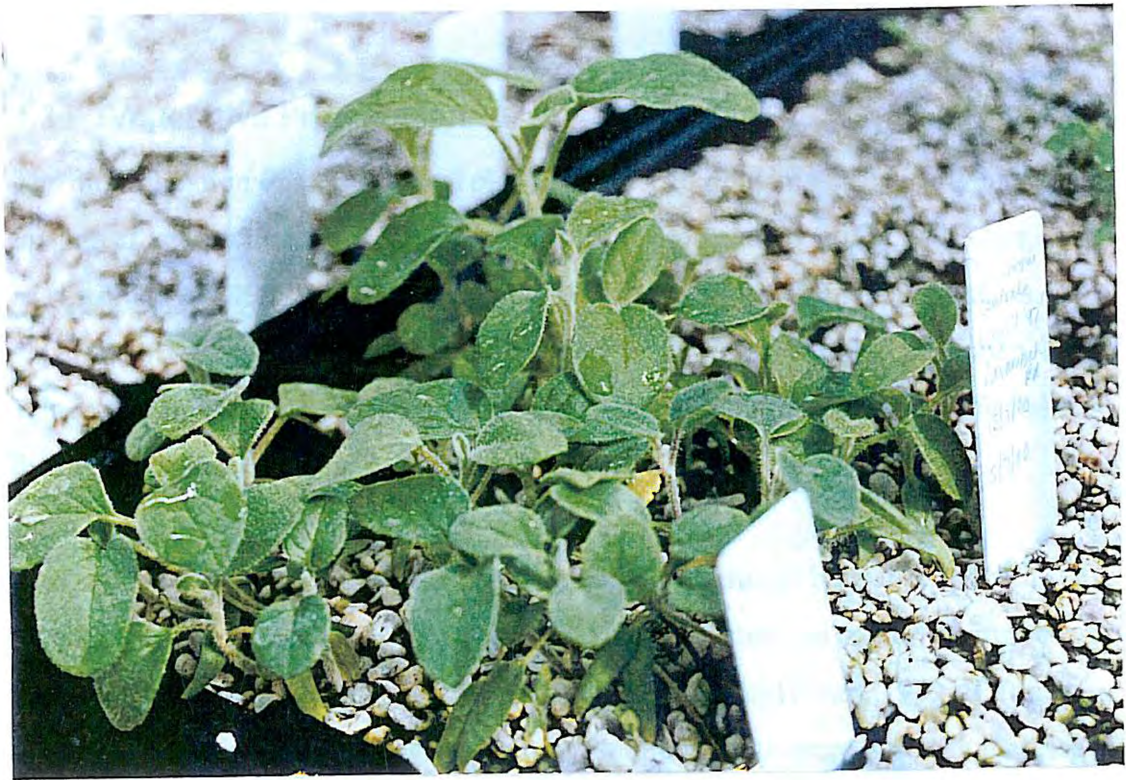
Table 4.1 Results from seed germination trial using three different smoke-related methods showing final germination percentage.

Germination technique	% Germination
Method 1-Aerosol smoke applied to seed tray	35 %
Method 2-Direct application of aerosol smoke to seed	17 %
Method 3-Smoke water applied to seed trays and smoke water imbition of seed	15 %

simple chi-square test (Pearson method) was performed on the results from the rmination trial using the three different methods. The chi-square value was 4.96 th 2 d.f. ($P = 0.084$). This indicated that there was a significant difference between ; three methods with method 1 which was using aerosol smoke to seed trays being ; most successful for germinating *S. centrale* seeds. For this reason method 1 was osen to carry out seed germination on samples collected from wild populations of *S. ntrale.*

Figure 4.1 ***S. centrale*** **seedlings germinated from seed treated using aerosol smoking of seed trays in mini-greenhouses.**

Figure 4.2 ***S. centrale*** **seedlings pricked out after germination in mini-greenhouses.**



4.3 Vegetative Propagation

The multiplication of an individual plant into a series of plants, genetically identical to the parent and each other, is known as vegetative propagation (Elliot and Jones, 1980; Hartmann *et al.*, 1997). Vegetative propagation differs from sexual propagation, which involves pollination, fertilisation and gene segregation (Elliot and Jones, 1980). Where plants do not breed true to type, clonal propagation methods are preferred so that variation can be minimised (Newell, 2000).

Cuttings are segments of shoots containing lateral or terminal buds that are cut from a parent plant and induced to form roots by chemical, mechanical and/or environmental manipulation, thus producing independent plants (Elliot and Jones, 1980; Hartmann *et al.*, 1997). The ability of cuttings to form adventitious roots decreases with increase in the age of the parent plant, a phenomenon that has been demonstrated in many woody plants including apple, pear, oak, eucalypts and ivy (Bachelard and Stowe, 1963; Hartmann *et al.*, 1997). The juvenile tissues of most plants have a higher rooting potential than mature tissues (Hartmann *et al.*, 1997).

Propagating from juvenile plants could be problematic because adult characteristics of the plant will be unknown, such as flower colour, habit and form (Elliot and Jones, 1980). Severe pruning where the shoots are cut back to old wood and the resulting new growth is used for cuttings is a procedure that may induce a temporary return to juvenility (Elliot and Jones, 1980).

4.3.1 Materials and methods

Cuttings of *S. centrale* were collected from young, healthy (5 month old) plants from both the plantation and potted plants at the Waite campus. Stem cutting material was collected early in the morning to avoid water stress. Material was kept cool, out of direct sunlight and used within two hours after collection. Cuttings ranged from 7 to 12 cm in length with two or more nodes present on each. The basal cut was made just below a node and made on an angle to aid adsorption of plant growth regulators. Leaves on the lower portion of cuttings were removed, with two to four leaves retained on the upper part.

For each cutting the basal 2 cm portion was placed into a solution of plant growth regulator (Clonex), with active constituent Indole Butyric Acid (IBA) at either 3 000 ppm or 8000 ppm, for approximately 10 seconds. Cuttings were allowed to air dry and were inserted into a mixture of perlite: vermiculite (1:1) in pots (190 mm x 190 mm). Pots were initially watered before being placed under intermittent mist with bottom heat at 25°C.

Three cuttings were taken from 100 plants and randomly allocated to these treatments (3 000 ppm IBA, 8 000 ppm IBA or water). All cuttings were completely randomised to 5 blocks (trays) per treatment within the propagation structure. Root production was monitored daily by visual assessment and replanting after investigation on selected cuttings. Plants were given a final score based on survival (yes or no).

4.3.2 Results

All treatments resulted in adventitious rooting (Table 4.2). When a plant growth regulator was applied to a cutting the survival rate increased. The higher the level of IBA applied to the cutting resulted in a greater the chance of survival. Survival rate for control cuttings was 60%, however cuttings were observed to be less healthy (weaker root system, smaller plants) when treated with water, when compared to those treated with IBA (Figure 4.1). Survival rate of plants originating from cuttings was higher then for plants germinated from seed, for all treatments.

Table 4.2 Percentage survival of *Solanum centrale* cuttings using three different rooting treatments.

Treatment	Percentage survival (%)
Water	60 %
3 000 ppm IBA	90 %
8 000 ppm IBA	95 %

A simple chi-square test (Pearson method) was performed on the results from the cutting trial using the three different rooting treatments. The chi-square value was 4.71

with 2 d.f. ($P = 0.095$). This indicated that there was a significant difference between the water treatment and the IBA treatments. There was no significant difference found between the two IBA treatments.

Figure 4.3 Cuttings treated with 8 000 ppm IBA.

4.4 Discussion

All three germination methods tested on *S. centrale* had a success rate below 40 % indicating the difficulty of seed germination. Method 1 using aerosol smoke applied to seed trays resulted in a significantly higher germination of *S. centrale* seed. *S. centrale* seed has been observed to germinate in the wild after a bushfire (D. Symon, pers. Comm.) and species from fire-prone vegetation often show dormancy and germinate in response to fire-related cues. Research on other native species seed germination has had similar findings with a positive effect on germination success found with the use of smoke (Morris, 2000; Read and Bellairs, 1999; Roche *et al.*, 1997).

All three rooting treatments applied to cuttings taken from *S. centrale* were successful in producing adventitious roots. Of the three treatments applied water was found to be the least effective treatment for vegetative propagation. Using a plant growth regulator, (Clonex) with active constituent IBA, was found to significantly improve the success of vegetative cuttings when compared to a water treatment. The use of plant growth regulators to improve root development has also been found in other native species (Kumar, 2000;Wilkinson, 1994; Armstrong, 1991; Salmon, 1990).

A requirement for the breeding of out-crossing species is maintaining the desired genotype. The low seed germination levels found in *S. centrale* support the argument that vegetative methods of propagation should be explored further. In commercial situations the use of cuttings would be an advantage as superior plant genotypes could be selected readily and plants would be expected to mature earlier. Once superior genotypes of *S. centrale* have been developed for commercial production cutting propagation will be essential. Vegetative (clonal) propagation is more expensive than traditional seed germination so the benefit of clonal, superior material must outweigh the cost for this to be viable. Further refinement of the cutting procedure is needed for commercial production.

Chapter 5 Investigations into the extraction and measurement of steroidal alkaloids

5.1 Introduction

In the genus *Solanum* and closely related genera such as *Lycopersicon* (eg. tomato), species have been found to contain steroidal alkaloids in their tissues. Some of these alkaloids have been found to be toxic in the human diet (Hall, 1992). Kingsbury (1964) suggests that any species of *Solanum* should be considered potentially poisonous unless determined otherwise. Analysis of ripe fruits compared to immature fruit of some species has shown that levels of alkaloids in ripe fruits are reduced and appear safe (Everist, 1974). To address steroidal alkaloid content in *Solanum centrale* leaves and fruit were analysed.

5.2 Materials and methods

Fruit and leaf material of *S. centrale* used for extraction was frozen in liquid nitrogen and stored at -80°C prior to analysis for steroidal alkaloid content. Several methods were tested for the extraction and measurement of compounds, and involved two forms of chromatography, high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). All methods were based on previous work done on *Solanum tuberosum* (potato), as many years of research has been carried out on the presence and role of steroidal alkaloids in this species. Some comparative work was conducted with green tubers of *S. tuberosum*.

5.2.1 Extraction and clean up methods

Three methods of steroidal alkaloid extraction were tested.

Method 1 (based on Hellenäs, 1995)

Small amounts (between 0.2-1.5g) of the leaves/fruit were ground in liquid nitrogen in a mortar and pestle. The ground tissue was placed in a 1.5 ml eppendorf tube, and 1 ml of water/acetic acid/sodium bisulphite ($NaHSO_3$), 95:5:0.5 (v/v/w) added, and mixed on a rotating wheel for approximately 15-20 min. Following mixing, samples were centrifuged at 8 000 rpm for 10 min and the supernatant passed through a millex®-HA 0.45 µm filter unit. A Sep-Pak® C18 (reverse phase) cartridge column

(Waters, USA) was conditioned by elution of 1 ml 100% acetonitrile followed by 1 ml water/acetic acid/ $NaHSO_3$ solvent. One ml of material extract sample was passed through the column, followed by 1 ml water/acetonitrile, 85:15 (v/v), for washing. Alkaloids eluted from the cartridge with 1 ml of acetonitrile/phosphate buffer (10 mM ammonium phosphate in 60% acetonitrile, adjusted to pH 2.5). One ml then adjusted to 5 ml with the same phosphate buffer.

Method 2 (based on Hellenäs, 1986)
Approximately 10 g of leaf and fruit tissue was blended using a Polytron, Kinematica AG blender for 2 min with 25 ml of water/acetic acid/sodium bisulphite (100:2:0.5) (v/v/w). The volume was then adjusted to 50 ml with the same solvent and an aliquot clarified by centrifuging at 4 000 rpm for 20 min. Ten ml of clarified extract was put onto Sep-Pak® C18 (reverse phase) cartridge column previously activated by elution with 5 ml acetonitrile followed by 5 ml phosphate buffer (10mM, pH 2.5). The cartridge was then washed with 2 ml acetonitrile/phosphate buffer 15:85 (v/v) and glycoalkaloid eluted with 4 ml acetonitrile/phosphate buffer 30:70 (v/v). Four ml was then adjusted to 5 ml with the same solvent.

Method 3 (based on Carmen *et al.*, 1986)
Approximately 10 g of leaf and fruit tissue was placed in a Polytron, Kinematica AG Blender with 12 ml of the extraction solution containing 4.0 g of 1-heptanesulfonic acid, sodium salts, in 1% aqueous acetic acid. The sample was blended at high speed for 3 minutes and filtered through coarse paper to obtain a crude filtrate. To purify the sample extract, a Sep-Pak® C-18 (reverse phase) cartridge column was conditioned by elution with 5 ml methanol followed by 5 ml of the extraction solution. Ten ml of the sample extract was added to the conditioned LiChrospher® 100 RP-18 (5μm) column (Merck, Darmstadt, Germany) and allowed to pass through, followed by 5 ml of 20% acetonitrile in water. All previous eluates were discarded. Any steroidal alkaloids were eluted from the column with two 1.0 ml volumes of the filtered (0.7 μm) and degassed HPLC mobile phase containing 0.29 g of ammonium phosphate in 250 ml of acetonitrile. The flow rate through the Sep Pak® column is restricted to 1-2 drops/sec. The sample solution was mixed by shaking and 20 μl injected into the HPLC.

5.2.2 Steroidal alkaloid standards

Stock solutions of common steroidal alkaloids found in potatoes and tomatoes were purchased from Sigma-Aldrich Pty. Ltd and used as standards. Purchased standards included solasodine, solanidine, demissidine, tomatidine, α-tomatine, α-solanine, and α-chaconine. HPLC working standards were prepared by diluting the stock solutions in the HPLC mobile phase or methanol for TLC analysis. Adding the appropriate amounts of the stock standard solutions to 10 ml aliquots of samples extracted from fruits and subjecting them to analysis by HPLC estimated recoveries of alkaloids. Standards were used to spike samples to determine amounts of steroidal alkaloids present in plant tissue.

5.2.3 Extraction analysis

Two types of analysis were explored to analyse the steroidal alkaloid extractions, high performance liquid chromatography and thin layer chromatography.

5.2.3.1 High performance liquid chromatography

All high performance liquid chromatography (HPLC) was carried out using a Hewlett Packard HPLC 1100 System, with a diode array detector, degasser and quaternary pump. The column used was a Lichrospher® 100 RP-18 (5μm), Merck, Darmstadt, Germany. All wavelength readings were carried out between 200 and 210 nm. Samples were run through the column for varying periods ranging from 15-90 min using an isocratic gradient. 20μl of sample were injected onto column and the flow rate was set at 1 ml/min.

Three mobile phases were used to separate the extracted compounds. The first being 100 mM ammonium phosphate buffer, in 35% acetonitrile, adjusted to pH 3.5 with phosphoric acid (for glycoside determination) and the second, 10 mM ammonium phosphate buffer, in 60% acetonitrile adjusted to pH 2.5 (for aglycone determination) (Friedman and Dao, 1992). The third mobile phase used was based on Carmen *et al.* (1986) and involved 0.29 g of ammonium phosphate in 250 ml of HPLC grade water and 250 ml of acetonitrile and then filtered through 0.7 μm glass filter and degassed.

5.2.3.2 Thin layer chromatography

Thin Layer chromatography (based on Carmen *et al.*, 1986) was used to observe the sample and compare to standards. Ten μl of sample solutions were spotted on silica gel (60) pre-coated plates (Merck, Darmstadt, Germany) along with appropriate standards. Spots were dried for 2 min with aid of warm-air blower (hair dryer) and the spotted plate then developed in an equilibrated tank. The solvent system used to separate the compounds extracted was chloroform/methanol/1% ammonium hydroxide (2:2:1) (Carmen *et al.*, 1986; Friedman and McDonald, 1995). The developed plate was then fumed with iodine vapours and evaporated by leaving the TLC plate at room temperature.

5.3 Results

5.3.1 Extraction and clean-up methods

The three methods were tested on the leaves and fruit of *S. centrale* as well as green potato tubers. All three methods were used in both HPLC and TLC analysis. When separated on TLC plates the result was a streaking effect, making interpretation difficult. Method 3 was chosen as the preferred extraction method for separation of compounds in both HPLC and TLC analysis. However, samples still needed further purification to gain more clarity and distinguish steroidal alkaloids that may be present.

5.3.2 Steroidal alkaloid standards

All standards were run using both HPLC and TLC analysis to try to distinguish unknown peaks in extractions of leaf and fruit material. Problems occurred when samples were spiked with standards as standards were not eluted from the column and did not appear on chromatograms (Figures 5.1 and 5.2).

5.3.3 Extraction analysis

5.3.3.1 High performance liquid chromatography

Samples extracted using method 1 and 2 resulted in high backpressure when run through the column using HPLC analysis, indicating impurities in the sample. This eventually led to blockages in the column and for this reason these samples were not

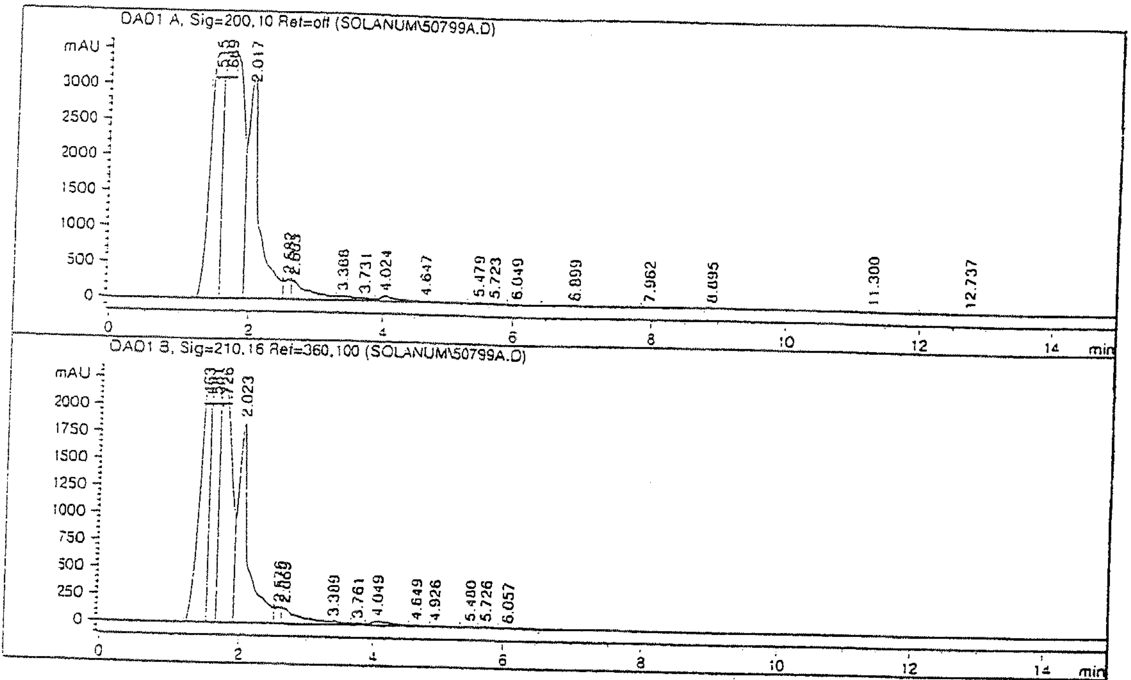
continued. All figures displayed in results are from samples extracted using method 3. Similar problems with backpressure were faced using method 3 when running samples through the column. As shown in Figure 5.3 and 5.4 even though samples still appear to contain impurities (peaks between 0-2 min) samples extracted from the green fruit and leaves resulted in some separation of compounds when run through the column and therefore elution of some compounds represented by unknown peaks shown in chromatograms. No separation (no peaks present on chromatogram) occurred when samples of fruit at the yellow and raisin stage of development were run through the column (Figure 5.5 and 5.6). Samples of potato extract using the same procedure applied to bush tomatoes were run through the column to test the methods used (Figure 5.8). Standards of α-solanine and α-chaconine known to be present in potato tissue were run on their own (Figure 5.7) and then used to spike a sample of potato extract (Figure 5.9). Impurities were also indicated when separating the potato extract, however two compounds were eluted from the column (as represented by the two peaks at around 4 min) at approximately the same time as the two standards α-solanine and α-chaconine. When the potato sample was later spiked with the two standards the same two peaks were produced.

5.3.3.2 Thin layer chromatography

Samples extracted from the fruit and leaves of *S. centrale* were run next to steroidal alkaloid standards and potato extracts. All standards were separated on the plate and observed visually. Potato extracts when separated produced two spots at similar distances on the plate to the α-solanine and α-chaconine standards. When both the leaf and green fruit extract of *S. centrale* were separated streaking occurred making the identification of spots difficult (Figure 5.10).

Figure 5.1 Chromatograms measuring a 20µl sample of extracted *S. centrale* green fruit at 200 nm and 210 nm using HPLC analysis.

Figure 5.2 Chromatograms measuring a 20µl sample of extracted *S. centrale* green fruit spiked with 20µg/ml α-solanine and 20µg/ml α-chaconine standards at 200 nm and 210 nm using HPLC analysis.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DAD1 B, Sig=210,16 Ref=360,100 (SOLANUM\50799A.D)
mAU
min

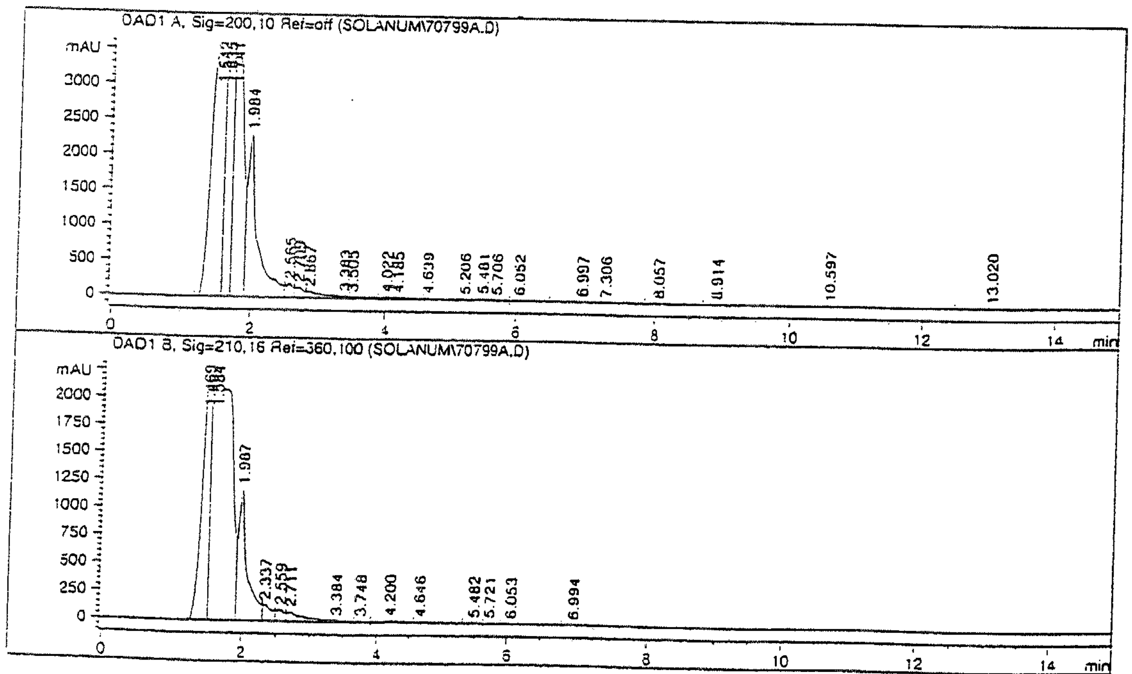
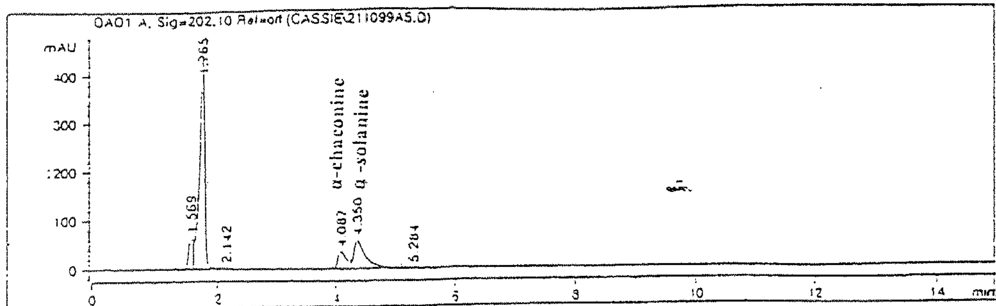

DAD1 A, Sig=200,10 Ref=off (SOLANUM\70799A.D)
DAD1 B, Sig=210,16 Ref=360,100 (SOLANUM\70799A.D)
mAU
min

Figure 5.3 Chromatograms measuring 20μg/ml α-solanine and 20μg/ml α-chaconine standards at 200 nm and 210 nm using HPLC analysis. Peak 1 measured after 4.0 min (α-chaconine) and peak 2 measured after 4.3 min (α-solanine).

Figure 5.4 Chromatograms measuring a 20μl sample of extracted *S. centrale* yellow fruit at 200 nm and 210 nm using HPLC analysis.

Figure 5.5 Chromatograms measuring a 20μl sample of extracted *S. centrale* raisin fruit at 200 nm and 210 nm using HPLC analysis.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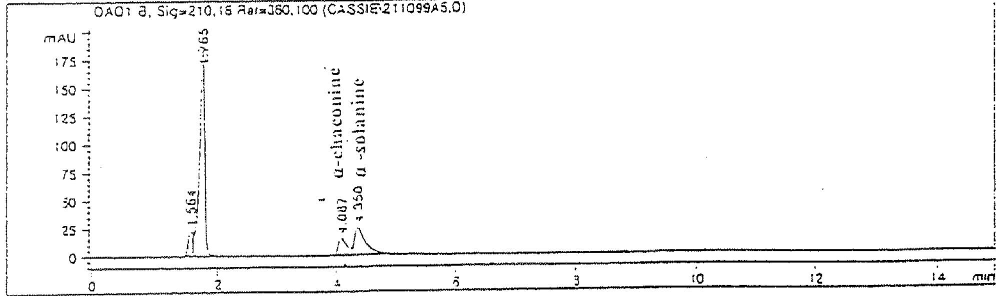
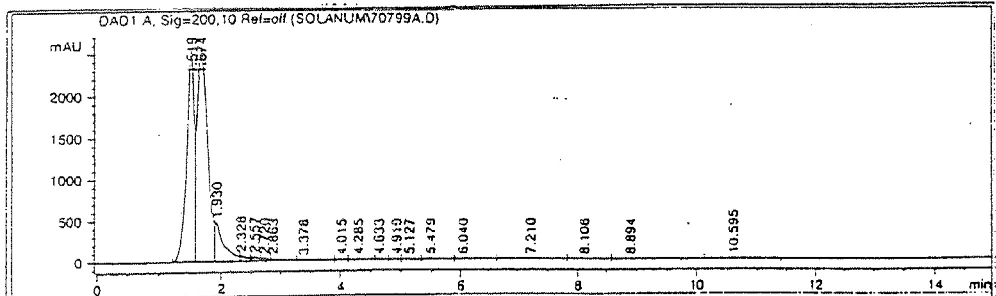
mAU
1.569
1.765
2.142
4.087
4.350
5.284
α-chaconine
α-solanine
min


DAD1 B, Sig=210,16 Ref=360,100 (CASSIE\211099A5.D)
mAU
1.564
1.765
4.087
4.350
α-chaconine
α-solanine
min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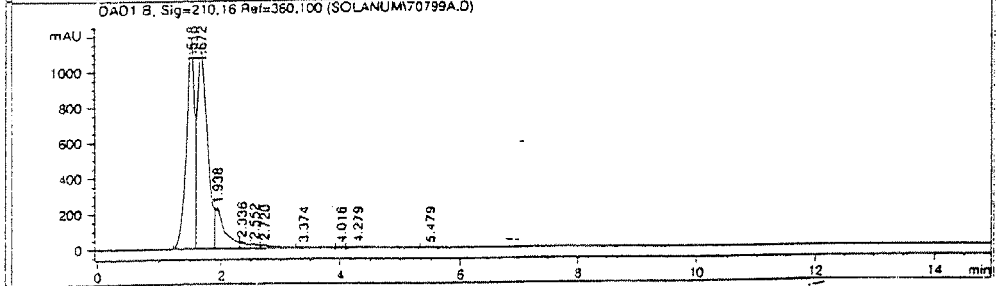
mAU
1.519
1.674
1.930
2.328
2.557
2.720
2.863
3.378
4.015
4.285
4.633
4.919
5.127
5.479
6.040
7.210
8.106
8.894
10.595
min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1.518
1.672
1.938
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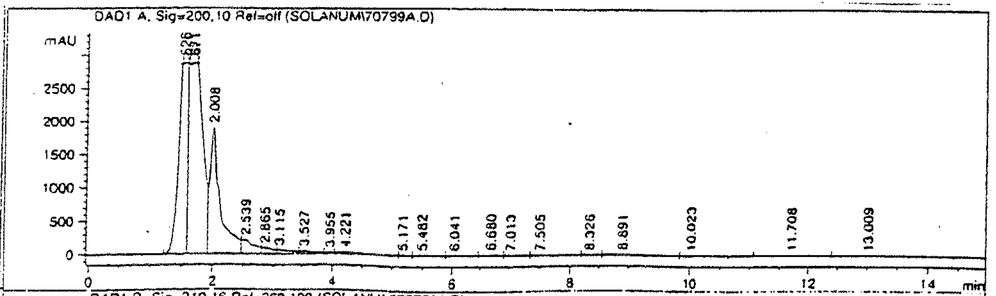
2.552
2.720
3.374
4.016
4.279
5.479
min


DAD1 A, Sig=200,10 Ref=off (SOLANUM\70799A.D)
mAU
1.626
1.671
2.008
2.539
2.865
3.115
3.527
3.955
4.221
5.171
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6.680
7.013
7.505
8.326
8.891
10.023
11.708
13.009
min

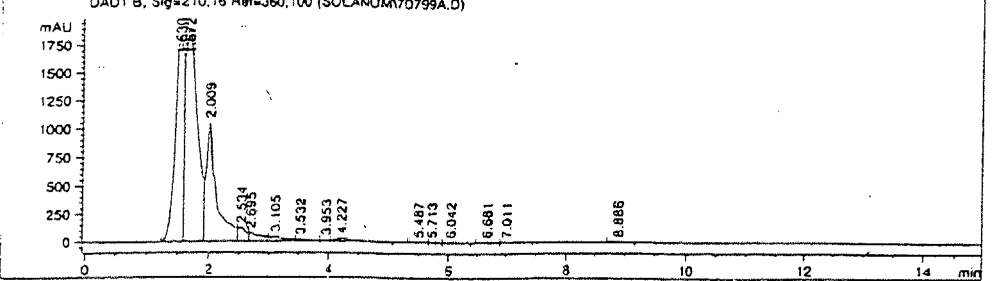
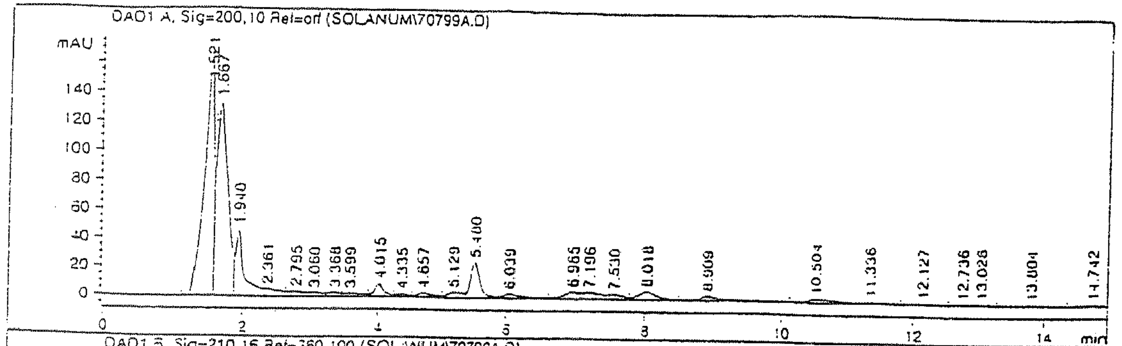
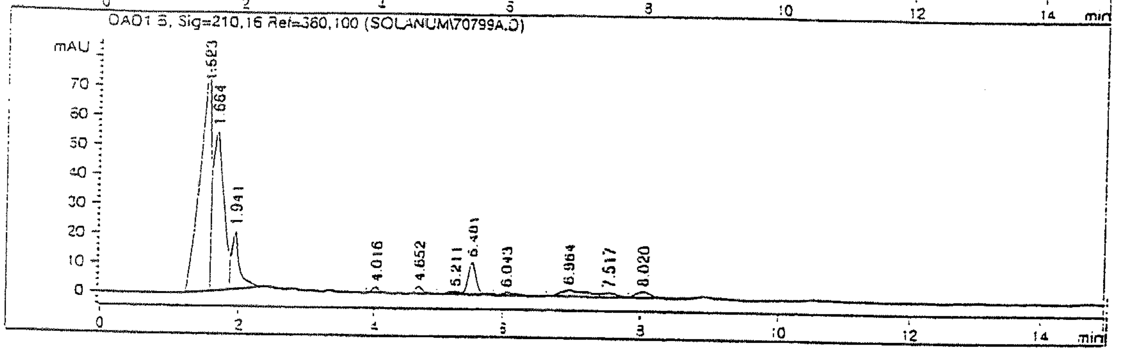

DAD1 B, Sig=210,16 Ref=360,100 (SOLANUM\70799A.D)
mAU
1.630
1.672
2.009
2.534
2.695
3.105
3.532
3.953
4.227
5.487
5.713
6.042
6.681
7.011
8.886
min

Figure 5.6 Chromatograms measuring a 20µl sample of extracted *S. centrale* green fruit at 200 nm and 210 nm using HPLC analysis. Peak measured after 5.4 min.

Figure 5.7 Chromatograms measuring a 20µl sample of extracted *S. centrale* leaves at 200 nm and 210 nm using HPLC analysis. Peak measured after 3.7 min and 5.7 min.


DAD1 A, Sig=200,10 Ref=off (SOLANUM\70799A.D)
mAU
min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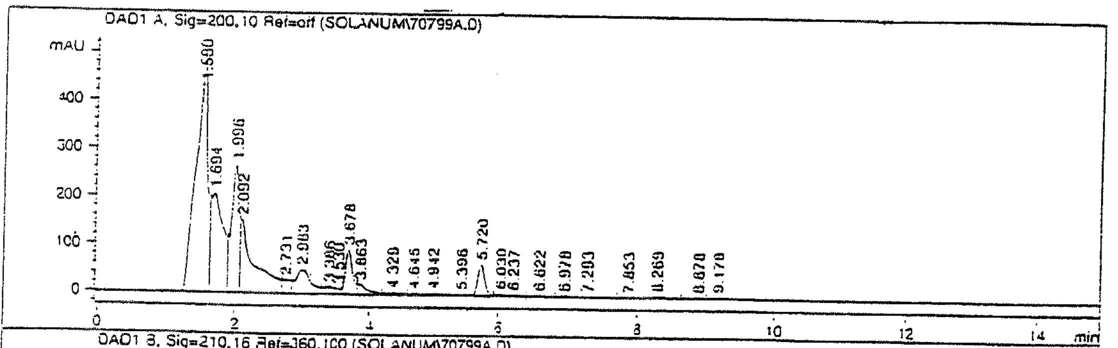
mAU
min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mAU
min

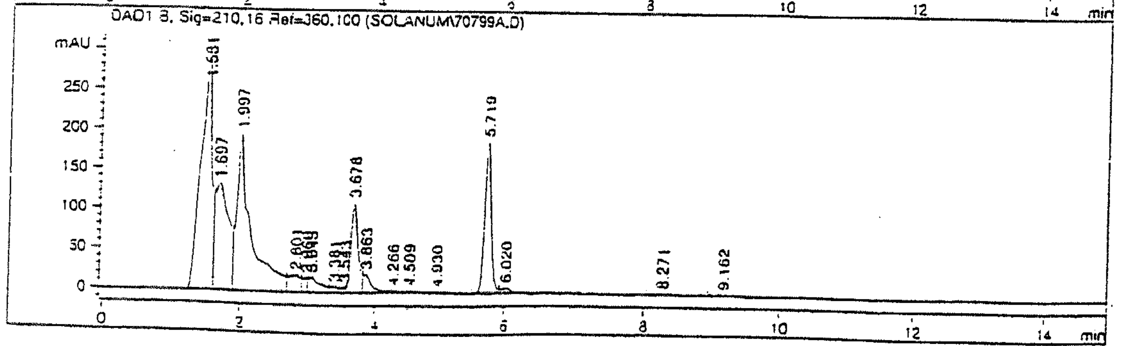
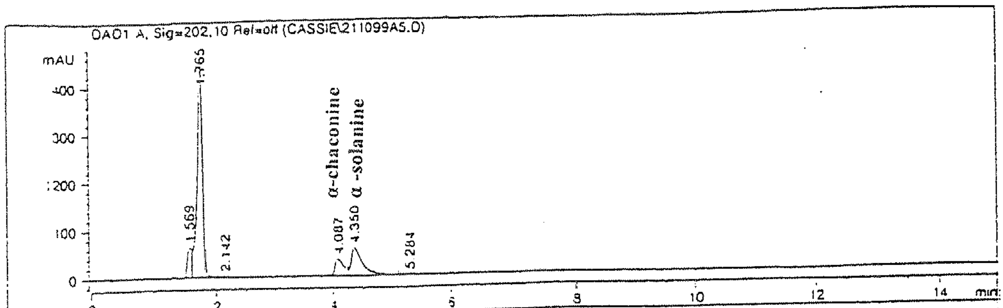

DAD1 B, Sig=210,16 Ref=360,100 (SOLANUM\70799A.D)
mAU
min

Figure 5.8 Chromatograms measuring 20µg/ml α-solanine and 20µg/ml α-chaconine standards at 200 nm and 210 nm using HPLC analysis. Peak 1 measured after 4.0 min (α-chaconine) and peak 2 measured after 4.3 min (α-solanine).

Figure 5.9 Chromatograms measuring extracted green potato samples at 200 nm and 210 nm using HPLC analysis. Peak 1 measured after 3.97 min and peak 2 measured after 4.17 min.

Figure 5.10 Chromatograms measuring extracted green potato samples spiked with 20µg/ml α-solanine and 20µg/ml α-chaconine standards at 200 nm and 210 nm using HPLC analysis. Peak 1 measured after 3.95 min and peak 2 measured after 4.14 min.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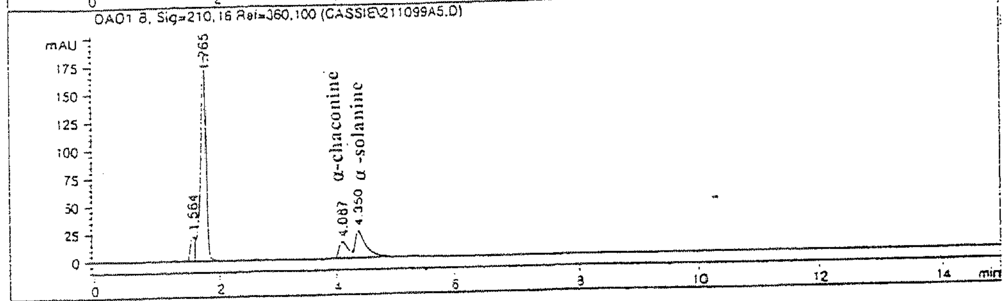
mAU
α-chaconine
α -solanine
min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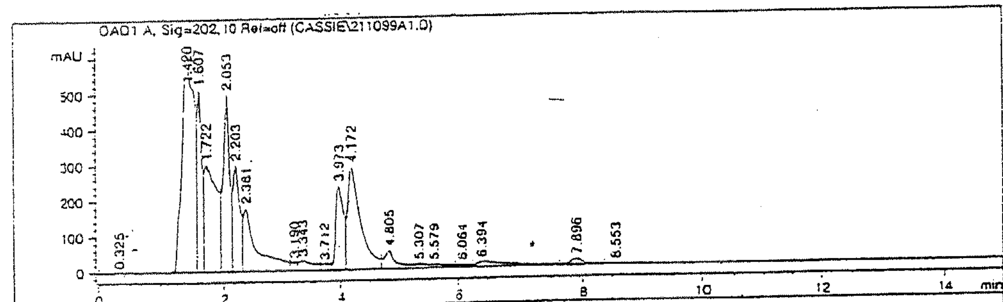
mAU
α-chaconine
α -solanine
min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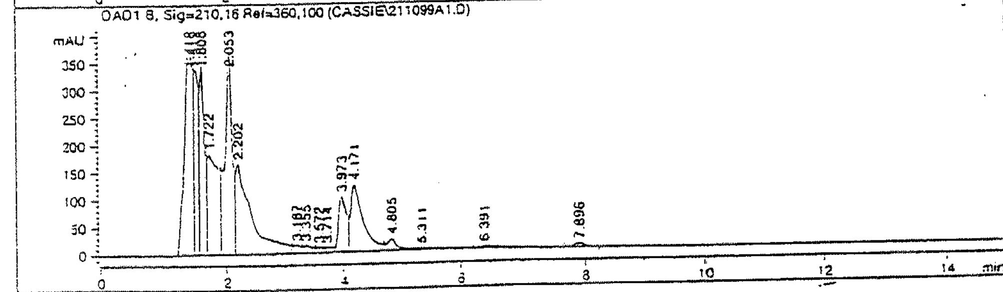
min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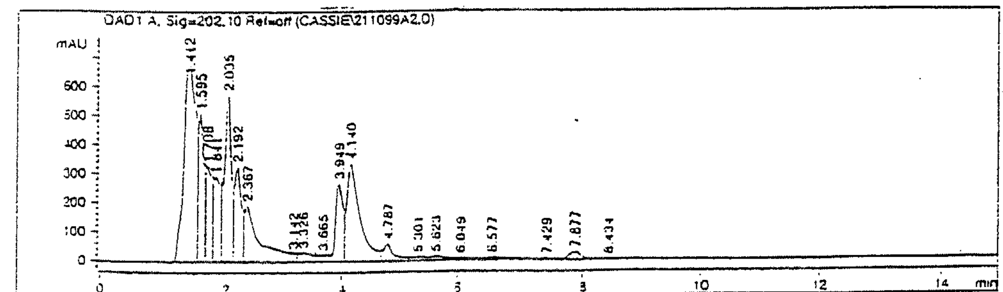
min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mAU
min

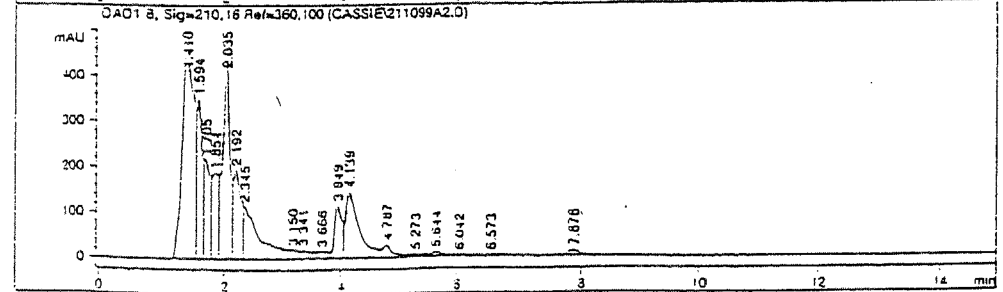
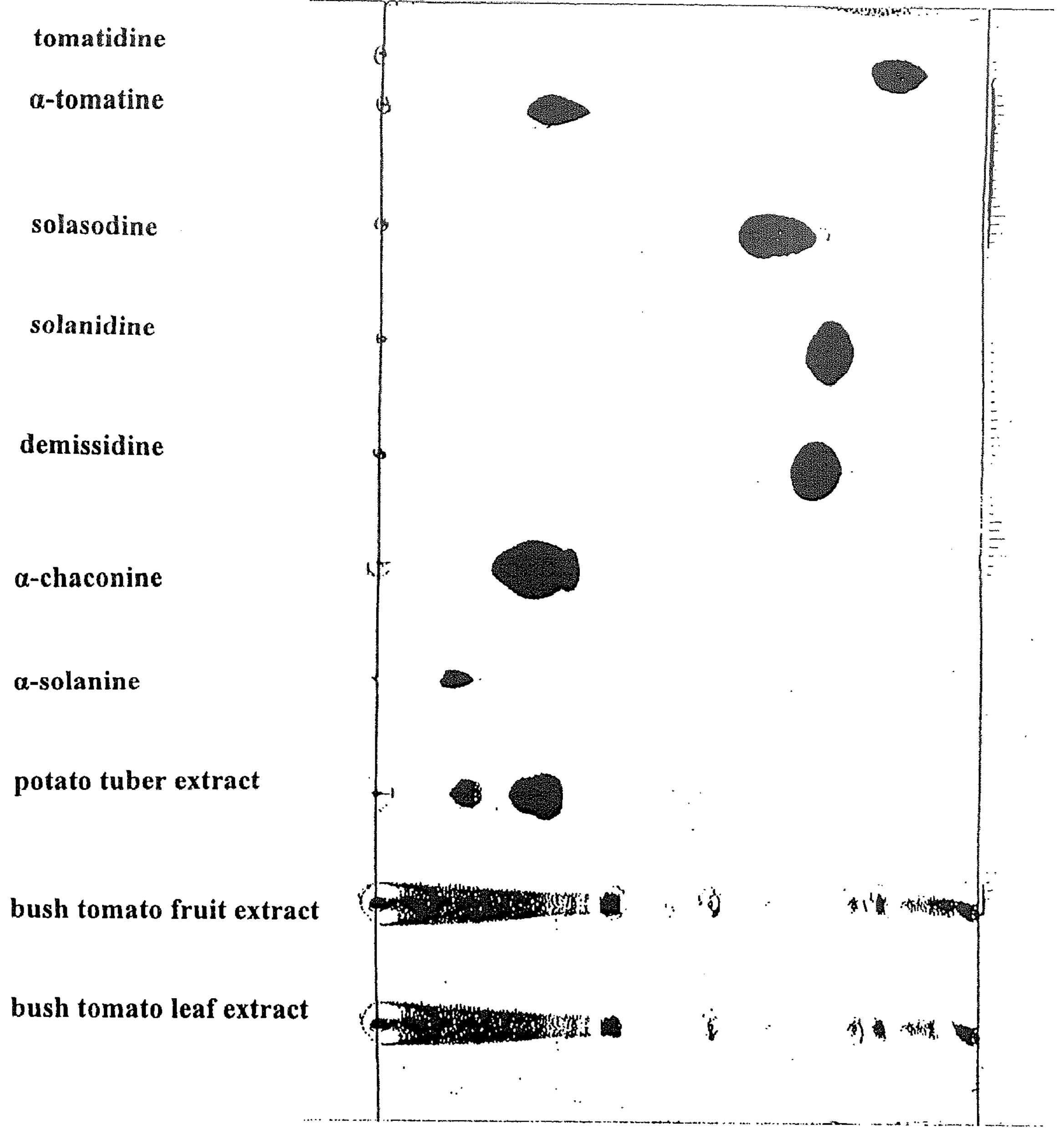

DAD1 B, Sig=210,16 Ref=360,100 (CASSIE\211099A2.D)
mAU
min

Figure 5.11 TLC plate showing fruit sample from *S. centrale* at green stage of development and a potato tuber sample run next to various steroidal alkaloid standards.



tomatidine
α-tomatine
solasodine
solanidine
demissidine
α-chaconine
α-solanine
potato tuber extract
bush tomato fruit extract
bush tomato leaf extract

5.4 Discussion

When food has been consumed for many years without evidence of adverse affects, the confidence in food safety is often taken for granted. Due to this the presence of alkaloids was investigated in this chapter. For some bushfoods a presence or absence of alkaloids has been established, few individual alkaloids have been identified (Bick, 1996). Some Australian plants investigated for alkaloids include; *Acacia* spp., *Acronychia* spp., *Araucaria* spp., *Backhousia* spp., *Citrus* spp., *Mentha* spp., *Santalum* spp. and *Solanum* spp. (Bick, 1996).

The HPLC and TLC results suggested that steroidal alkaloids are present in the leaves and green fruits of *S. centrale*, but not in the mature fruit. This supports research carried out on the fruits of other *Solanum* species where the concentration of various alkaloids (in most cases) were found to be less in ripe than green fruits (Bradley *et al.*, 1978; Everist, 1974). Toxic quantities of either solasodine or other compounds in the green (or ripening) fruits have been found in some Australian species of *Solanum*, e.g. *S. ellipticum*, *S. esuriale*, *S. laciniatum* and *S. simile* (Bradley *et al.*, 1978). As mentioned in Chapter 2 potatoes, another member of *Solanum* have been found to contain levels of solanine alkaloids that were unsuitable for human consumption (Finlay *et al.*, 1998). No identification of possible steroidal alkaloids was found for the *S. centrale* material analysed. These preliminary investigations require refinement and confirmation, but provide encouraging information regarding the implications for human health.

Almost all the HPLC methods used in this study and other literature use UV detection between 200 to 215 nm. This is a highly sensitive area of the UV spectrum causing problems if samples are not purified enough for analysis. Other compounds contaminating extracted samples can interfere with how the steroidal alkaloids move through the column and are eluted and expressed in analysis. Some of the following reasons back up observations that samples were over contaminated. Impurities in samples can cause extraneous peaks in chromatograms and in isocratic systems the elution of these impurities may result in drifting, unstable baselines (Snyder *et al.*, 1997). Even with the protection of column safeguards the column backpressure was

high and this could be linked to impurities in the samples injected onto the column. Other materials such as lipids and proteins may be present in the sample creating high backpressure. In the case of isocratic solutions, compounds that have the capacity factors of several orders of magnitude will not be eluted from the column for a long time if at all (Snyder *et al.*, 1997). This may explain why spiked samples did not display peaks for the standards introduced.

Due to problems faced with the sensitivity of HPLC analysis methods using TLC were employed. This was tested to evaluate whether alkaloids were present. Problems of sample impurity were also faced here. Plates displayed a streaking effect making identification of individual compounds impossible. It did however support the argument that steroidal alkaloids are likely to be present in the leaves and the green fruit of *S. centrale* and therefore indicating the need for further research in this area. Further studies into identification and quantification of steroidal alkaloids are necessary to commercial production in terms of food safety requirements. Identifying the concentration of alkaloids in the leaves and fruit of *S. centrale* may also provide a valuable chemical marker for a selection program in the future.

Chapter 6 Investigations of breeding system and development of hybridisation techniques for *Solanum centrale*

6.1 Introduction

One problem associated with the domestication of *S. centrale* relates to inefficient pollination of the species, as evidenced by poor fruit set in many plants under commercial conditions. Little is known about the floral biology of *S. centrale*, and the principles of hybridisation methodology in general are not well established. The aim of the work discussed in this chapter is to elucidate techniques for controlled hand pollination of *S. centrale*, and to make preliminary observations on its floral biology for use in future breeding system investigations and breeding programs.

6.2 Reproductive biology of *Solanum centrale*

Flowers of *Solanum* species are generally hermaphrodite. They produce little to no nectar, with the only reward offered to pollinators being pollen. The flowers are rarely scented but the human nose sometimes detects a faint sweet scent. There is often a very strong colour contrast between a dark-purplish corolla and the yellow anther cone (Faegri, 1986). If the flowers were female only and were without anthers they would possibly not attract pollinating vectors. Open flowers of *S. centrale* have a characteristic central androecium of pore-opening anthers (Figure 6.1). Pollen does not fall out spontaneously. It must be shaken out by vibrating the anthers ("buzz pollination") something native bee species do systematically, whereas introduced honeybees (*Apis mellifera*) do not. During anther vibration the pollen forms a cloud, powdering the insect. The pollen is used as a food source. (Faegri and Van der Pijl, 1979; Faegri, 1986).

Figure 6.1 Open *S. centrale* flowers with the characteristic androecium of yellow pore-opening anthers.

6.3 Materials and Methods

6.3.1 Plant material

Details of the plant material used for these studies are presented in Chapter 3. Between 1998 and 2000, controlled hand pollination experiments were conducted on *S. centrale* plants located in the plantation at the Waite Campus, Adelaide University, South Australia.

6.3.2 Sequence of floral development

An initial visual assessment was made of the sequential steps involved in floral development (Table 6.1), from bud burst to floral senescence. For ten plants, one mature bud (purple corolla) per plant was tagged and observed every morning (0900 h) and afternoon (1500 h) until flower senescence. After bud burst, observations of the state of the anthers (physical appearance, state of pore: open or closed), stigma (colour changes), and petals (physical position and appearance) were recorded. Any insect visitors to the flowers were recorded, as was the behaviour of these visitors.

6.3.3 Pollen viability

Mixed pollen was collected from ten plants and tested for viability using the fluoroscein diacetate test (Peterson and Taber, 1987).

6.3.4 Timing of stigma receptivity

The time when the maximum number of pollen grains was retained on the stigma (peak stigma receptivity) was determined in order to maximize the efficiency of artificial pollination and conservation of pollen supplies. The following three stages of floral development based on the determined sequence of floral development were tested:

Stage 1: anthesis (flower opening) with anthers closed

Stage 2: flower open, anthers open and bright yellow, with slightly brown tips, tip of stigma green.

Stage 3: start of flower senescence, anthers dull yellow in colour, stigma yellow-green.

For each stage of floral development tested, five flowers from each of five plants were bagged at the purple-bud stage and left until anthesis. Thus, for each floral stage twenty-five flowers were observed. Anthers were removed (flowers emasculated) prior to dehiscence and stigmas either pollinated at stage 1 or left for later pollinations at stages 2 or 3. In each case, a bulk mixture of cross pollen was used, collected from at least ten plants prior to pollination. Flowers were re-bagged after pollination and left for 24 hours before pistils were harvested for analysis of pollen germination on the stigma. Using a fluorescence microscope, the number of germinated pollen grains at the stigmatic surface was counted (see appendix for method).

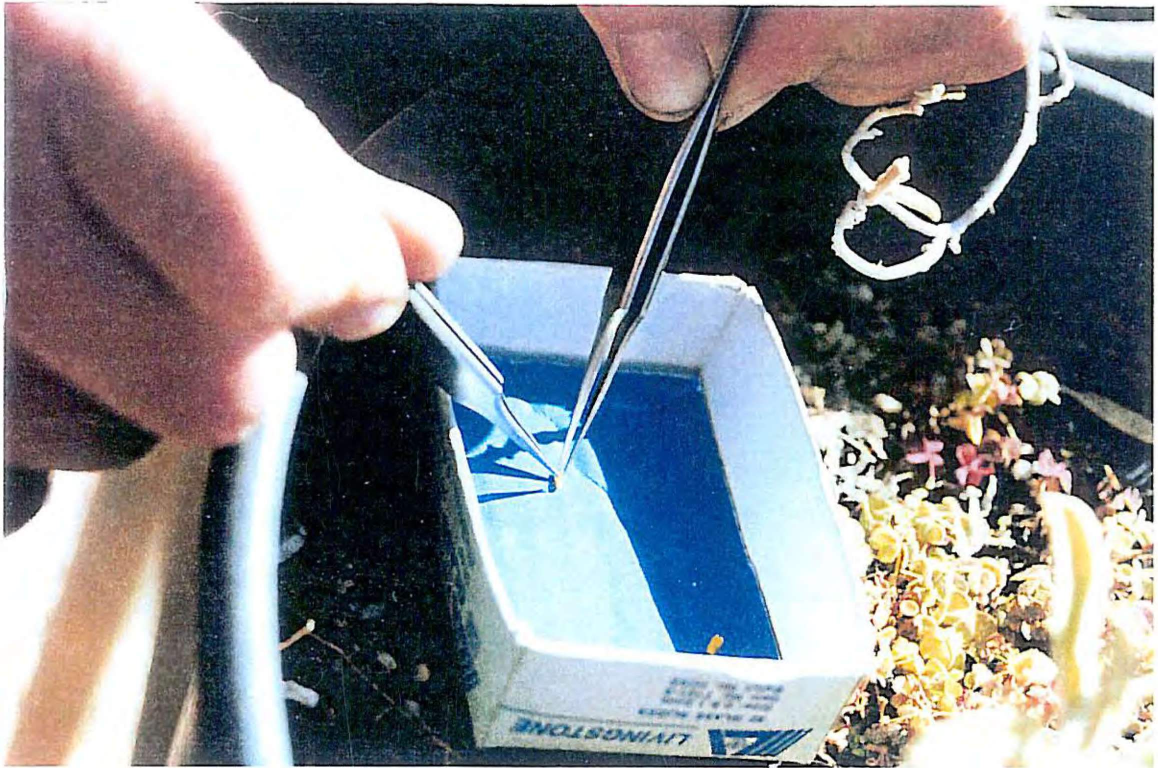
6.3.5 Techniques used for emasculation and pollen transfer

The removal of anthers and use of various sieving methods to collect pollen resulted in low amounts of pollen being collected and poor pollen viability. This approach was abandoned in favour of the collection of anthers where viable pollen could be collected by holding the anther horizontally, with a pair of forceps over a clean microscope slide and tapping the anthers with the side of a second pair of forceps (Figure 6.2). This method ensured that only mature pollen was collected, as immature pollen was too moist to be tapped out and remained in the anthers. Black and blue plastic was placed under the microscope slide to make visualisation of the pollen grains easier. Dragging the stigma across the slide or dabbing the stigma onto the slide achieved hand pollination. This was performed until pollen was clearly visible on the stigma surface (Figure 6.3). To protect the pollen from being knocked or blown off, it was useful to wedge the slide flat into a slide box with the plastic underneath. After pollination, the flower was re-bagged until harvest or fruit set.

Only flowers in bud were bagged, using small paper (glassine) bags to exclude pollinators (including wind), and sealed with a twist tie (Figure 6.4). Open and mature flowers were removed prior to bagging. Flowers were checked morning and night. As flowers opened, they were emasculated using a pair of jeweller's tweezers (Figure 6.5). Emasculated flowers were then re-bagged until pollination.

Figure 6.2 Technique for collecting pollen from anthers of *S. centrale*. Forceps were used to tap anthers, and pollen is collected on a microscope slide.

Figure 6.3 Hand pollination of *S. centrale* was achieved by dragging the stigma across the slide until pollen was clearly visible on the stigma surface.


LIVINGSTONE

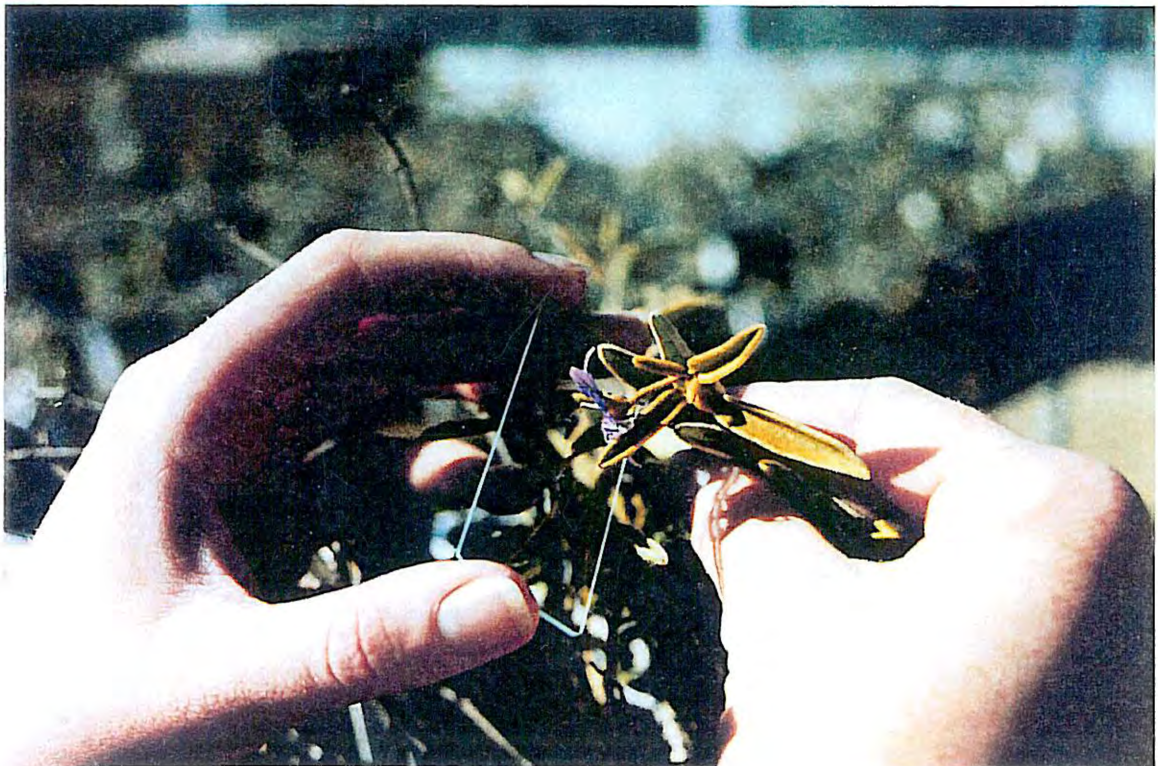


Figure 6.4 Paper bags were placed over *S. centrale* flowers in bud to exclude pollinators and then sealed with a twist tie.

Figure 6.5 *S. centrale* flowers were emasculated using a pair of jeweller's tweezers.

6.3.6 Compatibility

A series of controlled hand pollinations were carried out using both cross- and self-pollen in order to determine whether *S. centrale* is self-compatible. Data recorded included fruit and seed set numbers. For each pollination treatment, five branches were bagged at the bud stage on each of eight plants. On two separate occasions (stages 2 and 3) between 4 and 13 flowers were pollinated at the peak time for stigma receptivity. Double pollination was carried out so that regardless of changing weather conditions, at least one pollination event occurred when the stigma was fully receptive. After pollination, flowers were re-bagged and left for one week. Bags were then removed and preliminary fruit set recorded. To assess natural pollination, five to ten flowers per plant were tagged and not manipulated. Flowers were also bagged and not manipulated to determine whether self fruit-set was possible in the absence of manipulation. Fruit was allowed to develop naturally for 4 weeks after which fruit and seed set per fruit were recorded.

6.4 Results

6.4.1 Sequence of floral development

S. centrale flowers were examined in the morning and the afternoon and they remained open for approximately 48 hours. Following budburst the petals reflexed to expose the male and female reproductive parts, remaining in this configuration for approximately 24 hours. After this time the corolla began to close upward becoming level with the ovary and then by approximately 48 hours, the corolla closed over, encasing the male and female organs (Table 6.1).

Table 6.1 Visual stages of floral development of *S. centrale* flowers from bud burst to senescence

Time	Floral Stage	Status of floral organs: Corolla	Anthers	Stigma
(a) Opened by 9am				
Day 1 (AM)	open	reflexed	closed	green tip
Day 1 (PM)	open	reflexed	open, brown tips, bright yellow	green tip
Day 2 (AM)	open	reflexed	browning, dull yellow	yellow-green tip
Day 2 (PM)	open	level with ovary	browning, dull yellow	yellow-green tip
Day 3 (AM)	closed	closed	enclosed in corolla	enclosed in corolla
(b) Opened by 3pm				
Day 1 (AM)	closed	closed	-	-
Day 1 (PM)	open	reflexed	open, brown tips, bright yellow	green tip
Day 2 (AM)	open	reflexed	browning, dull yellow	yellow-green tip
Day 2 (PM)	open	level with ovary	browning, dull yellow	yellow-green tip
Day 3 (AM)	closed	closed	enclosed in corolla	enclosed in corolla

Equal numbers of flowers opened during both morning and afternoon. Anther dehiscence occurred only in the afternoon.

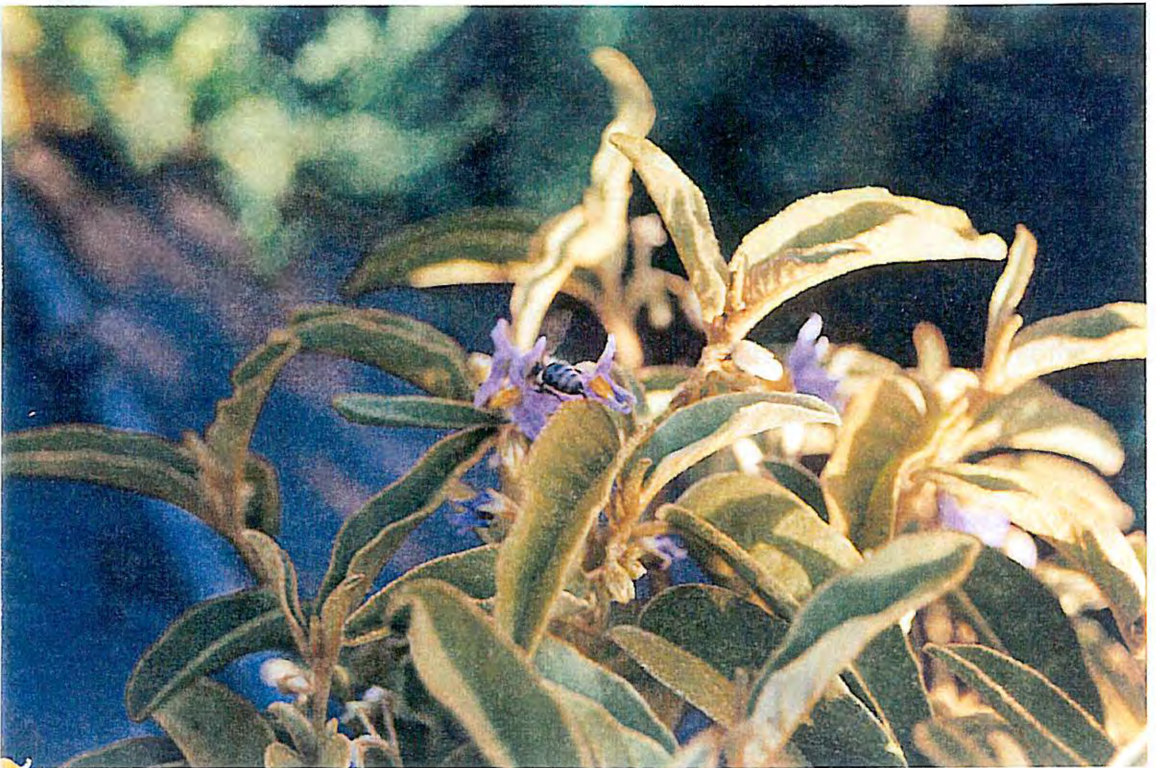
As described previously (Chapter 2), many bees including solitary and social bees, use floral sonication to harvest pollen (including bees in at least seven families and over 50 genera) (Buchmann *et al.*, 1977). The large blue-banded bee, *Amegilla* sp. (Hymenoptera: ANTHOPHORIDAE), a native solitary bee, was observed 'buzz' pollinating *S. centrale* in the Waite campus plantation.

The honeybees *Apis mellifera* (Hymeoptera: APIDAE), while not responsible for pollination of *S. centrale,* were seen visiting many flowers on plants in the plantation. They were also observed moving towards the calyx (Figure 5.6). Ant activity was also intense at this time. On closer observation, it was observed that sticky nectar was present on the calyx while flowers were still in bud. This was an attraction for both ants and honeybees.

Two flower morphs were observed in the Waite campus plantation of *S. centrale*; (1) flowers with a long style protruding past the level of the anthers and (2) flowers with short styles, the stigma located well below the level of the anthers. The majority of the plants had long-styled flowers. The short-styled flower is referred to as the 'thrum flower' and the long-styled flower is referred to as the 'pin flower' (Sedgley and Griffin, 1989). As the length of the style varies between flowers, the spatial arrangement of style and stigma is different in flowers of different genotype. This is referred to as heterostyly. Within a flower of a heterostylous species, a physical reduction in pollen transfer and a genetic incompatibility mechanism often occurs (Sedgley and Griffin, 1989).

Both heterostyly and self-incompatibility occur in *Primula, Durio zibethinus, Averrhoa carambola* and a number of species of *Cordia* (Knight, 1965; Opler *et al.*, 1975; Chin and Phoon, 1982).

Figure 6.6 Honeybees observed visiting extra-floral nectaries located on the calyx of *S. centrale* flowers.



6.4.2 Pollen Viability

Data from the fluoroscein diacetate test for pollen viability indicated that at least 99% of pollen was viable upon release from the anthers.

6.4.3 Timing of stigma receptivity

Forty percent of stigmas at stage 1, and 70% of stigmas at stages 2 and 3 of floral development, were receptive to pollen (Table 6.2). Based on these preliminary data it was concluded that, when using artificial pollination methods, the most receptive floral stage occurred when anthers had opened and were showing signs of browning at the tips (Table 6.1).

Table 6.2 Percentage of stigmas with a germinating pollen grain present for various stages of floral development.

Stage of floral development	Flower opening time	Stigmas with a germinating pollen grain present (%)
(1) stigma exposed, anthers closed	morning, day of flower opening	44
(2) stigma exposed, anthers open	afternoon, day of flower opening	70
(3) stigma exposed, anthers open, senescence commenced	afternoon, day after opening	70

6.4.4 Compatibility

As shown in Table 6.3, cross-pollination was more successful than self-, however the majority of plants were able to produce fruit with either self- or cross-pollen. In general, a large number of flowers were produced compared with relatively low amount of fruit set. Some plants failed to produce fruit at all. In general, cross-pollination was found to produce the largest fruit containing the highest numbers of seed.

Table 6.3 Summary of fruit and seed results from investigations into the breeding system of *Solanum centrale*.

Plant	Cross (hand-pollinated)			Self (hand-pollinated)			Self (unmanipulated)			Open (unmanipulated)			Summary of Breeding System
	% fruit set	seeds/fruit (mean±se)	fruit diameter (mm) (mean±se)	% fruit set	seeds/fruit (mean±se)	fruit diameter (mm) (mean±se)	% fruit set	seeds/fruit (mean±se)	fruit diameter (mm) (mean±se)	% fruit set	seeds/fruit (mean±se)	fruit diameter (mm) (mean±se)	
1	44.4	53.8±8.5	11.5±0.6	40.0	20.7±6.6	8.3±1.8	0.0	0.0	0.0	32.7	23.3±3.5	8.6±0.4	mixed
2	75.0	55.3±25.8	9.7±3.3	7.7	4.0*	6.0*	0.0	0.0	0.0	0.0	0.0	0.0	partially self-incompatible
3	60.0	21.3±3.3	8.0±1.0	40.0	38.0±9.0	12.5±0.5	20.0	7.0*	7.0*	15.4	25.0±11.0	9.5±1.5	mixed
4	22.2	17.0±7.0	10.0±1.0	0.0	21.3±8.3	9.0±0.6	0.0	0.0	0.0	7.4	4.0±3.0	6.5±0.5	self-incompatible
5	18.2	22.0*	9.0*	38.5	32.0±7.0	10.0±1.0	0.0	0.0	0.0	10.3	22.5±2.5	9.5±0.5	mixed
6	0.0	0.0	0.0	11.1	21.0*	10.0*	0.0	0.0	0.0	0.0	0.0	0.0	self-compatible, low fertility
7	42.9	63.7±9.4	12.7±1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	self-incompatible
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	low fertility
Mean	32.9	29.1	7.6	17.2	17.1	7.0	2.5	0.9	0.9	8.2	9.4	8.5	
% total plants that set fruit	75.0			63.0			13.0			50.0			

*single fruit

6.5 Discussion

This is the first, albeit preliminary, investigation of the reproductive biology of *S. centrale* in terms of artificial hybridisation. From this study, techniques of pollen collection and pollen transfer have been evaluated and can now be used in controlled breeding programs for the development of improved genetic resources for the bushfood industry. To maximise pollen germination, and subsequent seed set, hand pollination should be conducted at the time of peak stigma receptivity. For maximum efficiency pollen should be placed on the stigma when anthers have opened and the tip of the anther is starting to brown.

In general, smaller and fewer fruit were produced following self-pollination compared to cross-pollination, suggesting that there may be a self-incompatibility mechanism operating in *S. centrale*. This is especially likely in view of the fact that 99% of pollen is viable on release. Erwin (1931) measured the effect of pollination on the set of fruit in *Capsicum* spp. He found that only 46 percent of self-pollinated flowers set compared to 71 percent that were left to open pollination by bees. Similar findings were made in *Lycium* spp. found to be both self- and cross-compatible, however fruit production, seed production and pollen tube number following outcross pollination were significantly higher than following self-pollination (Miller and Venable, 2000; Agiular and Bernardello, 2001; Miller and Venable, 2002). This is an area that could be further investigated using some of the techniques developed in this study.

A factor that may limit the success of a controlled breeding program for *S. centrale* is high levels of variability observed in the fertility of the plants in the Waite Campus plantation. Similar variability has been reported in commercial plantings (A. Beal pers. comm.). Such variability has the potential to seriously affect yield and quality. Plant quality could be improved by the collection of seed from only selected, high yielding plants or populations, preferably with the ability to self-pollinate to produce large fruit.

The hermaphrodite sexual system possessed by most flowering plants creates the potential for close inbreeding through self-pollination. While many species strongly

limit self-fertilisation with self-incompatibility, about one-third of hermaphrodite taxa are self-compatible (Richards, 1986). Some self-compatibility has been displayed by *S. centrale*. The balance between outcrossing and self-fertilisation will depend, in part, on the relative deposition of self- and cross pollen on stigmas (Holsinger, 1992). This can be influenced by both intrinsic and extrinsic factors, especially features of floral morphology and display. An examination of co-variation between the level of outcrossing and aspects of floral morphology and display in a population of the self-compatible, perennial, *Solanum centrale* would be beneficial to better understand this variation within the specie.

Production of large numbers of flowers combined with low reproductive success could be due to a number of reasons such as pollen donation, pollinator limitation, pollinator attraction, and selective abortion (Stephenson, 1981; Sutherland and Delph, 1984). Efficiency of natural pollinators is an area that may benefit from future research. Natural pollination should be encouraged by maintaining the environment surrounding plantations to promote pollinators. This can be done by maintaining plant shelter and food plants, avoiding insecticides and other cultural changes to help insects. Low fruit set could be due to low pollinator numbers and/or infrequent visits of potential pollinators. If either of these problems were rectified, fruit set would increase (Sutherland and Delph, 1984). Further research would be useful in determining whether availability of resources/pollinators is limiting fruit set. Research aimed at attracting this bee to nest in or adjacent to *S. centrale* plantings may improve fruit set and productivity.

Despite appearing morphologically hermaphrodite, Horovitz (1978) suggested that it is possible that not all flowers in a population are "equisexual". A further suggestion has been made that many flowers may function only as pollen donors (Sutherland and Delph, 1984). Detailed investigations of floral ontogeny in *S. centrale* are required to determine if flowers exhibit differential fertility, which would significantly affect the efficiency of a breeding program.

Long styled and short styled flowers were observed in *S. centrale*. As an example, *A. carambola* clone flowers have either, long styles and short anther filaments or short

styles and long anther filaments. No fruit set was found to occur when short-style flowers self- or cross- with other short-style flowers. Very low fruit set occurs in similar situations involving long-style flowers. A short-style flower by long-style flower pollination (or vice versa) gives maximum fruit set. A similar observation was made for the European primrose, *Primulus vulgaris* where half the plants were found to have long-styled (pin-eyed) flowers and the other half short-styled (thrum-eyed). The pollen grains in the short styled plant were twice as large compared to those of the long-styled plant. This mechanism was considered to assist cross-pollination by larger pollen having the resources to travel twice the distance to grow and achieve fertilisation (Lewis, 1979).

Flowers have evolved intricate structures presenting difficulties in the extraction of pollen or nectar. The honeybee and other bees have been observed visiting the flowers of other Solanaceae plants such as *Capsicum* spp. (Erwin, 1931). The appearance of honeybee activity around flowers of this species may indicate the presence of extrafloral nectaries present on parts of the flower. Anderson and Symon (1985) reported the occurrence of such structures on other *Solanum* species with similar bee and ant activity noted. Extrafloral nectaries are nectaries on a plant organ, including parts of the flower that do not function as sources of nectar rewards for pollinators. They are structurally complex and seem to function during the bud stage when ants actively visit them. For *S. centrale*, extrafloral nectaries occur on the outer surface of the calyx and have been found to range in number between 0 and 10 with an average of 2 to 3 (Anderson and Symon, 1985). Extrafloral nectaries exude a sticky, sugar-rich substance at different stages of plant development (Anderson and Symon, 1985; Marginson *et al.*, 1985). Extrafloral nectaries, and their associated insects, offer considerable potential for exploitation as biological control systems against agricultural pests. They may also be a problem as ants may protect pests (e.g. mealy bugs) from parasites and predators and may discourage pollinators.

Chapter 7 Morphological Diversity

7.1 Introduction

Variation between individuals within a population may be found in morphological, anatomical, karyological, biochemical and molecular characters and determined genetically and environmentally except at the DNA level which is genetically (Kreitman, 1991). The conservation and management strategies for a species are determined by knowledge of the amount and distribution of variation in natural populations (Schaal *et al.*, 1991; Dawson *et al.*, 1993). The long- and short- term survival of a species is dependent on the amount of genetic variation present (Schaal *et al.*, 1991; Black-Samuelson *et al.*, 1997). Generally, populations with low genetic variability have a reduced potential to adapt to environmental changes (Ellstrand and Elam, 1993) and a higher risk of extinction (Schaal *et al.*, 1991).

Population variation is most easily obtained by morphological or phenotypic variation (Harper, 1977, Karp *et al.*, 1996). This has an advantage over other techniques, as it does not require breeding or laboratory studies and work can be done directly from the field collection. Another advantage is that phenotypic characters are often ecologically adaptive and therefore such variation can indicate genotypic variation, local differentiation, or ecotypes (Schaal *et al.*, 1991). In this chapter, the morphological variability was investigated within a single, large, wild population and between ten isolated wild populations in Australia.

7.2 Materials and Methods

7.2.1 Plant material

Seed was collected from Utopia Station, Northern Territory, and ten different wild populations around Australia. All material was grown at the Waite campus, University of Adelaide, South Australia. Measurements were taken of 100 plants from Utopia Station, and of 10 plants from each of the other populations. Plant material, the sampling procedure and the areas covered are described in Chapter 3.

7.2.2 Morphological characters and measurements

Morphological characters used for the identification of *S. centrale* included leaf shape, leaf colour, prickliness, growth habit, flower colour, and the shape, size and yield of fruit. (Table 7.1).

Table 7.1 Morphological characters measured for *Solanum centrale.* Colour codes were determined using Royal Horticultural Society (RHS) colour charts.

Code	Character	Description
LSH	Leaf shape	1. simple, entire, oval-ovate 2. simple, entire, keeled 3. highly sinuated, blunted lobes, keeled 4. undulating, open, oval-ovate 5. undulating, keeled, oblong
LCO	Leaf colour	1. pale green leaves (148C) 2. green leaves (146C) 3. slightly rusty leaves (162A) 4. rusty leaves (163B) 5. highly rusty leaves (163A)
PRI	Prickliness	1. no prickles present 2. slightly prickly 3. prickly 4. highly prickly
GRH	Growth habit	1. upright habit 2. sprawling habit
FLC	Flower colour	1. pale-purple flowers (87B) 2. purple flowers (88A) 3. dark-purple flowers (89C)
FSH	Fruit shape	1. round 2. ovate 3. diamond
FSI	Fruit size (diameter)	1. small <10 mm 2. medium 10-20 mm 3. large >20 mm
YIE	Yield (gms per plant, fresh weight)	1. low <250 g 2. medium 250-650 g 3. high >650 g

Five different leaf shapes were observed in *S. centrale* individuals. These are illustrated in Figure 7.1 and 7.2 and each character is described in Table 7.1. Leaf colour of fully expanded six-month-old leaves was measured using the Royal Horticultural Society (RHS) colour charts and based on the rustiness of the leaves shown in Figure 7.3 (RHS codes are shown in Table 7.1). Prickliness was measured because of its importance when harvesting the fruit and the variation of prickles that

was observed between individuals. The range of prickliness is shown in Figure 7.4 and 7.5. Two patterns were observed for growth habit, one being an upright growth habit and the other a sprawling habit. Three flower colours were observed in this species and classified using Royal Horticultural Society (RHS) colour charts, examples are shown in Figure 7.6 (RHS codes are shown in Table 7.1). The three types of fruit shape were round, ovate and diamond. Fruit size was classified as small <10 mm, medium 10-20 mm and large >20 mm. Yield for each plant was measured in terms of grams per plant of fresh weight material and allocated as low <250 g, medium 250-650 g and high >650 g.

Hybridisation between other members of *Solanum* is unknown, as the other species in the general area were *S. quadriloculatum* and *S. coactiliferum*. However, these are both morphologically distinct from *S. centrale,* and were not present in the actual study sites.

Figure 7.1 Examples of different leaf shapes (LSH) found in *Solanum centrale* as defined in Table 7.1. From L-R Leaf types 1- simple, entire, oval-ovate, 3- highly sinuated, blunted lobes, keeled and 2- simple, entire, keeled.

Figure 7.2 Examples of different leaf shapes (LSH) found in *Solanum centrale* as defined in Table 7.1. From L-R Leaf type 5- undulating, keeled, oblong and 4- undulating, open, oval-ovate.

0 1 2 3 4 5 6 7 8

0 1 2 3 4 5

Figure 7.3 Variation of leaf rust-coloured hair cover (LCO) of *Solanum centrale*. From top-bottom Leaf colour types 3- slightly hairy leaves, 4- hairy leaves and 5- highly hairy leaves as defined in Table 7.1.

Figure 7.4 ***S. centrale*** **displaying the 1- non-prickly (top) and 2- slightly prickly (bottom) character.**

Figure 7.5 ***S. centrale*** **displaying the 3- prickly (top) and 4- highly prickly (bottom) character.**

120

Figure 7.6 Examples of the three flower colours observed in *S. centrale.* From top-bottom 1- pale-purple, 2- purple, and 3- dark-purple flowers.

7.2.3 Data analysis

Cluster analysis and two ordination techniques: principal component analysis (PCA) and multidimensional scaling (MDS) were employed to assess morphological variability. Cluster analysis was carried out to generate a dendrogram using the SAHN clustering procedure and UPGMA method (NTSYS-pc version 2.0) (Rohlf, 1993). The data matrix was then standardised to allow for variables measured using different units, therefore reducing distortion. To measure the amount of distortion between the distance matrix and the dendrogram generated from the cluster analysis the cophenetic value was calculated (Sokal and Rohlf, 1962; Romersburg, 1984). The grouping of individuals was assessed using MDS ordination, and the contribution of each character towards discriminating between individual phenotypes was determined by PCA (Romero *et al.*, 2000). PCA was carried out using Multivariate Statistical Package version 3.12D (Kovach Computing Services, Anglesey, Wales).

7.3 Results

7.3.1 Morphological variability within Utopia population

Principal components with an eigenvalue greater than one were extracted. Kaiser's rule states that the minimum eigenvalue should be one if the correlation matrix is used (Rohlf, 1993). For this analysis the first three principal components account for 21.4%, 15.1% and 13.8% of the variance respectively. The eight morphological characters collectively explain 50.3% (Table 7.2). Since the principal components with an eigenvalue greater than one only described 50% of the variability all characters were analysed. Figure 7.7 is an example of PCA on two axes showing that the further from the centre of the axes the measured traits are placed, the more significant the character in terms of explaining variability between phenotypes of *S. centrale*.

A dendrogram showing the dissimilarities between individuals was produced from the morphological data. No real clustering could be identified (Figure 7.8). To allow the trends of characters across the ordination space to be examined, the MDS of the characters was plotted onto a three-dimensional space, based on the best-fit direction. The result of the MDS analysis (Figure 7.9) supports the dendrogram. As a measure of

good fit (Kruskal and Wish, 1978), the stress value of the MDS was calculated as 0.6 and this value indicates that the MDS gives a useful three-dimensional representation of the dissimilarities among the individuals (Clarke and Warwick, 1994). The cophenetic correlation value between the dendrogram and the distance matrix was 0.72, indicating a poor fit between the two, which means that the groupings produced by this analysis could not be distinguished from random events (Figure 7.10) (Rohlf, 1993).

Table 7.2 Coefficient loadings of characters on the first three components of PCA for *S. centrale* obtained from Utopia Station, Northern Territory.

Character	Component		
	1	2	3
GRH	0.082	-0.085	0.622
LEC	0.228	0.288	-0.377
LES	0.150	0.485	-0.333
PRI	0.111	0.697	0.314
FLC	-0.177	-0.158	-0.499
FRS	-0.498	0.400	0.072
FRH	-0.584	0.004	0.058
YIE	-0.535	0.065	-0.059
Eigenvalues			
Total	1.712	1.210	1.101
% of variance	21.405	15.120	13.756
% cumulative variance	21.405	36.526	50.282

Figure 7.7 PCA variable loadings on two axes for the eight morphological characters examined in plants from the Utopia Station population. Character names correspond to Table 7.1 and 7.2.

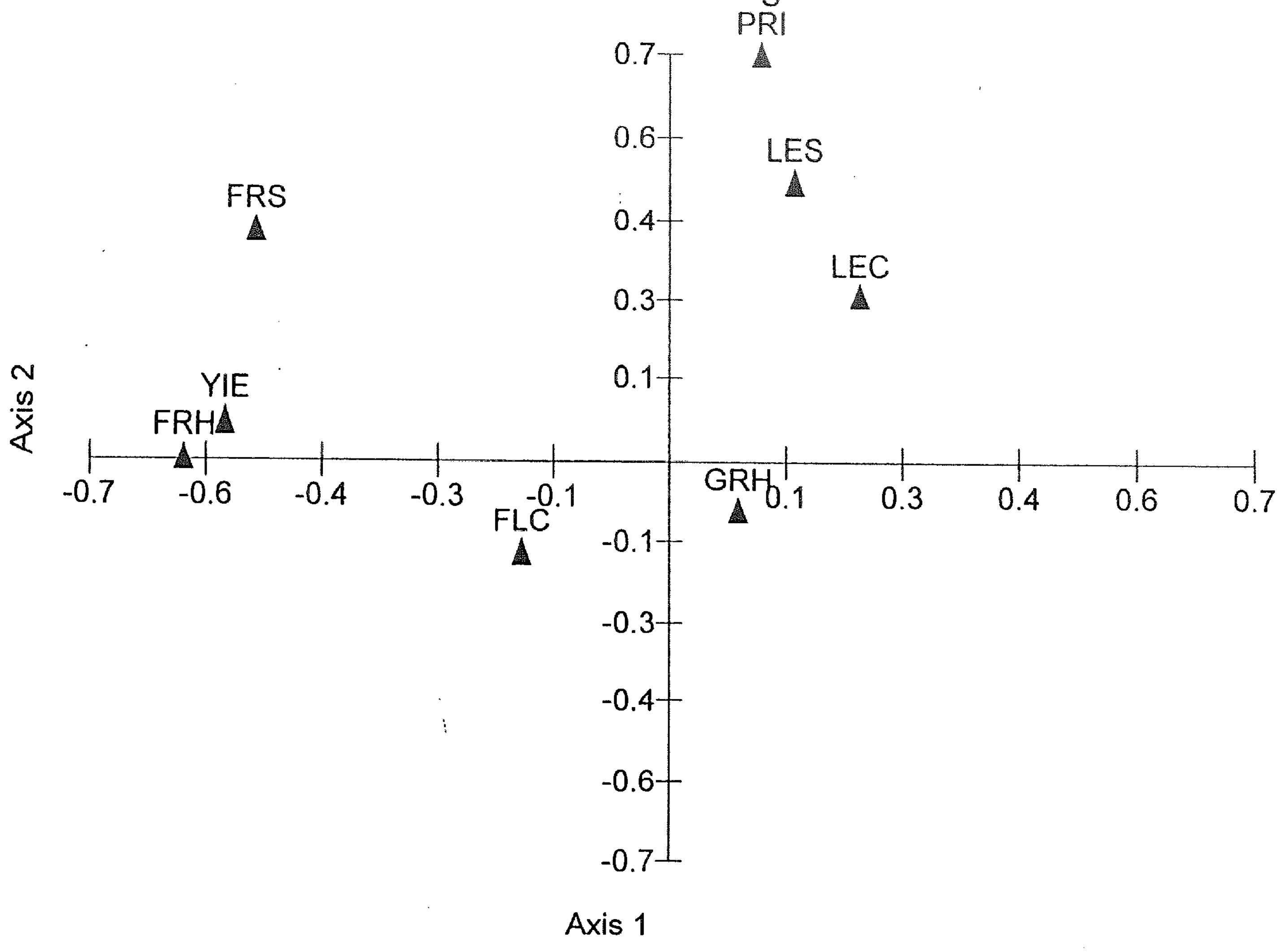
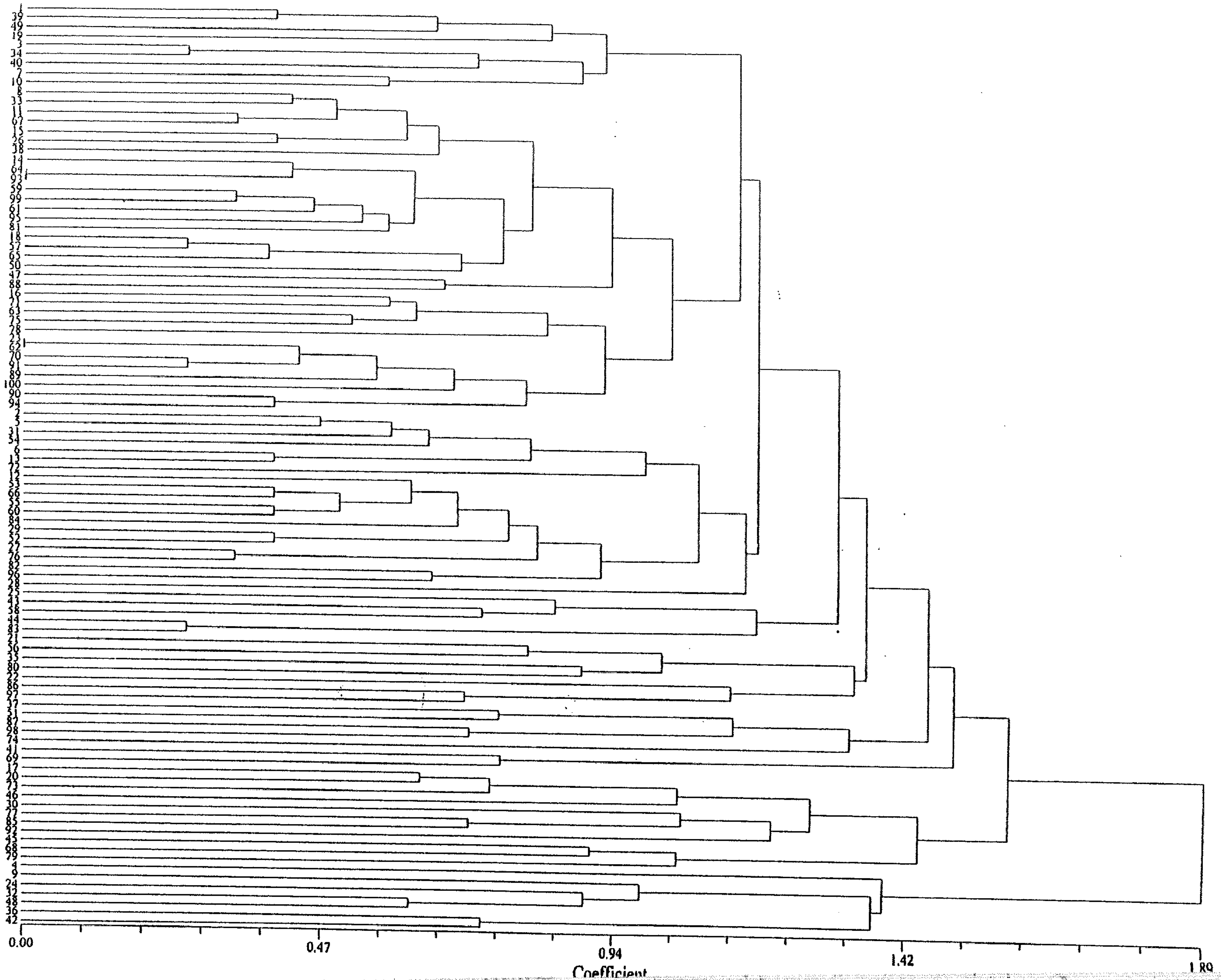

PCA variable loadings
PRI
LES
LEC
FRS
YIE
FRH
GRH
FLC
Axis 2
Axis 1
0.7
0.6
0.4
0.3
0.1
-0.1
-0.3
-0.4
-0.6
-0.7
-0.7
-0.6
-0.4
-0.3
-0.1
0.1
0.3
0.4
0.6
0.7

Figure 7.8 UPGMA dendrogram showing morphological variation and relatedness of 100 *S. centrale* individuals from Utopia Station.



0.00
0.47
0.94
1.42
Coefficient

Figure 7.9 Three-dimensional scatter-plot showing morphological variation and relatedness of 100 *S. centrale* individuals sourced from Utopia Station using MDS.

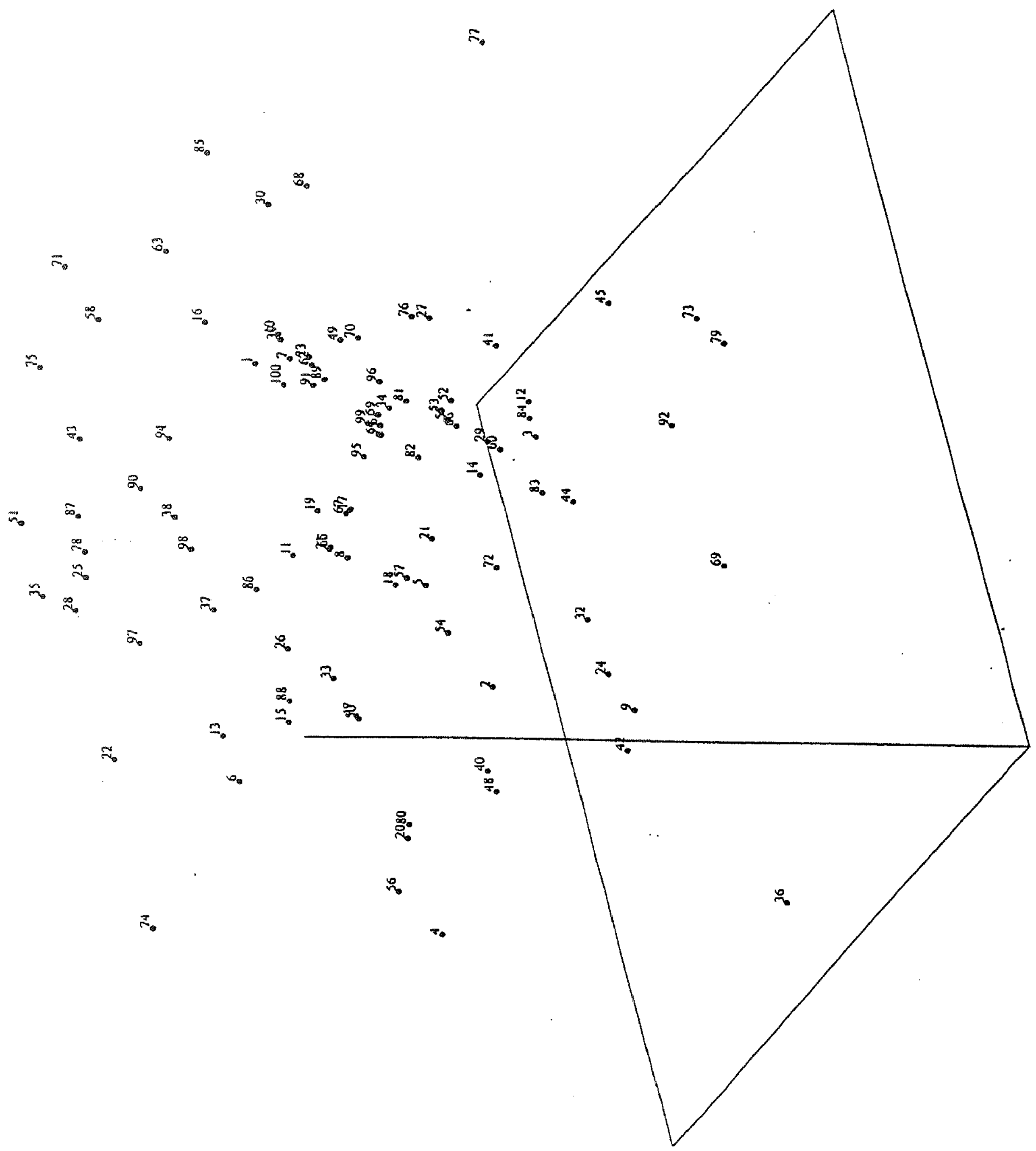
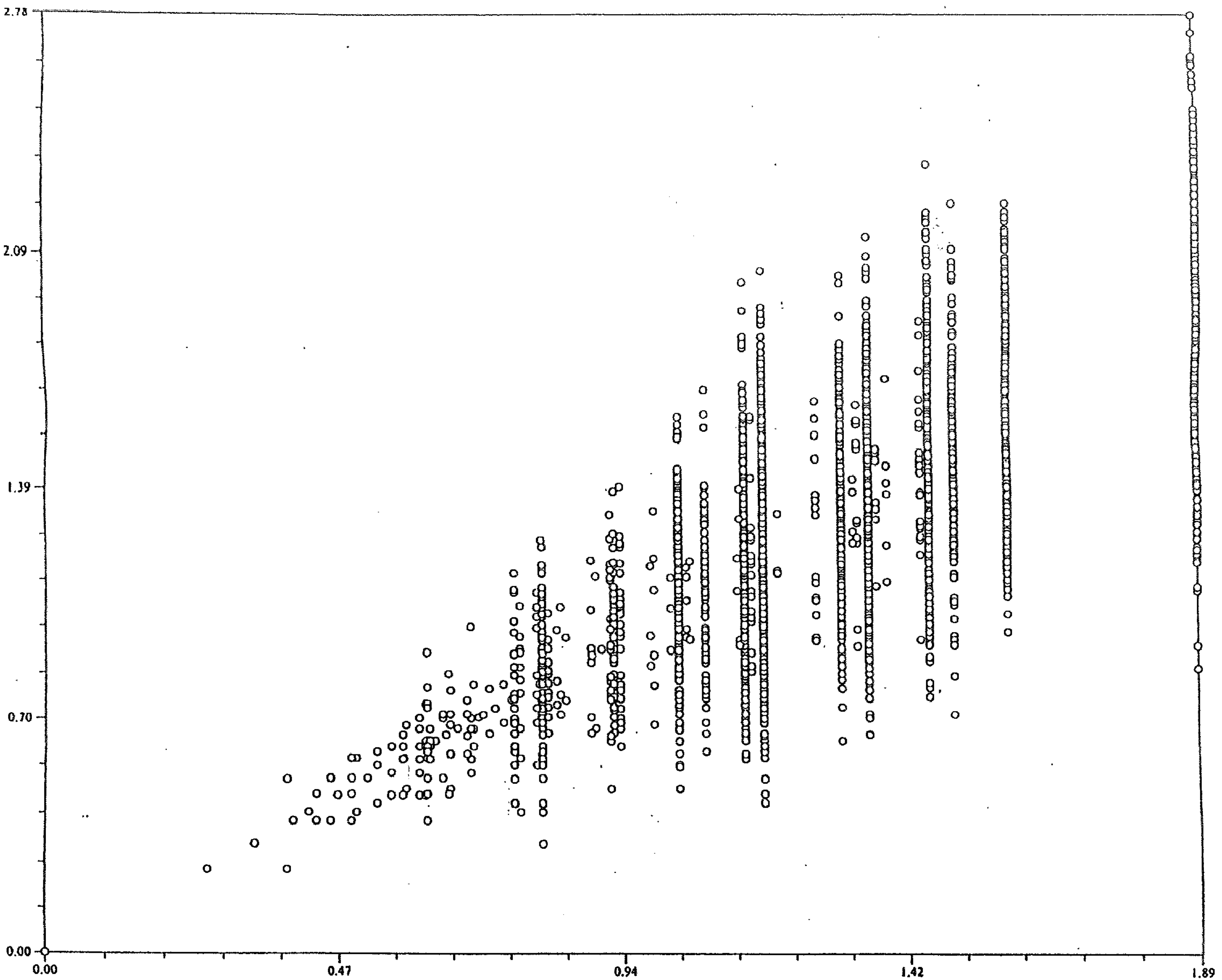


Figure 7.10 Plot of morphological similarities against cophenetic values based on UPGMA clustering for the Utopia Station population.


2.78
2.09
1.39
0.70
0.00
0.00
0.47
0.94
1.42
1.89
Similarity matrix
Cophenetic Matrix

7.3.2 Morphological variability between ten Australian populations

The first four principal components with an eigenvalue greater than one were extracted and these accounted for 28.9%, 20.8%, 14.7% and 13.4% of the variance, respectively. Collectively, they explained 77.8% of variance for the eight morphological characters (Table 7.3). Successive principal components were not used in the analysis as they gave only a slight increase in total variance. Figure 7.11 shows the PCA analysis on two axes once again illustrating that the further from the centre of the axes the more of a contribution the measured character has in terms of variability found in *S. centrale.*

The dendrogram of morphological data (Figure 7.12) displays 10 clusters. These 10 clusters correspond to the locations from which the morphological measurements were taken, except in the case of two Northern Territory populations (SH and TA) where some overlap is displayed. The result of the MDS analysis (Figure 7.13) however displays the clusters of the two Northern Territory populations as being separate along with other populations. As a measure of good fit (Kruskal and Wish, 1978) the stress value of the MDS was calculated as 0.38, indicating that this MDS gives potentially useful three-dimensional representation of the clustering based on morphological measures. The cophenetic correlation value between the dendrogram and the distance matrix was 0.81, indicating a good fit between the two, which means that the groupings produced by this cluster were unlikely to be due to random events (Figure 7.14) (Rohlf, 1993).

Table 7.3 Coefficient loadings of characters on the first four components of PCA for *S. centrale* obtained from ten different locations in Australia.

Character	Component			
	1	2	3	4
GRH	0.580	-0.139	-0.046	0.139
LEC	0.306	0.417	0.251	0.461
LES	-0.377	-0.267	-0.050	0.456
PRI	0.279	0.411	0.231	-0.450
FLC	-0.074	-0.019	0.825	0.216
FRS	-0.249	0.531	-0.179	0.419
FRH	0.492	-0.047	-0.335	0.346
YIE	0.204	-0.531	0.234	0.128
Eigenvalues				
Total	2.312	1.664	1.172	1.074
% of variance	28.897	20.801	14.651	13.420
% cumulative variance	28.897	49.698	64.349	77.769

Figure 7.11 PCA variable loadings on two axes for the eight morphological characters measured during the *S. centrale* study on ten different populations. Characters names correspond to Table 7.1 and 7.3.

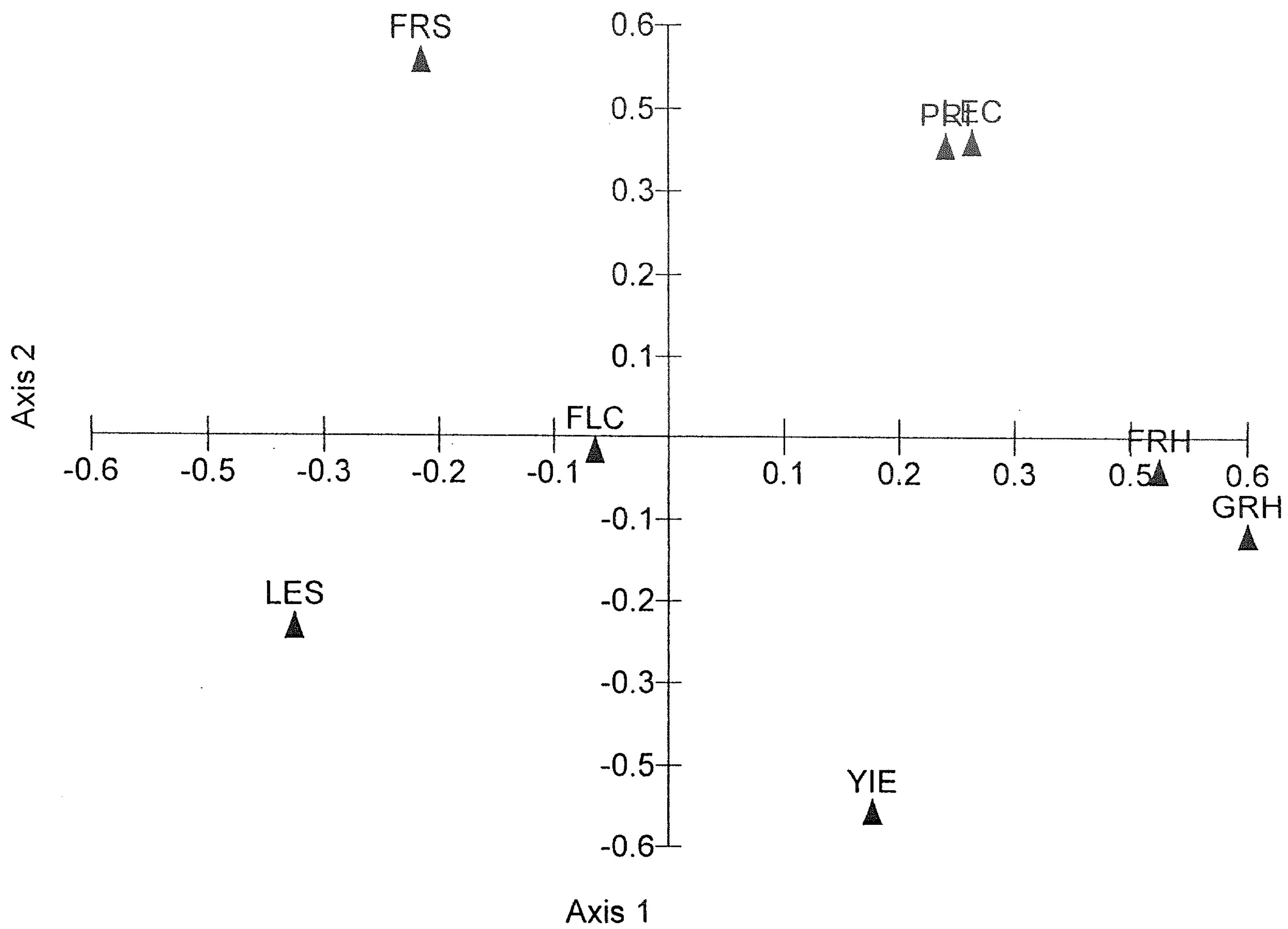

PCA variable loadings
FRS
PRI
EC
FLC
FRH
GRH
LES
YIE
Axis 2
Axis 1
0.6
0.5
0.3
0.2
0.1
-0.1
-0.2
-0.3
-0.5
-0.6

Figure 7.12 UPGMA dendrogram of 100 *S. centrale* individuals sourced from ten different regions within Australia.

UT = Utopia Station
ST = Stirling Swamp
LA = Laramba
TA = Tanami Road
GP = Gas Pipe
SH = Stuart Highway
JR = James Range
KC = Kings Canyon Turnoff
NE = Newman
EC = Ethel Creek

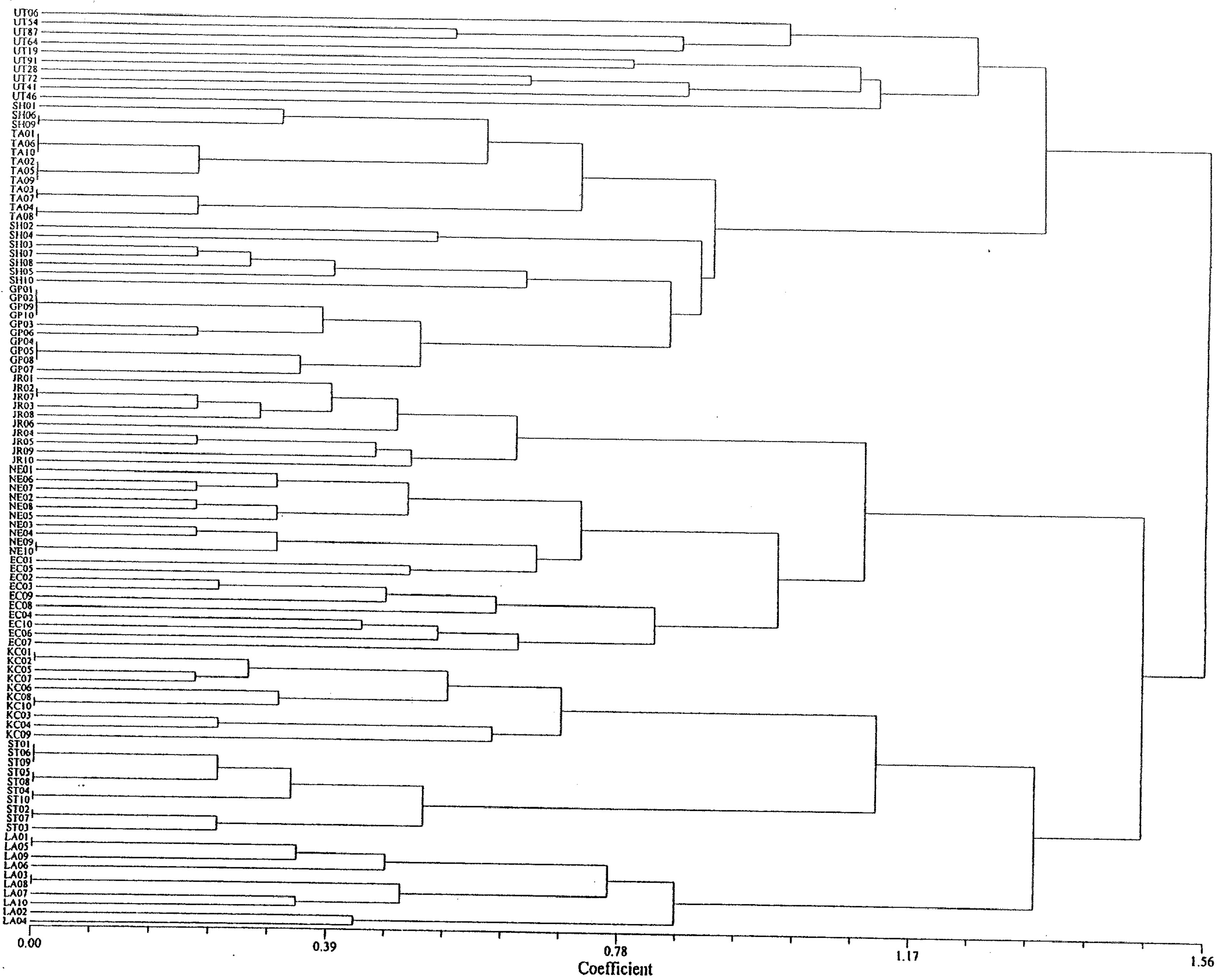
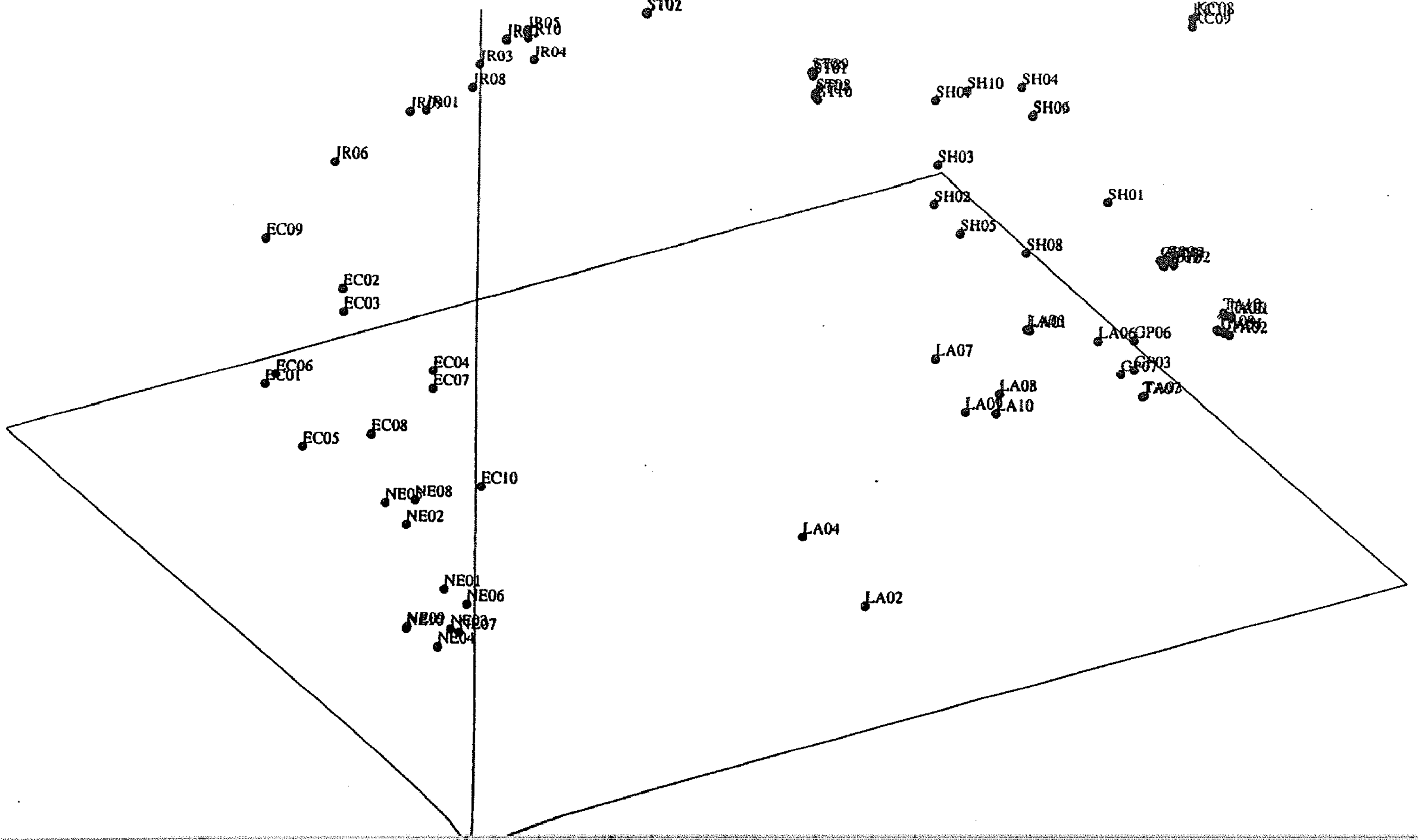

UT06
UT54
UT87
UT64
UT19
UT91
UT28
UT72
UT41
UT46
SH01
SH06
SH09
TA01
TA06
TA10
TA02
TA05
TA09
TA03
TA07
TA04
TA08
SH02
SH04
SH03
SH07
SH08
SH05
SH10
GP01
GP02
GP09
GP10
GP03
GP06
GP04
GP05
GP08
GP07
JR01
JR02
JR07
JR03
JR08
JR06
JR04
JR05
JR09
JR10
NE01
NE06
NE07
NE02
NE08
NE05
NE03
NE04
NE09
NE10
EC01
EC05
EC02
EC03
EC09
EC08
EC04
EC10
EC06
EC07
KC01
KC02
KC05
KC07
KC06
KC08
KC10
KC03
KC04
KC09
ST01
ST06
ST09
ST05
ST08
ST04
ST10
ST02
ST07
ST03
LA01
LA05
LA09
LA06
LA03
LA08
LA07
LA10
LA02
LA04
0.00
0.39
0.78
1.17
1.56
Coefficient

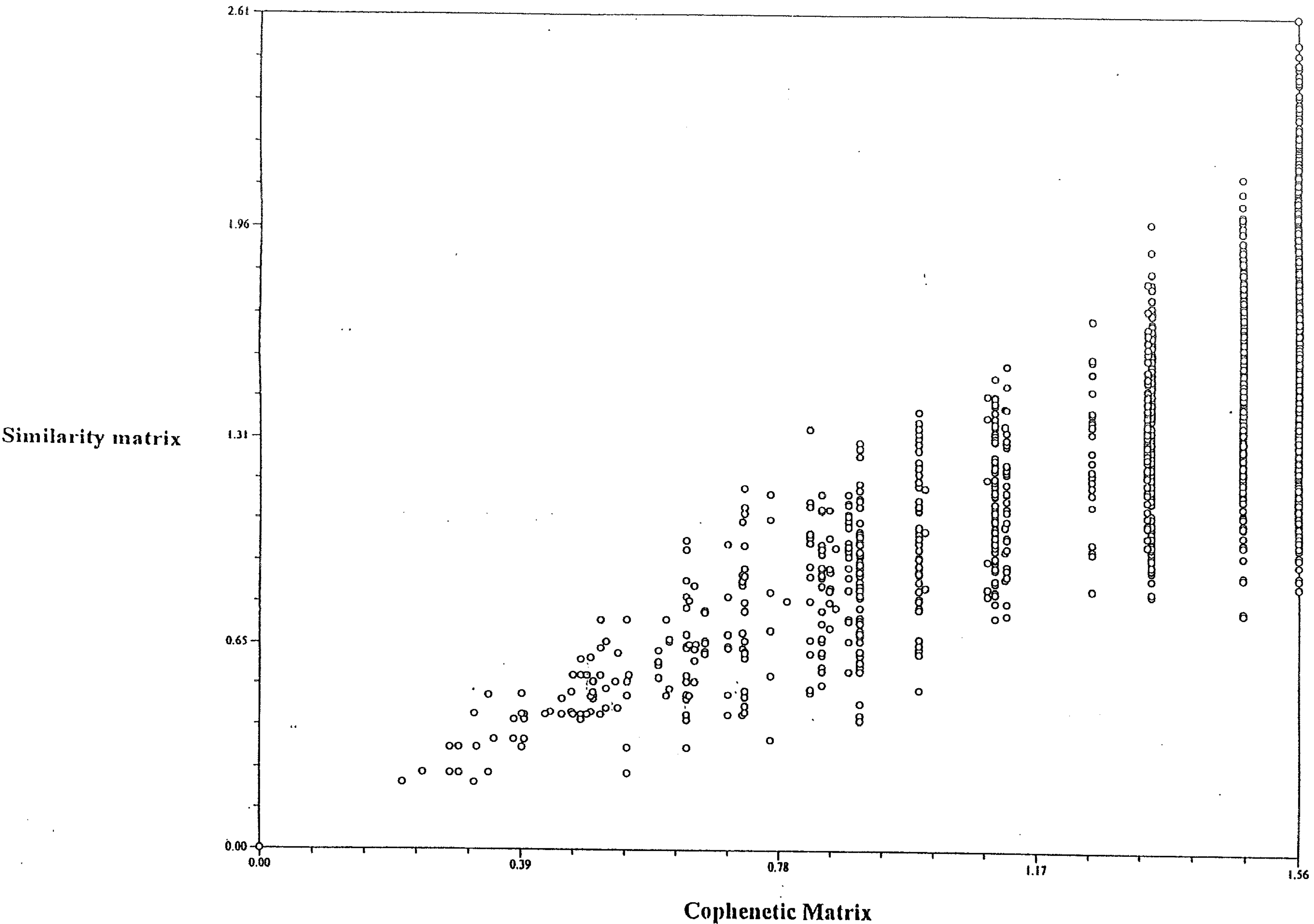
Figure 7.13 Three-dimensional scatter-plot, constructed using MDS ordination, of 100 *S. centrale* individuals collected from ten different populations within Australia.

UT = Utopia Station
ST = Stirling Swamp
LA = Laramba
TA = Tanami Road
GP = Gas Pipe
SH = Stuart Highway
JR = James Range
KC = Kings Canyon Turnoff
NE = Newman
EC = Ethel Creek



UT91
UT19
UT46
UT06
UT28
UT72
UT64
UT87
UT54
KC08
KC09
ST03
ST02
JR05
JR10
JR03
JR04
JR08
JR06
SH10
SH04
SH03
SH02
SH01
SH05
SH08
EC09
EC02
EC03
EC06
EC01
EC04
EC07
EC05
EC08
EC10
NE08
NE02
NE01
NE06
NE04
LA07
LA06
GP06
GP03
LA08
LA10
LA04
LA02

Figure 7.14 Plot of morphology similarities against cophenetic values for the ten different populations of *S. centrale* based on UPGMA clustering.


2.61
1.96
1.31
0.65
0.00
0.00
0.39
0.78
1.17
1.56
Similarity matrix
Cophenetic Matrix

7.4 Discussion

This Chapter has shown that large variations in morphology of *S. centrale* can occur in plants derived from seed harvested from different regions within Australia. This highlights the fact that selection of superior genotypes is required for the future commercial production of the bush tomato to ensure economic and commercial viability.

According to Belbin (1991), individuals that are close on an ordination plot would be similar in terms of species composition, whereas if they were at opposite ends of the distribution, they would have little or nothing in common, indicating that individuals within clusters have similar morphologies.

7.4.1 Morphological variability within Utopia population

Within the isolated population, plants of *S. centrale* collected from Utopia Station population in the Northern Territory of Australia showed a wide range of morphological variability. As described in Chapter 3, seeds from this population were collected over a larger geographical area than the other populations used in this study. The dendrogram and MDS ordination indicate a high degree of variability, which suggests out-crossing and random gene flow (Soltis and Soltis, 1990). Most of the 100 individuals were found to be different within the population. The cophenetic correlation and stress level calculated from the MDS also indicate that there was no significant clustering within this heterogenous population.

7.4.2 Morphological variability between ten Australian populations

Morphological variation within the ten different populations collected from different regions within Australia was less than the variation between populations. The UPGMA dendrogram and MDS ordination revealed clusters that in nearly all cases corresponded with the ten different locations. Due to this it is highly likely that the populations are significantly different. The significance of the clustering is supported by the cophenetic correlation and value of the stress level from MDS.

There was some overlap on the dendrogram between three of the populations sourced from the Northern Territory that was not observed in the MDS ordination. The

geographical distance between the three regions (TA, GP and LA) is around 40 kilometres as shown in Figure 3.2. This suggests either that some gene flow is occurring between the populations, possibly by seed distribution, or that the populations have been separated only relatively recently. Seed dispersal may have occurred by animals such as emus or humans (Noble, 1975).

The two Western Australian populations formed distinct clusters in both UPGMA and MDS analysis, being located close to each other suggests a link to geographical distance and morphology. A similar reasoning can be applied to these populations as was applied to those in the Northern Territory. This is opposite to a study carried out by Keiper and McConchie (2001) on the morphological variation among *Sticherus flabellatus* (Gleicheniaceae) populations of the Sydney region. The greatest similarities and dissimilarities exhibited by populations over the entire area studied did not reveal and geographical trend. Therefore, concluding that each individual population exhibited morphological variation that distinguished it from every other population, as opposed to representing a geographical region.

Variation shown by the morphological traits measured may reflect phenotypic plasticity, genetic differences between the populations studied, and/or primarily neutral variation that has drifted to different means in different populations (Godt and Hamrick, 1996). In this present study the true environmental, genotypic and genotype x environment components of this variation cannot be ascertained. The results however, do indicate that certain phenotypes of *S. centrale* are associated with specific environments and the integration of various traits (Dingle, 1990).

In many plant species the development of ecotypes is common (Jain, 1990) and has been associated with enhancing fitness (Soltis and Soltis, 1990). This may be due to variable selection for those characters that are particularly suited for growth within a local region (Slatkin, 1985, 1987; Jelinski and Cheliak, 1992). Schmid-Hempel (1990) suggests that to determine how the range of phenotypic traits exhibited within each population are adapted, a number of individuals would need to be studied over their lifetime and the number of progeny produced observed.

By simultaneously examining a number of traits in *S. centrale* a characteristic pattern of variability has been revealed for each population in this study (Jain, 1990).

This study was limited to the use of eight morphological characters because when the measurements were completed on all plants there were some redundancies of descriptions found. Previous morphological studies have suggested concentrating on easy, simple and generally used observations (Jacquard *et al.*, 1986). Environmental and cultivation factors make morphological identification inconsistent and therefore the study of morphology alone may be insufficient for assessing the diversity of a species (Bachmann, 1994). A reliable, consistent and reproducible method for confirming genotypes is required. For this reason, Chapter 8 describes the use of molecular techniques in addition to morphology to assess the diversity of *S. centrale*.

Chapter 8 Genetic Diversity of populations of *S. centrale*

8.1 Introduction

The rate of gene flow, mode of pollen and seed dispersal, type of mating system (selfing or crossing), population size, and geographical distance contribute to genetic diversity within and between plant populations (Loveless and Hamrick, 1984; Ellstrand and Marshall, 1986; Kaufman *et al.*, 1998). Darmency (1997) found that the mating system of plants appears to play a major role in genetic diversity by determining the rate of exchange of genes. Lower interpolation and higher intrapolation differences in genetic variation have been found when species are predominantly outcrossing (Huff *et al.*, 1993; Maguire and Sedgley, 1997). In addition, Warwick (1991) found that a reduced level of heterozygosity and fewer genotypes were produced when the species is mainly self-pollinated compared to out-breeders.

S. centrale displays a high degree of outcrossing and, as described in Chapter 6, exhibits some self-incompatibility. High levels of variability were observed in the fertility of *S. centrale* plants. Such variability has the potential to affect yield and quality when the plants are used as a commercial crop. The diversity of *S. centrale* has not been evaluated before and this chapter describes the genetic diversity of *S. centrale* within one isolated population and between ten different Australian populations.

A number of methods are available for analysing molecular diversity in plant populations. The relative merits of these methods have been discussed in different reviews (Bachmann, 1994; Karp *et al.*, 1996) and from these the RAPD-PCR method has been chosen for this study. RAPD-PCR has been used successfully in population genetics in many plants, including *Banksia cuneata* (Maguire and Sedgley, 1997), *Cedrela odorata* (Gillies *et al.*, 1997), *Erodium paularense* (Martin *et al.*, 1997) wild bean (Cattan-Toupance *et al.*, 1998), *Grevillea barklyana* (Hogbin *et al.*, 1998), and *Olea europea* (Mekuria *et al.*, 1999). This technique was used to evaluate the level of genetic variation in *S. centrale*.

8.2 Materials and methods

8.2.1 Plant material

Plant material used in this study has been described in Chapter 3.

Field (1997) developed a technique for collecting olive leaves that ensured DNA of high quality and adequate yield could be obtained for subsequent analysis. This technique was adopted for *S. centrale* and involved collecting young, fully-grown leaves fresh from the field, wrapping them tightly (to exclude as much air as possible) in thin plastic film and storing at 4°C until DNA extraction.

8.2.2 DNA isolation

High level of polyphenols can interfere with the recovery of high purity DNA (Fontanazza *et al.*, 1994; Burr, 1997). To address this problem with the leaves of *S. centrale* the method to extract DNA was based on successful methods involving plants that have high phenolics such as *Banksia* (Maguire *et al.*, 1994) and *Vitis* (Steenkamp *et al.*, 1994).

DNA was extracted using the method reported by Mekuria *et al.* (1999). Approximately 2-2.5 g of stored leaves were weighed, ground with liquid nitrogen into a fine powder in a pre-chilled mortar and pestle, and transferred to 7.5 ml of cold (0°C) extraction buffer containing 3% (w/v) cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 0.1 M ethylenediaminetetra-acetic acid (EDTA), pH 8.0, and 1.0 M Tris HCl, pH 8.0. Fifteen µl (0.2% (v/v)) of 2-mercaptoethanol and 15 mg of polyvinylpyrollidone (PVP-40T) were added just before the leaves were ground.

The mixture was incubated at 60°C for 30 min and inverted every ten min during this time. After cooling on ice, an equal volume of chloroform/iso-amyl alcohol [24:1 (v/v)] was added, mixed gently for 10 min, and centrifuged at 3 000 x g for 20 min at room temperature. The upper, aqueous layer was removed and gently mixed with 2/3 volume of cold (0°C) isopropanol to precipitate the DNA, which was spooled onto a glass rod, and transferred into 20 ml of wash buffer [76% ethanol (v/v), 10mM ammonium acetate (NH_4Ac)]. The DNA was washed until white and then

resuspended in 1 ml of TE Buffer (10 mM Tris hydrochloric acid, 0.1 mM EDTA, pH 8.0).

RNA was digested with 2 μl of 10 mg/ml DNase-free RNase A (AMRESCO®, Solon, OHIO, USA), prepared as described in Sambrook *et al.* (1989) for 30 min at 37°C. The solution was diluted with 2 ml of TE buffer and 1 ml of 7.5 M NH_4Ac and placed on ice for 20 min at 4°C, followed by centrifuging at 10 000 x g for 20 min at 4°C. Two volumes of cold ethanol (-20°C) were added, and DNA was allowed to precipitate on ice for 20 min. The solution was centrifuged at 8 000 x g for 10 min at 4°C and, after draining the tube, the pellet was dissolved in 1 ml of TE buffer. To determine the concentration and quality of the DNA extracted, absorbency measurements were taken at 230, 260 and 280 nm in a Shimadzu UV-VIS Recording Spectrophotometer UV 160A, and ratios of 260/280 and 260/230 were calculated. DNA was used for further analysis when the ratios were greater than or equal to 1.8 indicating that the presence of protein and polyphenols was low (Johnson, 1994).

8.2.3 Polymerase chain reaction (PCR) technique

Polymerase chain reactions (PCR) were performed on thermal cyclers (PTC-100™ Programmable Thermal Controller, MJ Research Inc.) that required the reaction mixture to be overlaid with mineral oil to prevent evaporation. For these machines, oil was placed around tubes within wells to facilitate faster heat transfer between heating block and sample. Reactions were carried out in 0.5 ml Eppendorf tubes.

Where possible, a master mix was made containing all PCR reaction components except for DNA. This master mix was aliquoted into individual reactions before the addition of different DNA samples. In order to reduce the chances of contamination, controls containing all reagents except for template DNA were included for each primer as a negative test for contamination. For use in PCR, stock DNA was diluted to 40 ng/μl with TE buffer.

PCR reactions were carried out in 20μl containing 40 ng of genomic DNA, 1.5 mM $MgCl_2$, 1 x PCR Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatine), 200 μM each of dGTP, dATP, dTTP, and dCTP, 0.25 μM 10-

mer primer, and 1 unit *Taq* DNA Polymerase (GIBCO BRL®, Life Technologies Inc., Gaithersburg, MD, USA), and overlaid with an equal volume of paraffin oil to prevent evaporation. The PCR program consisted of an initial denaturation (strand separation) of 2 min at 95°C, 39 cycles of 1 min at 95°C, 10 sec at 50°C, 15 sec at 45°C, 20 sec at 40°C, 1 min at 35°C, 30 sec at 45°C, and 1 min 45 sec at 72°C, and a final extension step of 5 min at 72°C. (Collins and Symons, 1993). Duplicate, and if necessary triplicate, amplifications were conducted to ensure reproducible results.

8.2.4 Agarose gel electrophoresis

DNA amplification fragments were separated by gel electrophoresis. Eight µl of each 20 µl PCR reaction was combined with 2 µl of 10 x loading buffer (Watson and Thompson, 1988) and mixed thoroughly. A 100 bp molecular-weight marker ladder (GeneWorks, Adelaide, DMW 100M) was loaded into wells on each side of the gel to aid interpretation of band identity. Samples were run on 1.75% SeaKem® GTG® agarose gels (Rockland, Maine USA) in TBE buffer (Sambrook *et al.*, 1989) at a constant current of 80 mA for 90 min.

PCR products were stained with ethidium bromide (0.5 µg/ml), visualized under UV light, and photographed with Polaroid 667 film. A video capture program, Tekcap (Version 1.0, Tekram Corporation), was used to capture digital images of the stained gels. Paint Shop Pro (Version 5.0, Jasc Software Inc., US) was then used to save the images in bitmap format for analysis.

8.2.5 Primer screening

Six samples of DNA were chosen at random and subjected to RAPD-PCR using primers from Operon Technologies, Almeda, CA, USA, or made by the Nucleic Acid and Protein Chemistry Unit, University of Adelaide, Australia. Sixty 10-mer oligodeoxyribonucleotide primers were screened using this DNA and protocol. From the results of this screening, six primers that produced the most consistent and distinct polymorphisms were chosen for further analysis. PCR reactions with these six primers were repeated at least twice. If bands were not reproducible for a primer, a third PCR was run and only reproducible bands used in the analysis.

8.2.6 Data analysis

Gel-Pro Analyser (Version 3.1, Media Cybernetics, Maryland, USA) was used to derive the molecular weight of the amplification products from the digital images of the gels based on the 100 bp DNA ladder. Amplification fragments were scored as present (1) or absent (0) for all individuals, and the data recorded in a binary matrix.

Binary matrices were analysed using the program NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Version 2.02j, Exeter Software, Setauket, New York). Genetic similarities between the different plants were obtained by performing pair wise comparisons of all individuals using the simple matching coefficient (Sokal and Michener, 1958; Rohlf, 1993; Bradley *et al.*, 1996). Cluster analysis was performed using the unweighted pair-group method arithmetic average (UPGMA) and the SAHN algorithm to construct a dendrogram indicating genetic similarities (Belbin, 1991, 1994).

The cophenetic matrix was plotted against the similarity matrix using NTSYS-pc to evaluate the randomness or otherwise of the clusters by calculating the cophenetic correlation. To represent the relationships among individuals in two or three dimensions the similarity matrix was ordinated using non-metric multidimensional scaling. A minimum spanning tree was superimposed on the ordination to help detect outliers and to link neighbouring points.

8.3 Results

8.3.1 Primer screening

The six decamer primers selected for RAPD-PCR analysis produced a total of 127 bands, of which 83 (65%) were polymorphic and the remaining 44 monomorphic (Table 8.1). Monomorphic bands were those present in all individuals, while polymorphic bands were those bands present in at least one, but not all individuals. Individual primers produced between 18 (OPD-07) and 25 (OPZ-11) bands. A UV photograph of an ethidium bromide stained agarose gel with the PCR products obtained after using one of these primers (OPB-14) on 17 *S. centrale* individuals is

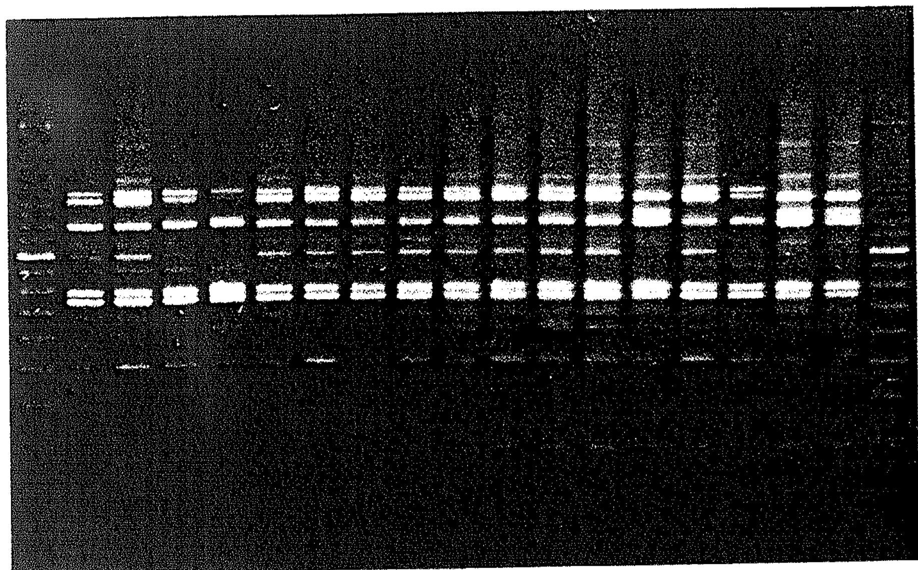
shown in Figure 8.1. This was carried out for all primers on all *S. centrale* individuals used in this project.

Table 8.1 The six decamer primers selected using the RAPD-PCR technique.

Primer	Primer sequence (5'-3')	Number of bands produced	Polymorphic bands
1. OPZ-11	CTCAGTCGCA	25	24
2. OPB-14	TCCGCTCTGG	20	11
3. OPB-20	GGACCCTTAC	24	11
4. OPD-07	TTGGCACGGG	18	15
5. GC-01	CAGGCCCTTC	21	11
6. GC-20	GTTGCGATCC	19	11
Total		127	83
Mean		21.1	13.8

Figure 8.1 Agarose gel photographed under UV light after staining with ethidium bromide. Amplification products, using primer OPB-14, were obtained from DNA extracted from 17 *S. centrale* individuals. Lanes 1 + 19 – 100 bp ladder, lanes 2-18 – *S. centrale* individuals.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



8.3.2 Genetic variability within Utopia population

The RAPD technique was used to investigate genetic variability within an isolated population of *S. centrale* derived from seeds collected from Utopia Station, Northern Territory. Using the analysis of 100 individuals, a dendrogram was constructed and is shown in Figure 8.2. The individuals in this population showed less than 94% similarity to each other and no individuals were found to be the same. Also, when the data were ordinated by multidimensional scaling (Figure 8.3), the 100 individuals in the population were found to be unique from each other with no real clustering as occurred in the dendrogram (Figure 8.2).

The measure of the goodness of fit between the dendrogram and the original similarity matrix is the cophenetic correlation coefficient. This was calculated and found to be around 0.6 indicating that the clusters could not be distinguished from random events (Rohlf, 1993) (Figure 8.4). The stress level for this analysis was calculated and found to be about 0.63 also indicating that there was no significant clustering amongst individuals.

Figure 8.2 Dendrogram showing the genetic similarities between 100 *S. centrale* individuals from the Utopia Station population, using six primers, the simple matching coefficient and UPGMA clustering.

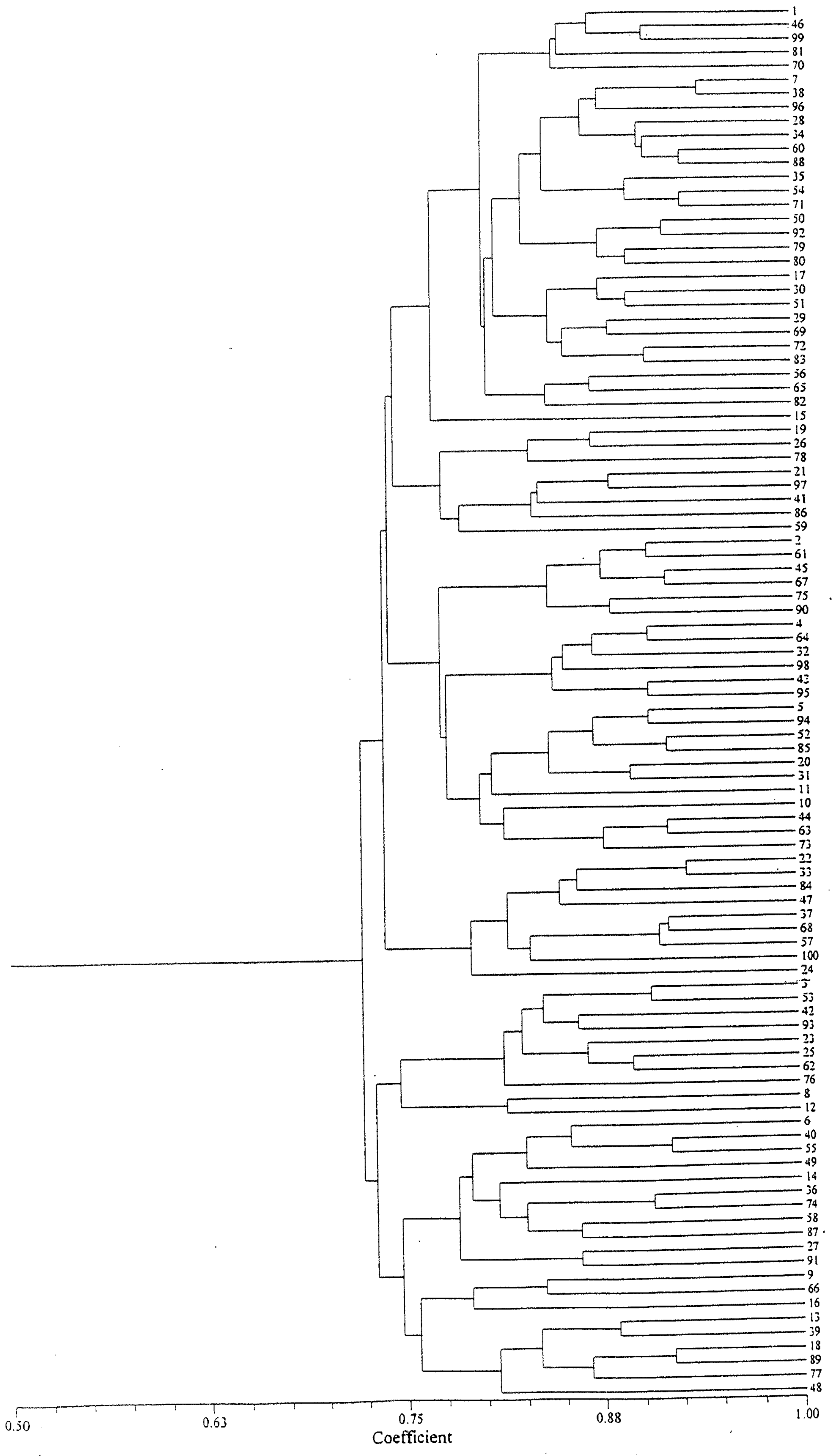

1
46
99
81
70
7
38
96
28
34
60
88
35
54
71
50
92
79
80
17
30
51
29
69
72
83
56
65
82
15
19
26
78
21
97
41
86
59
2
61
45
67
75
90
4
64
32
98
43
95
5
94
52
85
20
31
11
10
44
63
73
22
33
84
47
37
68
57
100
24
3
53
42
93
23
25
62
76
8
12
6
40
55
49
14
36
74
58
87
27
91
9
66
16
13
39
18
89
77
48
0.50
0.63
0.75
0.88
1.00
Coefficient

Figure 8.3 Three-dimensional multidimensional scaling plot for the 100 *S. centrale* individuals from the Utopia Station population examined by six primers for genetic similarity.

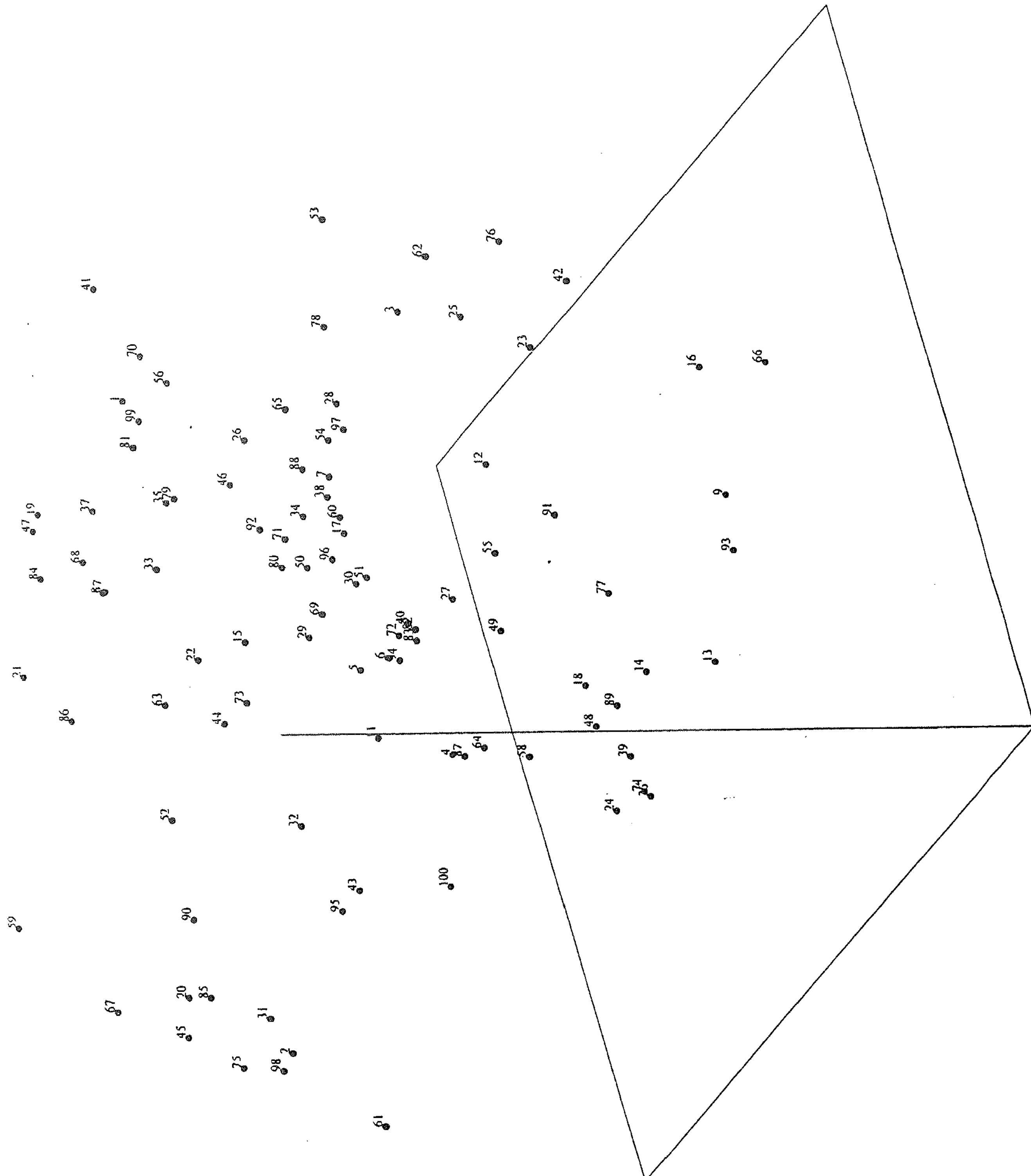
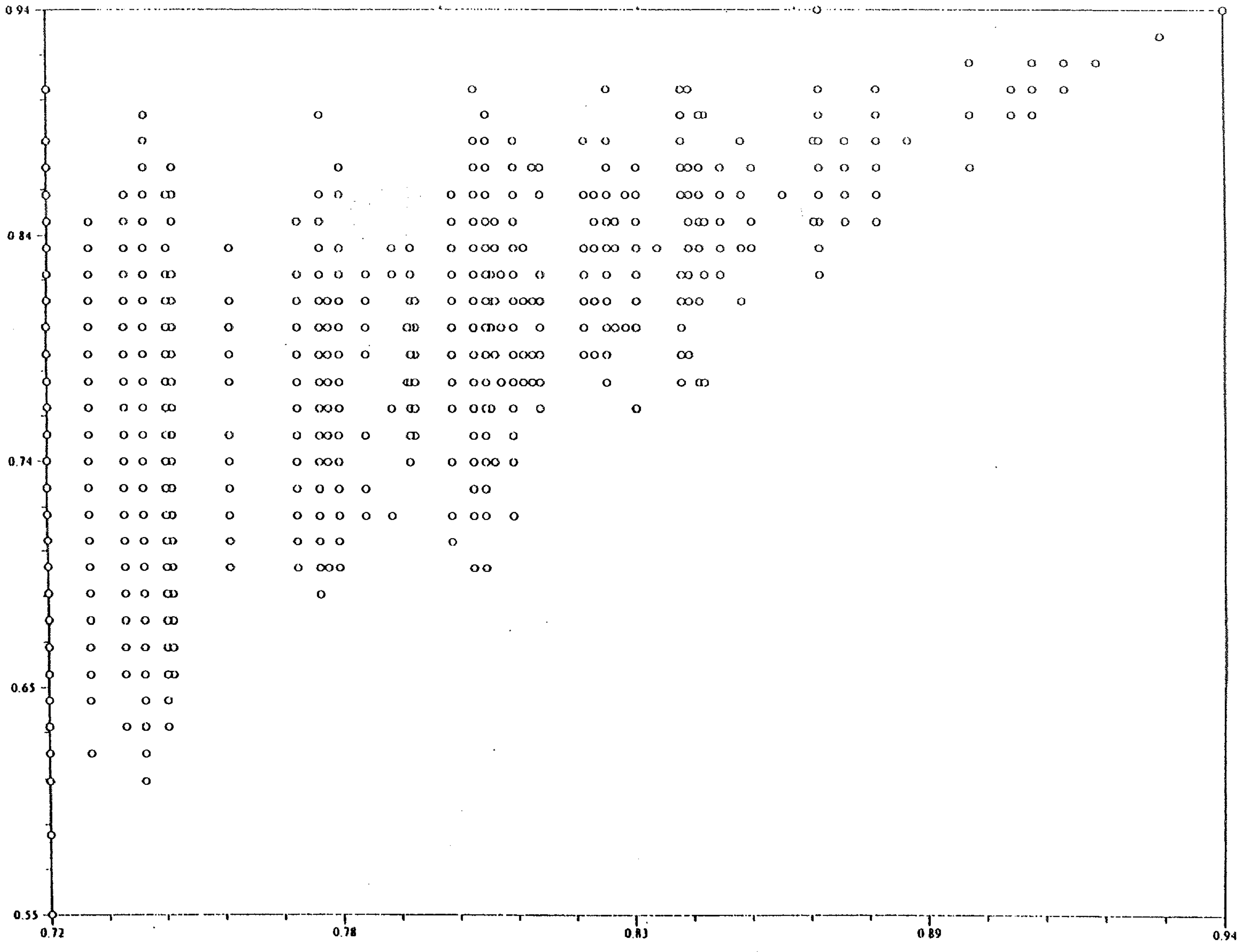


Figure 8.4 Plot of genetic similarities against cophenetic values based on UPGMA clustering for 100 *S. centrale* individuals from Utopia Station population.



0.94
0.84
0.74
0.65
0.55
0.72
0.78
0.83
0.89
0.94
Cophenetic Matrix
Similarity matrix

8.3.3 Genetic variability between ten Australian populations

Ten molecular clusters were identified from the UPGMA dendrogram (Figure 8.5) and these corresponded with the 10 different locations from which the seed material was sourced. The individuals showed less than 98% genetic similarity, and no individuals were found to be identical. When the data were ordinated by multidimensional scaling (Figure 8.6), the 100 plants in the study formed the same ten discrete clusters as the dendrogram (Figure 8.5).

The value of the cophenetic correlation coefficient was about 0.9 indicating a good fit, and therefore the presence of clusters was highly unlikely to have occurred due to random events (Figure 8.7). The stress level of about 0.4 supported this argument, also suggesting that the clusters were significant.

Due to overlapping of populations found when three-dimensional MDS plots were performed, a detailed analysis of some of these populations was undertaken. Three Northern Territory populations that appeared to overlap in the MDS plot were analysed separately. Figure 8.8 is a dendrogram of the three populations indicating three separate clusters also supported by the two-dimensional MDS plot (Figure 8.9). The stress level determined as 0.3 suggests that these are three significant clusters.

The two Western Australian populations were also found to overlap on the MDS plot, and analysed separately. Both the dendrogram and MDS analysis suggest that the two populations clustered separately (Figure 8.10 and 8.11) and the stress level of 0.15 supports this argument.

Figure 8.5 Dendrogram showing 10 molecular clusters derived from the genetic similarities of the 100 *S. centrale* individuals using the simple matching coefficient and UPGMA clustering. The 10 molecular clusters coincide with the 10 groups based on location of seed material.

Key
UT = Utopia Station
ST = Stirling Swamp
LA = Laramba
TA = Tanami Road
GP = Gas Pipe
SH = Stuart Highway
JR = James Range
KC = Kings Canyon Turnoff
NE = Newman
EC = Ethel Creek

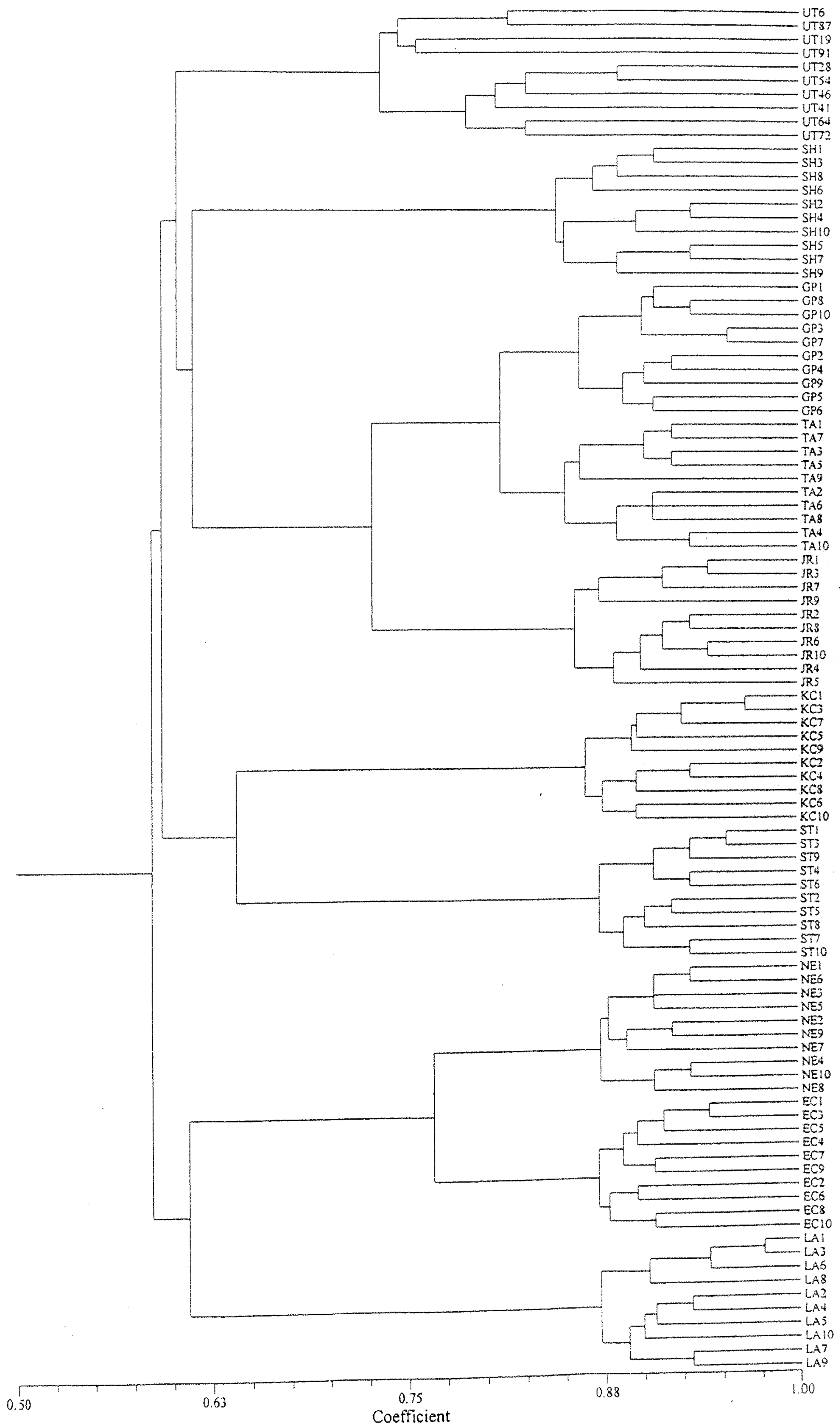
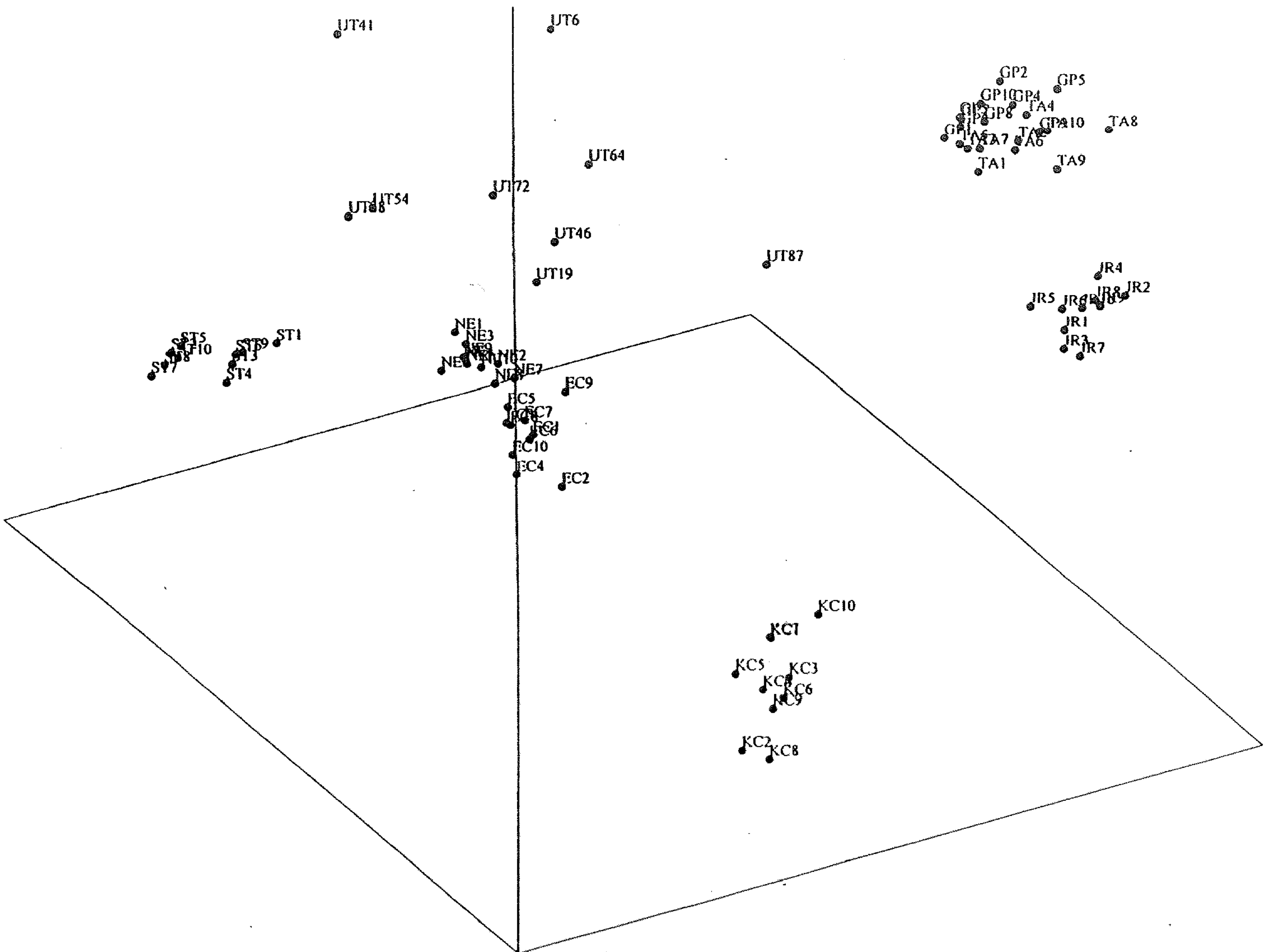

UT6
UT87
UT19
UT91
UT28
UT54
UT46
UT41
UT64
UT72
SH1
SH3
SH8
SH6
SH2
SH4
SH10
SH5
SH7
SH9
GP1
GP8
GP10
GP3
GP7
GP2
GP4
GP9
GP5
GP6
TA1
TA7
TA3
TA5
TA9
TA2
TA6
TA8
TA4
TA10
JR1
JR3
JR7
JR9
JR2
JR8
JR6
JR10
JR4
JR5
KC1
KC3
KC7
KC5
KC9
KC2
KC4
KC8
KC6
KC10
ST1
ST3
ST9
ST4
ST6
ST2
ST5
ST8
ST7
ST10
NE1
NE6
NE3
NE5
NE2
NE9
NE7
NE4
NE10
NE8
EC1
EC3
EC5
EC4
EC7
EC9
EC2
EC6
EC8
EC10
LA1
LA3
LA6
LA8
LA2
LA4
LA5
LA10
LA7
LA9
0.50
0.63
0.75
0.88
1.00
Coefficient

Figure 8.6 Molecular clusters derived from the genetic similarities of the 100 *S. centrale* individuals sourced from 10 different Australian populations using three-dimensional multidimensional scaling.

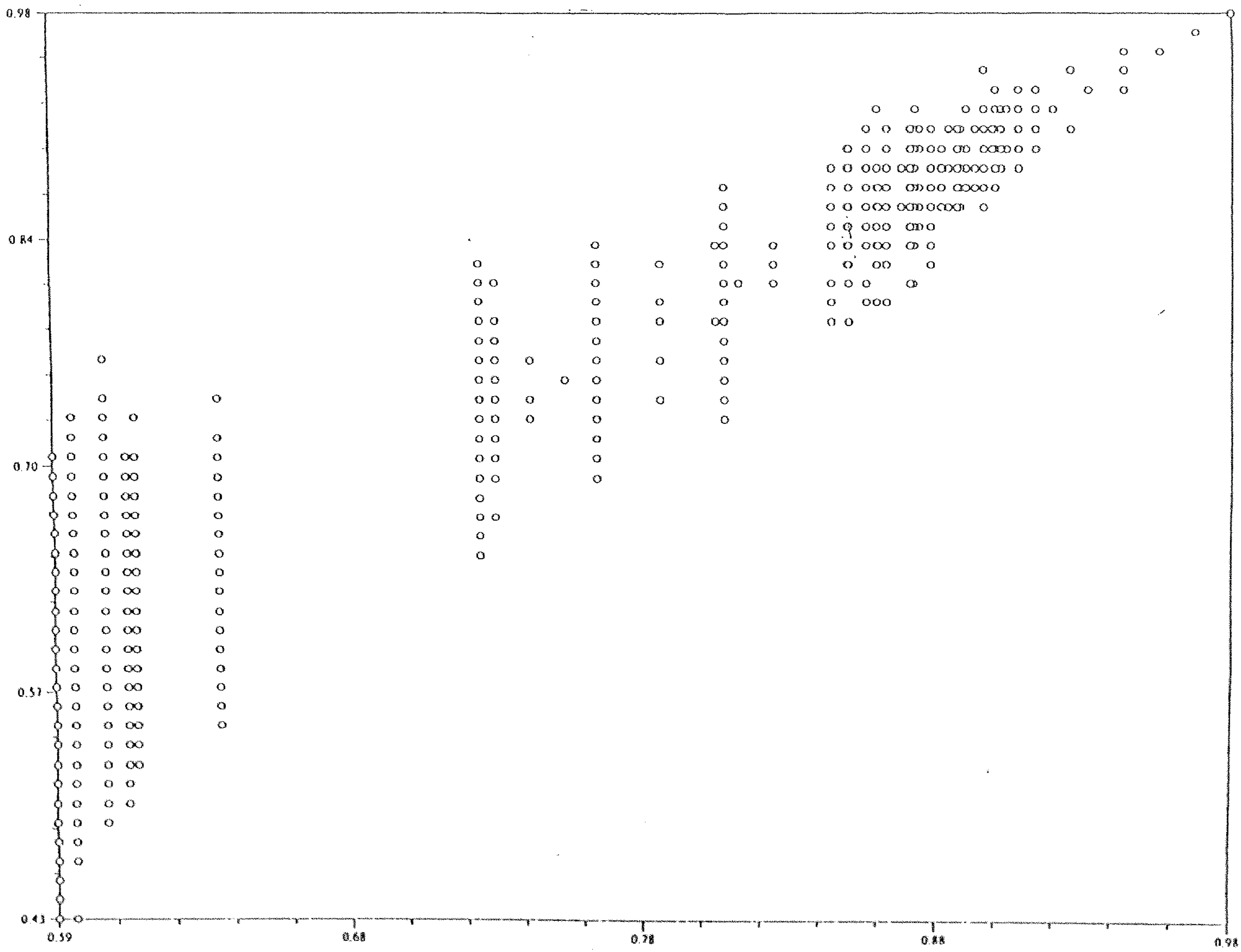
Key
UT = Utopia Station
ST = Stirling Swamp
LA = Laramba
TA = Tanami Road
GP = Gas Pipe
SH = Stuart Highway
JR = James Range
KC = Kings Canyon Turnoff
NE = Newman
EC = Ethel Creek



SH7
SH4
SH2
SH6
SH9
SH3
SH8
UT41
UT6
GP2
GP5
TA4
TA8
TA1
TA9
UT64
UT72
UT54
UT46
UT87
JR4
JR2
JR5
JR1
JR7
UT19
ST1
ST4
NE1
NE3
NE7
FC9
FC5
FC7
FC10
FC4
FC2
KC10
KC5
KC3
KC6
KC8

Figure 8.7 Plot of genetic similarities against cophenetic values based on UPGMA clustering for 10 different Australian populations.

Similarity matrix



Cophenetic Matrix

Figure 8.8 Dendrogram showing three molecular clusters derived from the genetic similarities of 30 *S. centrale* individuals using the simple matching coefficient and UPGMA clustering.

Key
GP = Gas Pipe
TA = Tanami Road
JR = James Range

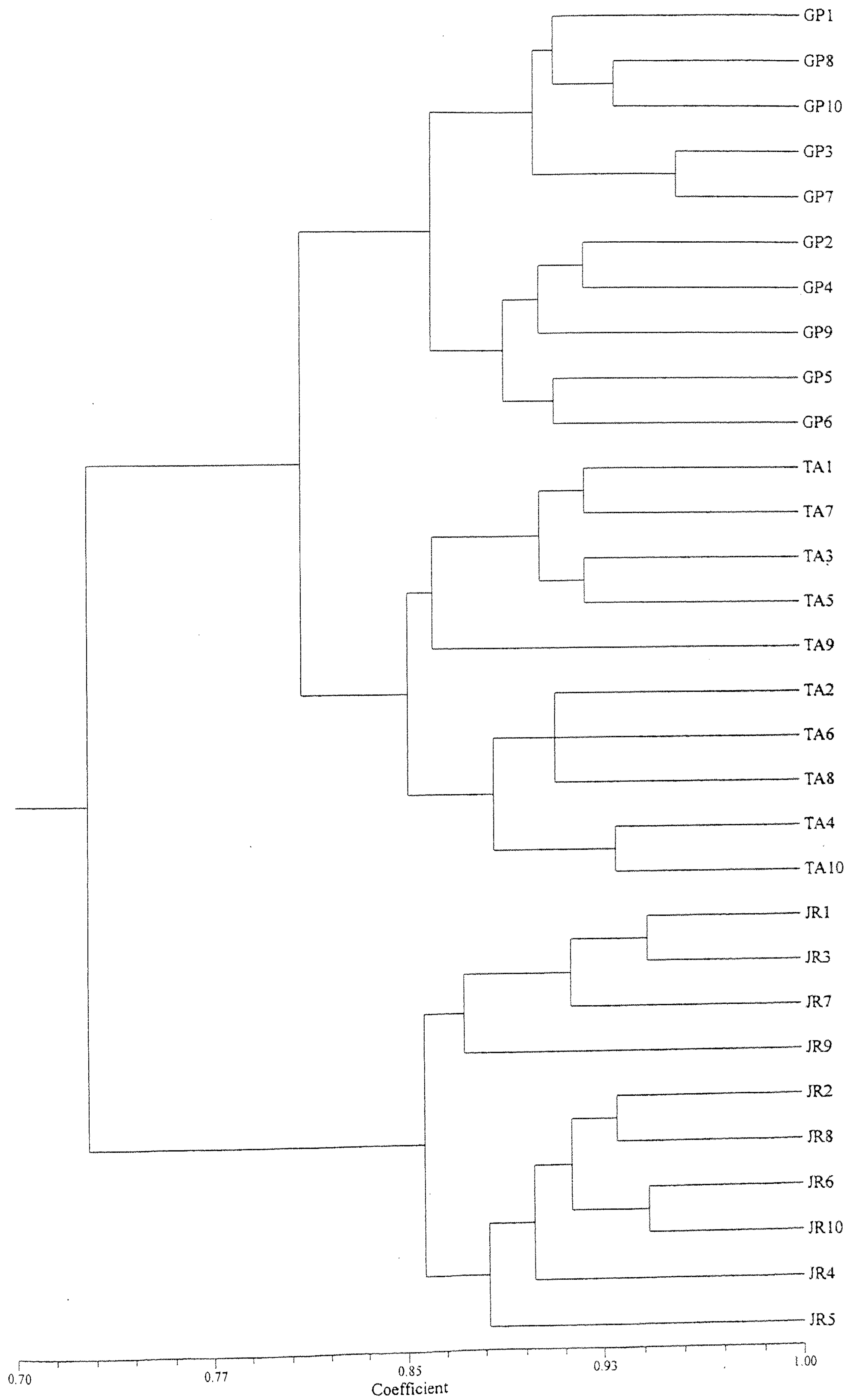

GP1
GP8
GP10
GP3
GP7
GP2
GP4
GP9
GP5
GP6
TA1
TA7
TA3
TA5
TA9
TA2
TA6
TA8
TA4
TA10
JR1
JR3
JR7
JR9
JR2
JR8
JR6
JR10
JR4
JR5
0.70
0.77
0.85
0.93
1.00
Coefficient

Figure 8.9 Molecular clusters derived from the genetic similarities of the 30 *S. centrale* individuals sourced from three different populations using two-dimensional multidimensional scaling.

Key
GP = Gas Pipe
TA = Tanami Road
JR = James Range

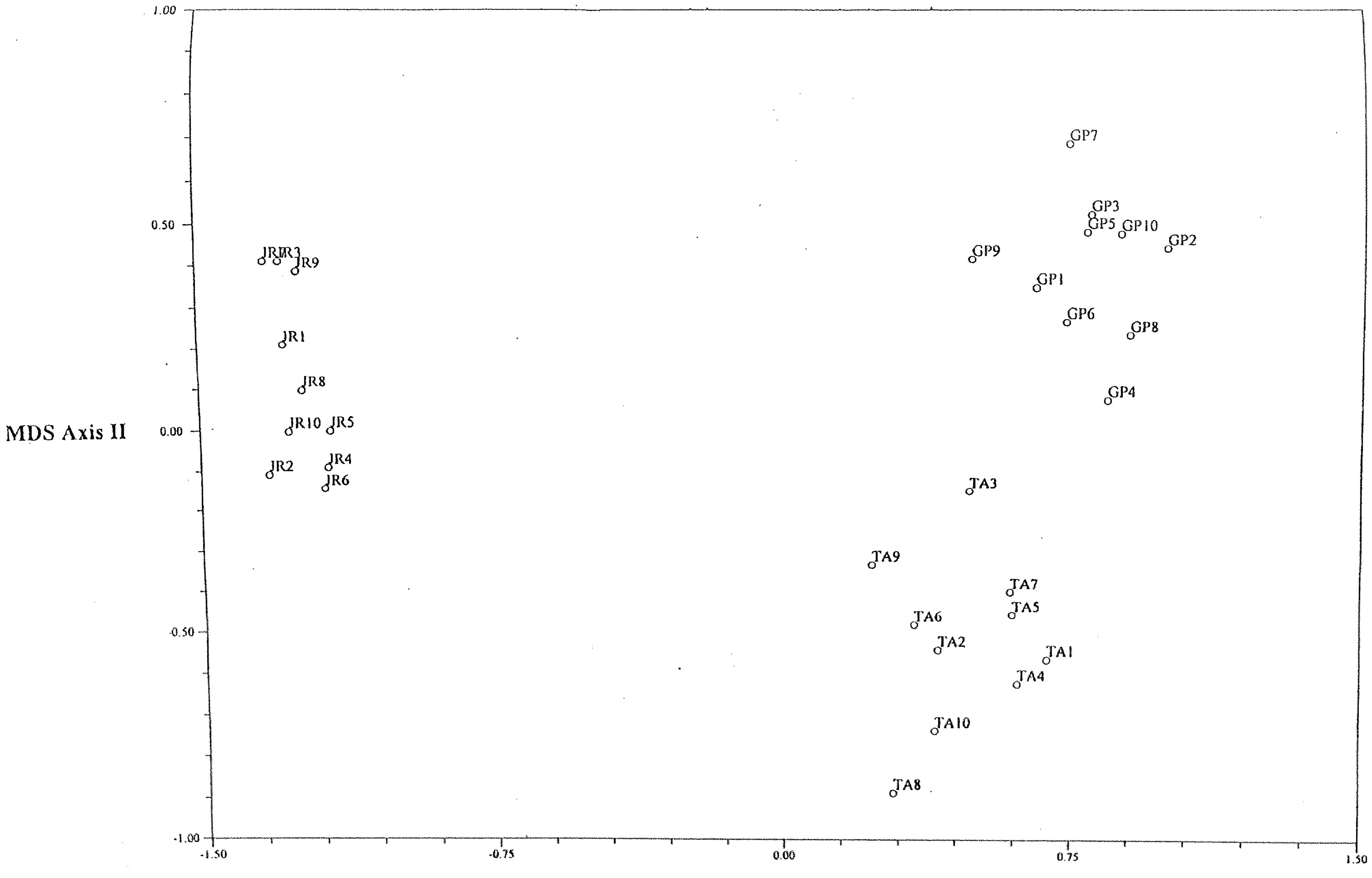

MDS Axis II
MDS Axis I
1.00
0.50
0.00
-0.50
-1.00
-1.50
-0.75
0.00
0.75
1.50
GP7
GP3
GP5
GP10
GP2
GP9
GP1
GP6
GP8
GP4
TA3
TA9
TA7
TA5
TA6
TA2
TA1
TA4
TA10
TA8
JR3
JR9
JR1
JR8
JR10
JR5
JR2
JR4
JR6

Figure 8.10 Dendrogram showing two molecular clusters derived from the genetic similarities of 20 *S. centrale* individuals sourced from Western Australia using the simple matching coefficient and UPGMA clustering.

Key
NE = Newman
EC = Ethel Creek

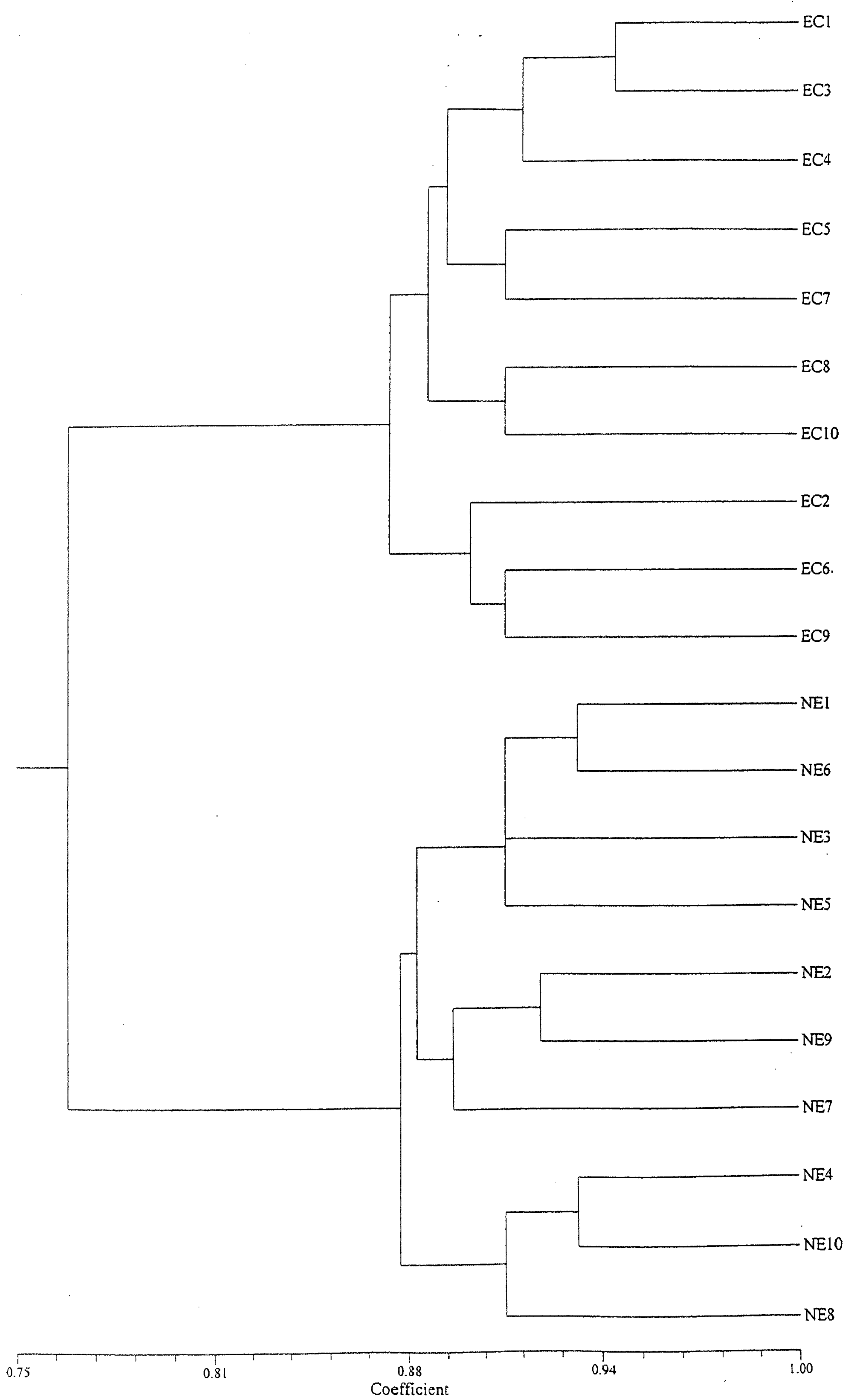
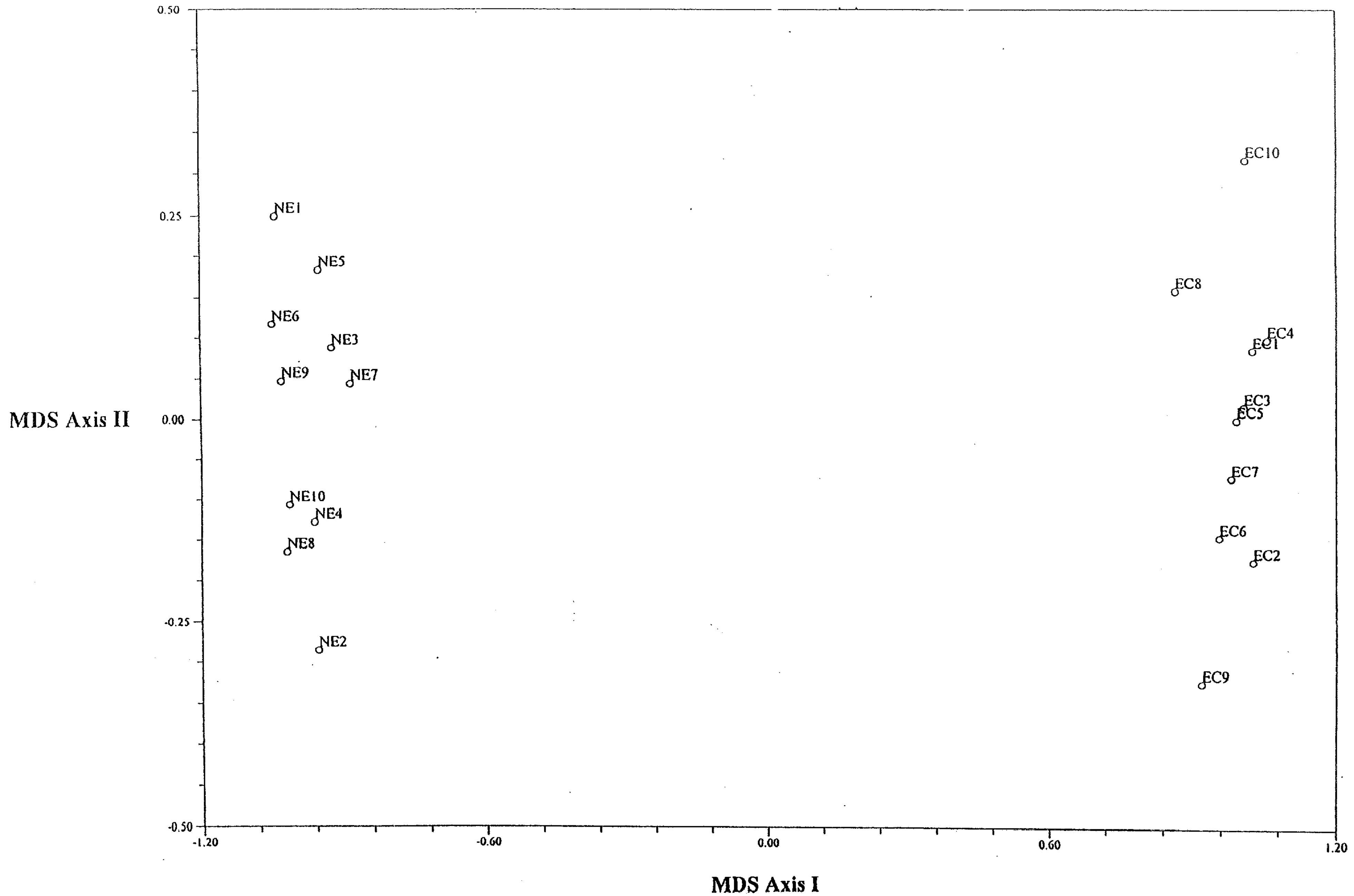

EC1
EC3
EC4
EC5
EC7
EC8
EC10
EC2
EC6
EC9
NE1
NE6
NE3
NE5
NE2
NE9
NE7
NE4
NE10
NE8
0.75
0.81
0.88
0.94
1.00
Coefficient

Figure 8.11 Two molecular clusters derived from the genetic similarities of 20 *S. centrale* individuals sourced from two different Western Australian populations using two-dimensional multidimensional scaling.

Key
NE = Newman
EC = Ethel Creek


MDS Axis II
MDS Axis I
0.50
0.25
0.00
-0.25
-0.50
-1.20
-0.60
0.00
0.60
1.20
NE1
NE5
NE6
NE3
NE9
NE7
NE10
NE4
NE8
NE2
EC10
EC8
EC4
EC1
EC3
EC5
EC7
EC6
EC2
EC9

8.4 Discussion

This Chapter has examined the spread and diversity of *S. centrale* from an isolated population on Utopia Station, Northern Territory and from a comparison of ten different wild populations located across Australia. The preservation of genetic diversity both within and among natural populations is a fundamental goal of conservation biology (Hamrick *et al.*, 1991). Knowledge of the genetic diversity present in natural populations is required to manage germplasm resources effectively, in terms of exploitation and conservation of biodiversity (Keiper and McConchie, 2000).

RAPD markers exhibited a high level of efficiency in detecting DNA polymorphism over a large number of randomly sampled loci, and proved to be very useful for detecting levels of genetic variation among natural populations of this species. The RAPD-PCR based method has been used to study genetic variation in garlic (Bradley *et al.*, 1996), beans (Skroch and Nienhuis, 1995) and collards (Farnham, 1996); also to study intra and inter specific variation in *Lens* (Abo-elwafa *et al.*, 1995), and for identification and characterisation of woody perennial cultivars of almond (Bartolozzi *et al.*, 1998), peach (Warburton *et al.*, 1996), grapevine (Loureiro *et al.*, 1998), fingerprinting studies of *Eucalyptus globulus* clones (Nesbitt *et al.*, 1997), *Eucalyptus nitens* ramets (Vaillancourt *et al.*, 1998), genetic variation between commercial olive cultivars (Mekuria *et al.*, 1999) and DNA fingerprinting for the classification of selected almond cultivars (Woolley *et al.*, 2000).

8.4.1 Genetic variability within Utopia population

This study revealed the range of genetic variability between individuals within an isolated population of *S. centrale*. Seed from Utopia Station was collected over a larger area (see Chapter 3) than from any of the other populations used in this study. The dendrogram indicated a high degree of genetic variability suggesting both out-crossing and random gene flow. No real clustering was apparent, and each individual out of 100 was different within the Utopia Station region. The cophenetic correlation, MDS, and stress level indicate that there was no significant clustering within this heterogeneous population. Therefore individuals are contributing relatively equally to

the gene pool, and it can be concluded that gene flow (both pollen and seed dispersal) in an isolated *S. centrale* population is uniform among individuals.

8.4.2 Genetic variability between ten Australian populations

Seed for ten different locations, sourced from wild populations in Australia was collected to look at variation between different populations of *S. centrale*. The DNA fingerprints from the seedlings were analysed using UPGMA clustering and MDS ordination, and both the dendrogram and MDS ordination showed the existence of ten molecular clusters. All ten molecular clusters corresponded with the ten different locations indicating that it is highly likely that the populations are significantly different and that it is highly unlikely that the clustering has occurred due to random events. Significant population differences were found by RAPD analysis indicating low levels of gene flow between the populations studied. Genetically isolated populations often form with the development of ecotypes (Jelinski and Chelaik, 1992).

While no individuals were found to be the same, some populations were found to overlap on the MDS plot. The two Western Australian populations appeared to overlap as well as three of the Northern Territory populations. All populations were found to be different when analysed in detail.

Utopia Station exhibited the most variability of the different populations. This could be due to less restriction on pollen flow across Utopia Station than between the 10 widely different locations. Physical boundaries between the regions and collections over a small area could be limiting diversity and therefore gene flow. The unique, heterogenous population collected from Utopia Station has a distance from boundary to boundary of approximately 10 kilometres. When compared to the smallest distance between the ten location which is approximately 40 kilometres. Physical barriers, such as rivers are present between collection sites limiting pollen dispersal and result in gene isolation. Pollen and seed dispersal mechanisms and mating systems have been found to influence genetic variation within and between populations (Loveless and Hamrick, 1984; Ellstrand and Marshall, 1986; Kaufman *et al.*, 1998).

Genetic variation is the resource from which populations draw for short-term adaptation to environmental change and for longer-term evolutionary change (Frankel and Soule, 1981). When higher levels of diversity are found in larger populations the capacity for that population to withstand changing environments is a greater. This may not be the case for some of the smaller populations of *S. centrale* who lack the potential to overcome the constraints of continuing habitat loss and fragmentation (Keiper and McConchie, 2000). A combination of genetic drift and a higher incidence of inbreeding due to colonisation events could be contributing to the lower levels of genetic variation in the smaller populations. This has further implications for these populations in maintaining genetic diversity in disturbed environments. Unknown are the affects on long-term population viability in reduced genetic diversity populations (Keiper and McConchie, 2000). The introduction of individuals carrying novel genes may partially restore the genetic diversity to depleted populations (Butler *et al.*, 1994). However, the introduction of these genotypes may reduce the overall population fitness due to evolution under widely different selective regimens (i.e. outbreeding depression) (Hamrick *et al.*, 1991).

The evolutionary potential of a species, the genetic structure of populations and gene flow are determined by plant breeding systems (Brown, 1979; Loveless and Hamrick, 1984; Korpelainen, 1995) As discussed in Chapter 6, *S. centrale* has the ability to self- and cross- pollinate. When a species has a mixed mating system the genetic diversity changes with time (Loveless and Hamrick, 1984), often by selective pressures from ecological conditions (Lloyd, 1974).

Chapter 9 Genetic marker for non-prickliness in *S. centrale*

9.1 Introduction

Some genotypes of *S. centrale* display prickles, which are extensions of some of the epidermal cells (Raven and Johnson, 2001). They range in length from one to five mm, if present occur on the stems and petioles, but never the leaves or flowers (Figure 9.1). It is not known whether the presence or absence of prickles confers an ecological advantage, but for the populations examined in the present study, prickly plants were about as abundant as non-prickly ones.

From a commercial aspect, prickliness in *S. centrale* is an undesirable trait because the prickles are a problem during plant handling and fruit harvesting, and scratches to the skin can become infected (A. Beal pers. comm.). The identification of a DNA marker to identify genotypes that are prickly or non-prickly could be used in a breeding program to select superior non-prickly cultivars.

Bulked segregant analysis (BSA) is used to identify genetic markers linked to specific genes of interest and was originally suggested by Michelmore *et al.* (1991). The main prerequisite is a population of plants that clearly segregate for the trait in question. Specific genomic regions are targeted against a random genetic background of unlinked loci (Williams *et al.*, 1990) when BSA is used with the RAPD-PCR technique no prior knowledge of the genomic DNA is required. This is in contrast to other procedures such as the development of near isogenic lines (Young *et al.*, 1988), chromosome walking (Michiels *et al.*, 1987) and chromosome jumping (Rommens *et al.*, 1989).

BSA has been successfully used in a number of breeding programmes to identify useful markers including, resistance against *Melampsora larici populina* in *Populus* (Cervera *et al.*, 1996; Villar *et al.*, 1996), various genetic linkages to genes of economic interest in *Prunus* (Chaparro *et al.*, 1994; Warburton *et al.*, 1996), stem growth index markers in radiata pine seedlings (Emebiri *et al.*, 1997), scab resistance in *Malus* (Koller *et al.*, 1994; Markussen *et al.*, 1995; Cheng *et al.*, 1996), pendula

gene in *Picea* (Lehner *et al.*, 1995), black leaf spot resistance *Ulmus* (Benet *et al.*, 1995) and sex determination in *Pistacia* (Hormaza *et al.*, 1994).

In this Chapter, samples of bulked DNA obtained from individuals displaying either prickliness or non-prickliness were used to identify a genetic marker linked to this character.

Figure 9.1 Top: *S. centrale* seedling showing prickly phenotype. Bottom: *S. centrale* seedling showing non-prickly phenotype.

9.2 Materials and Methods

9.2.1 Plant material

Seeds of *S. centrale* collected from Utopia Station, Northern Territory were grown at the University of Adelaide and the seedlings were found to segregate for prickliness. Twelve non-prickly plants and 12 prickly plants were visually identified and selected to use for BSA. Also as part of this study, seedlings of each type were cross-pollinated and grown to reproductive maturity. Fruit resulting from these crosses was collected and the seed germinated to observe the progeny.

9.2.2 DNA extraction

The preparation of DNA and determinations of concentrations and purity were performed following the methods described in Chapter 8.

9.2.3 Preparation of bulked DNA samples

DNA samples from 12 plants showing a high degree of prickliness were diluted to 20 ng/μL and mixed in equal proportions. A non-prickly bulk was formed in the same way from the DNA of 12 plants showing a complete absence of prickliness.

9.2.4 Screening primers

The two samples of bulked DNA were screened for polymorphisms between the prickly and non-prickly DNA bulks using 120, 10-mer oligoribonucleotide primers of arbitrary sequence obtained from kits OPA, OPB, OPC, OPD, OPF and OPZ (Operon Technologies, Almeda, CA, USA). The PCR mixture and amplification conditions were as described in Chapter 8, using a total of 40 ng/μL of the bulked DNA. The amplification products were separated on 1.75% (w/v) agarose gels (Seakem®GTG®agarose, FMC, Bioproducts, Rockland, Maine, USA) run in TBE buffer at 100 mA, and inspected after staining with ethidium bromide.

9.2.5 Confirmation of marker in progeny

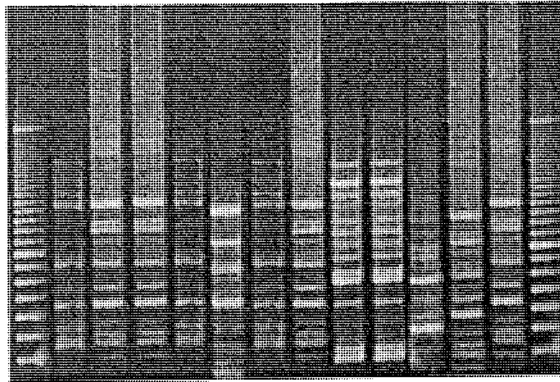
When a polymorphism was detected between the two bulks, it was confirmed by running two additional PCRs before further analysis. If the polymorphism was consistent, the primer was tested with the DNA from each individual that comprised

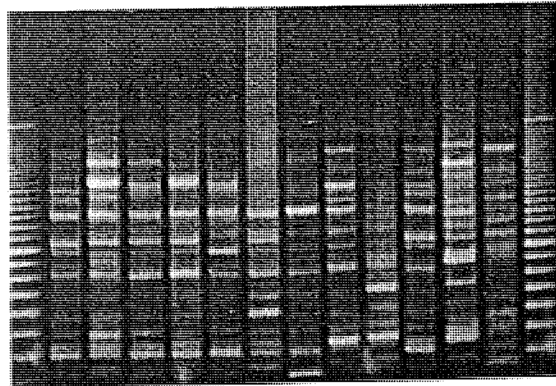
the bulked DNA samples, and 20 of the progeny that resulted from the cross-pollinations of prickly and non-prickly plants.

9.3 Results

The 120 10-mer primers produced an average of 11 bands per primer, with 6 primers giving no amplification products. The size of fragments ranged from 300 to 1600 bp. Putative evidence of a marker linked to prickliness was assumed when a reproducible PCR fragment occurred with one sample of bulked DNA but not with the other, and this was found for primers OPD-07, OPD-14, OPD-15, OPC-20, OPC-17, OPF-13, OPA-09, OPA-11, OPD-10 and OPD-19. When these primers were tested separately on each of the 12 prickly and 12 non-prickly individuals that made up the bulks, the fragment produced by OPD-15 (5'-CATCCGTGCT-3') was present in all non-prickly individuals and absent in all except one prickly individual (Figure 9.2). The results obtained for the other primers indicated that they were not reliable as markers.

Figure 9.2 PCR products obtained after amplification of the DNA from 12 non-prickly *S. centrale* individuals (lanes 2-13) and 12 prickly *S. centrale* individuals (lanes 16-27) with primer OPD-15. The RAPD fragment linked to the non-prickly character is located at about 680 bp. Molecular-weight markers (100-bp ladder, GIBCO-BRL) in lanes 1, 14, 15, 28.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
680 bp


15 16 17 18 19 20 21 22 23 24 25 26 27 28
680 bp

9.4 Discussion

The technique of bulked segregant analysis (BSA) combined with RAPD-PCR was used to identify a genetic marker linked to the trait of prickliness in *S. centrale*. The segregation of this character was identified in plants raised from seeds collected from Utopia Station, in the Northern Territory of Australia. Prickliness in commercial crops of *S. centrale* is considered to be undesirable, and the use of a molecular marker to identify the presence of the gene, either in seeds, or in very young plants before the character is expressed, would be a valuable aid to the improvement of this species.

Bulked segregant analysis overcomes several problems when identifying markers linked to particular genes. There is a minimal chance that regions unlinked to the target region will differ between the bulked samples of many individuals (Michelmore, *et al.*, 1991). Morphological traits can be controlled by a single gene (or a few major genes) and the comparison of bulks of the extreme individuals can rapidly identify markers linked to that trait (Michelmore, *et al.*, 1991). A total of 120 10-mer primers were screened using two bulked DNA samples and, although several of these appeared to be promising, further testing confirmed the reliability of only one of them, namely OPD-15. The marker was about 680 bp and was present in 100% of non-prickly individuals, and absent in 96% of the individuals that showed prickliness. Over the 44 individuals that were used in this program, 60% displayed the marker for the absence of the prickly character.

RAPD markers are reported to be difficult to reproduce between workers and laboratories (Jones *et al.*, 1997) and the production of a sequence tagged site (STS) can improve the reliability of the marker (Mekuria *et al.*, 2002). The conversion of the RAPD marker for non-prickliness to an STS marker is the subject of Chapter 10.

Chapter 10 Sequence tagged site for the RAPD marker linked to non-prickliness in *S. centrale*

10.1 Introduction

In Chapter 9, a RAPD band of about 680 bp was found to be linked to non-prickliness in *S. centrale.* Generating markers linked to traits of interest using the RAPD-PCR technique and BSA is effective as there is no requirement for sequence information of the species studied, and developing markers is time efficient. However, one of the problems associated with this technique is that RAPD markers can be difficult to reproduce reliably (Jones *et al.*, 1997). Hausner *et al.* (1999) also suggest that a single RAPD band may consist of several similar sized DNA fragments each with a different sequence, while polymorphisms are identified usually as present or absent without regard to the intensity of the band.

To improve the use of RAPD markers in breeding programs, longer more specific primers can be developed from the sequences of the RAPD fragments creating sequence-tagged sites (Olsen *et al.*, 1989), also referred to as sequence characterised regions (SCARs) and were developed by Paran and Michelmore (1993). This Chapter describes the conversion of the RAPD marker for the non-prickly trait into an STS marker.

10.2 Materials and Methods

10.2.1 Isolation and purification of RAPD fragment

The 680 bp RAPD fragment was excised from an agarose gel using a surgical blade (Swann-Morton, England) and purified using the QIAquick Gel Extraction Kit (QIAGEN®, Roche Molecular Systems, US). The section of gel was weighed, and three volumes of Buffer QG were added to 1 volume of gel (100 mg was assumed to have a volume of approximately 100 μL). The mixture was incubated at 50°C for 10 min (or until the gel was dissolved), and one gel volume of isopropanol was added to the sample and mixed. The sample was applied to a spin column and centrifuged for 1 min. The flow-through was discarded and 0.5 ml of Buffer QG was added to the spin

column and centrifuged for 1 min. The spin column was washed with 0.75 ml of Buffer QG, centrifuged for 1 min, and the flow-through was discarded again. Thirty μL of 10 mM Tris-Cl, pH 8.5 was added to the centre of the membrane and DNA collected by centrifuging for 1 min.

An aliquot of the purified DNA was reamplified using the same primer and PCR reaction conditions used previously and the presence of a single band of 680 bp was confirmed by separation on 1.75% agarose run in TBA buffer.

10.2.2 Cloning and transformation of DNA

The reamplified PCR product was cloned into the plasmid vector pGEM-T Easy using the pGEM®-T Easy Vector System I (Promega Corporation, Madison, WI, USA). Cloning was carried out using 3 μL of reamplified PCR product, 1μL of pGEM®-T Easy vector, 1 μL of T4 DNA ligase and 5 μL of 2 x Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, and 10% polyethylene glycol). The reagents were gently mixed and incubated at 4°C overnight.

The cloning reaction was diluted in 100 μL of 1 x TE buffer and then added to 200 μL of competent cells (made competent by CaCl method – see Appendix), mixed gently and incubated on ice for 30 min. The cells were heat shocked for 2 min at 42°C, and put back on ice. 1 ml of LB agar (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, bacteriological agar No.1 (1g/100ml)) was added and the cells were incubated at 37°C for 60 min.

LB agar (bacteriological agar No. 1 (1 g/100 ml), 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) was autoclaved at 121°C for 20 min, cooled to 50°C, made to 50 mg/ml with ampicillin, poured into 9 cm Petri dishes, and stored at 4°C in the dark. Just prior to use, the agar was coated with a mixture of 16 μL of 50 mg/ml X-Gal (Promega®, USA) and 33 μL of 25 mg/ml isopropylthiogalactoside (IPTG, Promega®, USA) to achieve blue/white colony screening of the recombinants, and allowed to dry at 37°C. After the transformation process was completed, the plates were spread with 100 μL of cells and incubated overnight at 37°C.

10.2.3 Mini plasmid preparation

Plasmid DNA was prepared using a standard alkaline extraction protocol (Promega Protocol and Application Guide, 1991, Promega Corporation, Madison, USA). Several white colonies were selected and transferred to 10 ml screw cap tubes containing 3 ml of LB broth and 3.0 μL of 100 mg/ml ampicillin. The tubes were placed on a rotating wheel and incubated at 37°C overnight.

Each bacterial culture was centrifuged for 5 min at 3000 rpm, and the supernatant removed from the tube, leaving the pellet as dry as possible. The pellet was suspended in 90 μL of GET (25 mM Tris HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose) by vortexing, mixed with 180 μL of freshly made 0.2 M NaOH/1% SDS solution by gentle inversion, and then 130 μL of 3M KAc, pH 4.6, was added and again gently inverted. The mixture was centrifuged at 14 000 rpm for 15 min, the supernatant transferred into a fresh tube containing 2 μL of RNase A (10 mg/ml, DNase-free) (AMRESCO®, Solon, OHIO, USA), and incubated at 37°C for 30 min.

400 μL each of tris-saturated phenol and chloroform were added, vortexed for 10 sec, and centrifuged at 14 000 rpm for 5 min. The supernatant was carefully removed and transferred to a new tube, and the DNA was precipitated with 2.5 vol of cold ethanol (-20°C). After mixing, the solution was left at room temperature for 10 min and centrifuged at 14 000 rpm for 15 min. The supernatant was carefully removed, the pellet washed with 100 μL of cold 70% ethanol (–20°C), centrifuged at 14 000 rpm for 5 min, and the supernatant again carefully removed. The pellet was dried on a heating block for 3 min at 50°C, dissolved in 30 μL of 0.1 mM EDTA, pH 7.0, and stored at -20°C.

The presence of an insert was confirmed by digestion with *Eco*R1. The reaction mixture consisted of one μL of plasmid DNA, 6.95 μL of sterile water, 2 μL of 10x universal buffer (1 M Tris acetate, pH 7.8, 5 M KAc, 1 M MgAc, 0.1 M spermidine, and 0.1 M dithioerythritol) and 0.05 μL of *Eco*R1 (40U/μL) (Boehringer, Mannheim, GmbH, Germany). The mixture was incubated for 1 hr at 37°C and subjected to electrophoresis on 1.5 % agarose in TBE buffer. Plasmid DNA containing an insert of the expected size was then prepared for sequencing.

10.2.3 Preparation of plasmid DNA for sequencing

The dideoxy terminator sequencing kit of Applied Biosystems was used to perform PCR on plasmid DNA. The reaction mixture contained 4μL of the terminator ready reaction mix (Series 3), 13.9 μL of sterile water, 2μL of plasmid DNA, and 0.1 μL (100 ng/ml) of either:

M13 forward primer (5'-TGTAAAACGACGGCCAGT-3') or,

M13 reverse primer (5'-CAGGAAACAGCTATGACC-3'),

The following program was used for amplification: 24 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

After amplification, the PCR products were mixed with 80μL of 75% isopropanol, vortexed, and left at room temperature for 15 min. The mixture was centrifuged for 20 min at 14 000 rpm and the supernatant was carefully removed and discarded. 250 μL of 75% isopropanol was added, vortexed, centrifuged for 5 min at 14 000 rpm and the supernatant again carefully removed. The pellet was dried at 50°C for 1 min and stored at -20°C. Sequencing was carried out at the IMVS Sequencing Centre, Molecular Pathology, Frome Rd, Adelaide, South Australia.

10.2.4 Design and analysis of sequence specific primers

A sequence specific primer pair was designed using PRIMER v. 0.5 (Whitehead Institute for Biomolecular Research, USA) and the primers were synthesised by Proligo Australia Pty Ltd.

The primer set was screened using individuals from the population of *S. centrale* segregating for prickliness (Section 9.5). The reagents for PCR were as described in section 8.2.3, using the new primers at a concentration of 0.25 μM. The following program was used to perform amplification: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 1 min, with a final extension of 10 min at 72°C. The PCR products were separated on 1.75% agarose gels in TBE buffer at 100 mA, and visualised under UV light after staining with ethidium bromide.

Verification of the sequence tagged site was carried out on all 24 individuals used to make the two bulked DNA samples tested with the 10-mer primer OPD-15 and the 20 progeny derived by crossing prickly and non-prickly parents.

10.2.5 Test of sequence homology

Sequences were aligned using ClustalX (Thompson *et al.*, 1997) and edited with BioEdit Sequence Alignment Editor version 4.8.1 (Northern Carolina State University, USA).

10.3 Results

10.3.1 Isolation and purification of RAPD fragment

Purification and reamplification of the RAPD marker linked to the non-prickliness trait in *S. centrale* produced a single band of about 680 bp. Five of these fragments were purified from separate PCR reactions and used for cloning.

10.3.2 RAPD fragment cloning and sequencing

The five purified RAPD fragments were cloned into pGEM®-T Easy vector, and three white colonies were selected from each of the five LB plates to produce 15 mini-plasmid preparations. After digestion with *Eco*R1, five positive recombinants were found, the remainder being false positives (Figure 10.1). The insert was detected after separation on agarose gels.

10.3.3 Sequence specific primers analysis

The full sequence of the RAPD marker was 678 bp (Figure 10.2), and one forward and one reverse primer were designed from the sequence to develop the STS marker. The sequences of these primers are:

Forward primer 5'-AGTATGGGAAGCACAATCCG-3', located between bases 14-33, and

Reverse primer 5'-AAAAAAGGTCCTTTTCTTTGGG-3', located between bases 452-431.

The expected size of the product amplified by this primer set would be 438 bp, that is, 240 bp shorter than the original RAPD marker.

When the primers were tested on individuals from the segregating population for prickliness the expected product of 438 bp was found in 11 of the 12 progeny designated as non-prickly based on morphology. It was also present in 1 of the 12 progeny designated as prickly (Figure 10.3).

10.3.4 Comparison of RAPD and STS sequences

STS fragments amplified from the genomic DNA of plants 1, 3 and 6 were purified, cloned, and sequenced following the protocols described in Section 10.2. After aligning the sequences of the STS and RAPD markers (Figure 10.2), it was found that the overlapping segments showed a homology of 95%. The discrepancy was due to eight base pairs spread throughout the STS sequence that were not detected in the RAPD sequence (Figure 10.2).

Figure 10.1 The RAPD marker of about 680 bp after digestion with *Eco*R1 separated on 1.75% agarose. Lanes 1 + 7 – 100 bp ladders, Lanes 2-6 – recombinant DNA showing insert of about 680 bp.

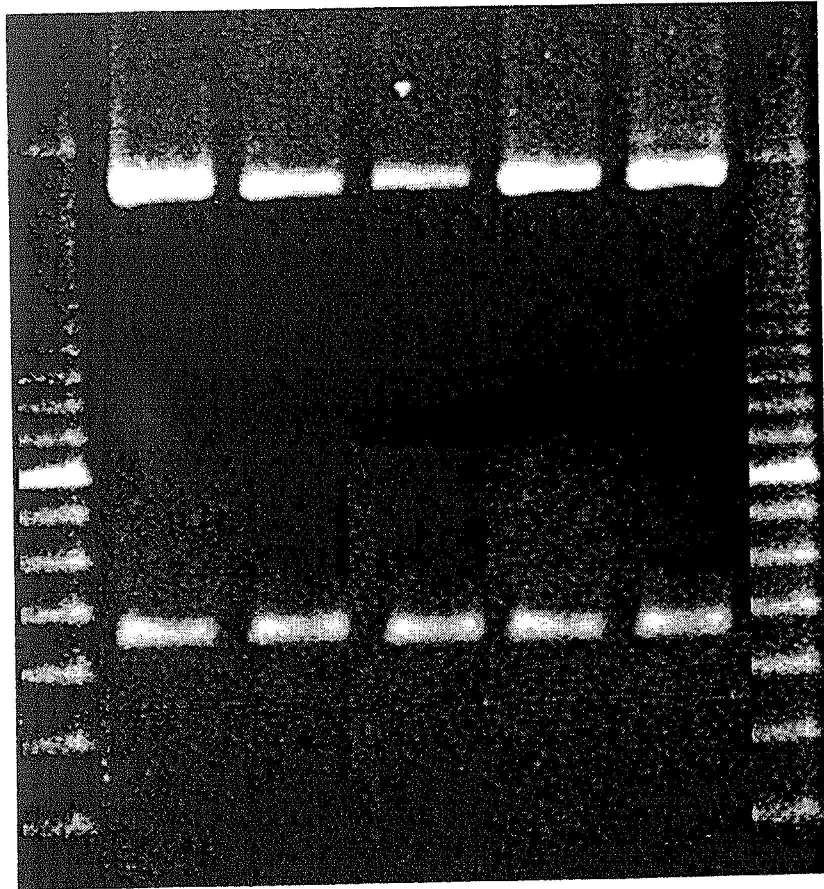
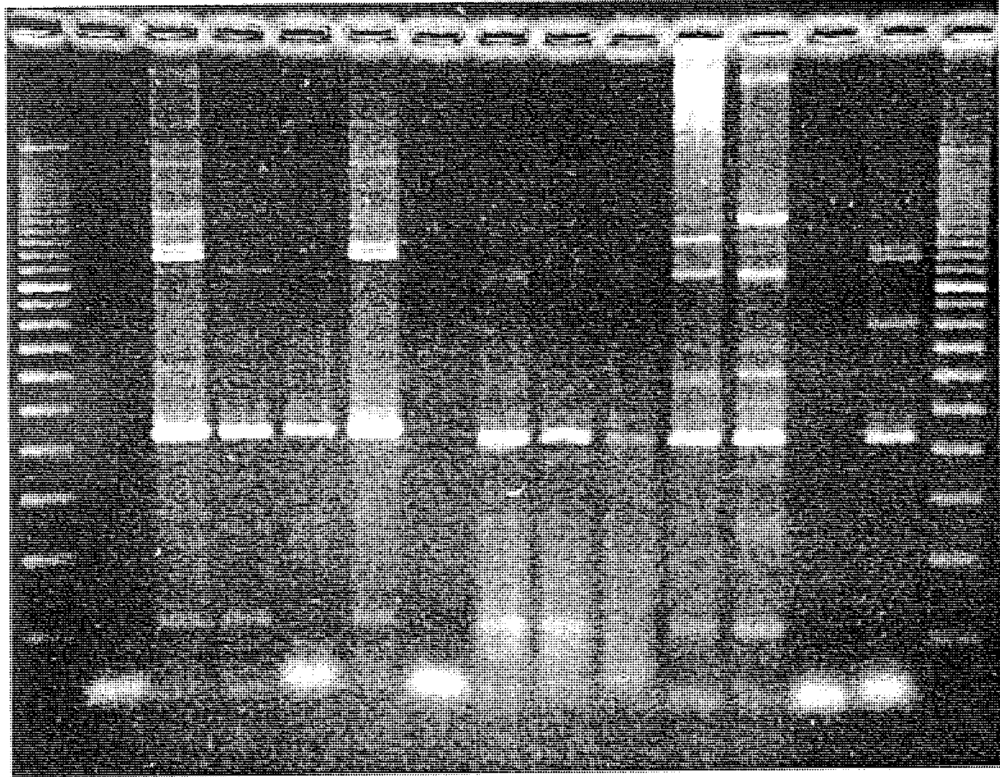

1 2 3 4 5 6 7
680 kb

Figure 10.2 Homology between the sequences of the STS marker and the original RAPD fragment.

```
                    10        20        30        40        50        60        70        80        90       100       110
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RAPD       CATCCGTGCTACAAGTATGGGAAGCACAATCCGAGAGATTGTTTGNCCNCTTCNAGTGTATGTTTAGAGTGTGGTGCAAAAGAACACGAGTTTAGACATTGTCCATGGGT
STS        -------------AGTATGGGAAGCACAATCCGAGAGATTGTTTGGCCAGTTTGAGTGTATGTTTAGAGTGTGGTGCAAAAGAACACGAGTTTAGACATTGTCCATGGGT
Clustal Co

                   120       130       140       150       160       170       180       190       200       210       220
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RAPD       TACTAAGGATTATGAGGATGACCGTTGACGTTCTCAACCATACCTCTTTATGGGTCACTAGACTTGGAGTAATGCTTCATAGGTATGTTAAAGGTTTTTGATTTTGAGGN
STS        TACTAAGGATTATGAGGATGACCGTTGACGTTCTCAACCATACCTCTTTATGGGTCACTAGACTTGGAGTAATGCTTCATAGGTATGTTAAAGGTTTTTGATTTTGAGGT
Clustal Co

                   230       240       250       260       270       280       290       300       310       320       330
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RAPD       ATATGCATTGATAGACCCAGGNCACCACTTTTTCTTTTATTACTNCTTTTGTNGCCAAAAGCTTTATGTNTAGTCCCGAATTGNTGTGNGAGCCTTATAAAGNGTCTACC
STS        ATATGCATTGATAGACCCAGGT-ACCACTTTTTCTTTTATTACTCCTTTTGTTGCCAAAAGCTTTATGTT-AGTCTCGAATTGTTGTGTGAGCCTTATAAAGTGTCTACC
Clustal Co

                   340       350       360       370       380       390       400       410       420       430       440
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RAPD       CTNATTGNTGAGTTCCATTGTTGCTAGAAGAGTCTATNAGAAATTGTCT-ATGTTCATTTTGCATAAAATCTTTCCTTGTAATTTTATTGGTGCGCAAGTGCCCAAAGAA
STS        CTTATTGGTGAGTTC-ATTGTTGCTAGAAGAGTCTATA-GAAATTGTCTCATGTTCATTTTGCATAAAATCTTTCCTTGTAATTTTATTGGTGCGCAAGTGCCCAAAGAA
Clustal Co

                   450       460       470       480       490       500       510       520       530       540       550
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RAPD       AAGGACCTTTTTTGCCACTTGTCAAGGGTATTTTAAGAAAGATCAATGAATGTGTTTGGCGGGAATTTCAGATTGTTTCTAGAGTGATGGTTTTAGGCATAGGATGAGGG
STS        AAGGACCTTTTT--------------------------------------------------------------------------------------------------
Clustal Co

                   560       570       580       590       600       610       620       630       640       650       660
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RAPD       ATTTGCTCGGTGGCCTCAATGAAGGGAGACGAAGGGTCCACATTGAATGTAGCAATTGTAGGAGGACTCGCCCTAGGTGCCCAAAATGTGGAAGAAATCATAATAACACA
STS        --------------------------------------------------------------------------------------------------------------
Clustal Co

                   670
           ....|....|....|....
RAPD       TGTCTGAGGAGCACGGATG
STS        -------------------
Clustal Co
```

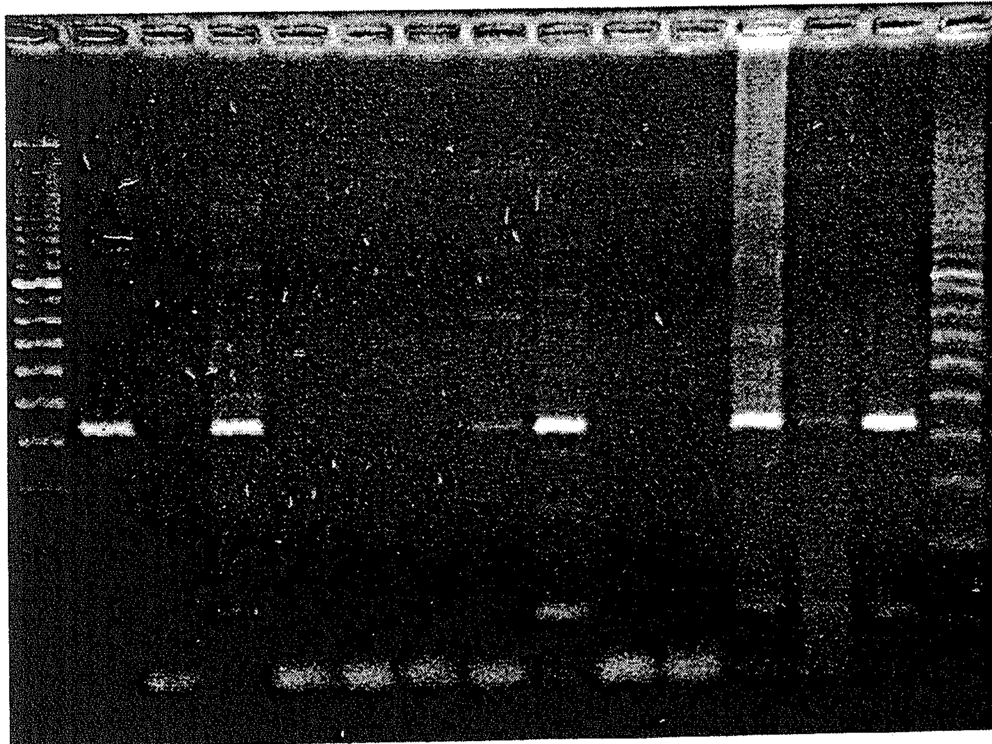
Figure 10.3 Using the designed forward and reverse primers genomic DNA was amplified and separated on 1.75% agarose. The non-prickly marker is the fragment of about 440 bp. Lane 1, 15, 16, 30 – 100 bp ladder, lane 2 - control, lanes 3-12, 14, 15, 17, 22, 25 – non-prickly progeny, lanes 13, 16, 18-21, 23-24, 26 - prickly progeny, lane 29 – original individual used to isolate, clone and sequence the fragment.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



480 bp

16 17 18 19 20 21 22 23 24 25 26 27 28 29 30



480 bp

10.4 Discussion

Molecular markers based on DNA sequences are the ideal means of identifying genotypes and following inheritance of economically important characters. STS markers are designed to identify a specific location on a chromosome, and are generally preferred to RAPD fragments because the primers are larger and more specific, and the products are more reproducible between laboratories (Olson *et al.*, 1989). These markers are inherited in a co-dominant fashion and are not affected by environment. This makes them suited for tagging characters of importance and following their inheritance for use in breeding programs and solving production problems (Vithanage, 1995). Some STS primers developed in one species maybe effective in detecting polymorphism in other related species (Rajapakse, 2001).

A RAPD marker of about 678 bp linked to non-prickliness was identified with the 10-mer primer OPD-15. This Chapter involved the isolation and cloning of the 678 bp marker and the production of an STS marker that could be used as a selection tool in future breeding programs involving *S. centrale*.

The method used to isolate and reamplify the RAPD fragment from agarose consistently produced one band of the expected size. The band was cloned and sequenced, and one pair of sequence specific primers was synthesised from the sequencing results. Primers designed by the PRIMER computer program were successful in amplifying a product that showed a consistent polymorphism for non-prickliness. The STS marker was subsequently cloned and sequenced and found to be 8 bp longer than that of the original RAPD fragment. Mekuria *et al.* (2001) also found small differences between a RAPD marker and the STS marker derived from it.

Twenty *S. centrale* individuals produced from crosses of the 24 individuals used in the bulks were used to test the designed primers. Based on a visual assessment of gels 92% of the non-prickly segregating progeny produced the STS marker and 100% of the other non-prickly individuals used in the bulk, while 95% of individuals from the crosses and the bulks showing prickliness did not produce the STS marker. The STS primers produced markers the same as OPD-15 for all of the individuals tested. One

individual that was visually assessed as being prickly produced the marker band with the STS primers and OPD-15. This may have been due to an experimental error such as a labelling mistake or the possibility of more then one gene being involved in the expression of non-prickliness.

Other products were amplified during PCR with the STS primers in addition to the expected product. Reasons for this include non- specific binding, incomplete extension by the polymerase (Love *et al.*, 1990), and slippage events during the PCR replication process, (Tautz, 1989).

Finding markers linked to morphological traits has been successfully used in a number of other horticultural crops such as: peach and pear (Hayashi and Yamamoto, 2002), macadamia (Vithanage *et al.*, 1995), grape (Thomas *et al.*, 1993), citrus (Kijas *et al.*, 1993) and rice (Monna *et al.*, 1994). Very little research in horticulture has been done on establishing genes linked to prickliness. However, a gene controlling the prickles in roses was located using similar techniques described in this study (Rajapakse *et al.*, 2001). This information provides initial tools for marker selection and gene introgression for the improvement of these commercial crops.

From the sequence of a RAPD marker linked to non-prickliness in *S. centrale* an STS marker was developed using sequence specific primers. The marker was present in 96% of non-prickly individuals and absent in 95% of prickly individuals. Future breeding programs can benefit from this marker, as it will accelerate the selection process for non-prickly individuals, which is desirable for the commercial development of the bush tomato industry in Australia.

Chapter 11 General Discussion

Isolation and Australia's unique landscapes and climates have had a large impact on the development of the native flora. For thousands of years Aboriginals utilised the native flora as a food source, and in recent years, 'bushfoods' have become something of a novelty in modern Australia.

The development of the bushfood industry offers a number of potential benefits such as an impact on the conservation of a species in some areas and a commercial supplement to marginal agricultural enterprises, both irrigated and dryland. There is also the potential to offer Landcare groups, Aboriginal communities, farmers, bush regenerators, and local councils, economic returns on revegetation programs with incentives to preserve wild populations.

Despite this, the reliance on wild populations presents a problem in the expansion and growth of the industry. There are potentially detrimental effects on species diversity and habitat due to selective or excessive harvesting. Moreover, the high cost of collecting produce, seasonal variability, and varying product quality inhibit commercial growth. To conserve the environment and provide improved consistency, quality and a reliable supply of product, production methods need to be developed.

Solanum centrale, the Australian bush tomato is an endemic plant (Latz, 1995) with potential as a commercial bushfood. This species is showing promise in both domestic and overseas markets as flavouring in value-added products (Graham and Hart, 1997). However, unreliable and variable supply of wild bush tomatoes limits the crop potential. To deal with this problem commercial plantations in Australia have been established to meet market demands. The establishment of these plantations has highlighted a number of domestication problems associated with commercial production.

This research aimed to assess the horticultural management of this species and factors influencing their productivity. One key area was the evaluation of the genetic diversity

of *S. centrale* within a single large population, and between several wild populations located in different regions of Australia. Following on from this a molecular marker for non-prickliness in the species was developed.

S. centrale is mainly found in arid, remote areas of central Australia (Black, 1934; Symon, 1981). Due to this, and the seasonal nature of growth the most accessible material on which to conduct this research was seed. Seeds were collected by various people (including the author), germinated at the University of Adelaide, and plant material used for morphological and genetic analysis.

As the bushfood industry is a relatively new industry, where the species has had very little domestication, there is a need to select for high quality and high yield. For this reason, one of the studies involved investigating the reproductive biology of *S. centrale* in terms of artificial hybridisation. Findings indicate that a factor limiting a controlled breeding program is the high levels of variability in fertility of the plants (Holsinger, 1992). This may be linked to the presence or efficiency of pollinators, and future research to determine whether availability of resources or pollinators is limiting fruit set may be beneficial (Stephenson, 1981; Sutherland and Delph, 1984). A step towards plant quality may be to collect seed only from high yielding plants or populations, with the ability to self-pollinate and produce large fruit. This study indicates that the species can outcross under natural and artificial conditions, suggesting that controlled pollinations could be used to generate plants with commercial potential. Another observation was the possibility of extrafloral nectaries present on parts of the flower of *S. centrale*. There is considerable potential for exploitation of extrafloral nectaries and their associated insects as biological control agents for the future (Anderson and Symon, 1985).

Steroidal alkaloids have been found in the tissues of the species of the genus, *Solanum*. Toxic in the human diet (Hall, 1992) it has also been suggested that any species of *Solanum* should be considered potentially poisonous unless determined otherwise (Kingsbury, 1964). For this reason, another area investigated in this project was the presence of steroidal alkaloids in the leaves and fruit of this species. A number of methods of extraction and analysis were tested with varying results. Using

these techniques, based on previous work undertaken on other *Solanum* species, compounds were observed but not identified. There is a need to improve clean-up procedure to aid in the analysis of the alkaloids present. Further work into identification and quantification of specific alkaloids found in the fruit of *S. centrale* is required for food safety reasons.

DNA fingerprinting analysis, using the RAPD technique has many applications including molecular markers useful for selection strategies (Brady *et al.*, 1996). The benefits of the RAPD technique are the small amounts of DNA required, ease of application and the advantage that markers can be visualised without the need for labelled radioisotopes. However, unless reaction conditions are rigidly standardised, reproducibility of results can be limiting when using RAPDs.

When using selection criteria for crop improvement, the broader the genetic diversity, the more likely it is that significant gains are made. The extent of genetic variability within an isolated population of *S. centrale* was explored. The population (100 plants in total) were collected from Utopia Station, Northern Territory.

Between the 100 *S. centrale* individuals, the RAPD data revealed a high level of genetic diversity and no two individuals had the same fingerprint. The genetic similarities found between the 100 individuals from one population varied from 72% to 95%, confirming the existence of high genetic diversity in the gene pool. As *S. centrale* is a relatively new crop, no previous genetic diversity study has been conducted using wild populations in Australia. Maguire and Sedgley (1997) and Huff *et al.* (1993) concluded that a high level of diversity is a feature displayed by plants that are predominantly outbreeding. *S. centrale* appears to be predominantly outcrossing and it was expected that gene flow would be random. This was confirmed by the absence of significant molecular clustering.

The size of the breeding population in any breeding program has a large effect on the identification of individuals that are superior to the parents. In the study of ten different populations sourced from around Australia, 10 significantly different molecular clusters were identified. Each of these clusters corresponded with the ten

different locations indicating that dispersal of pollen and seed between the sites is limited. There is a needed to improve the knowledge of gene flow in *S. centrale* by examining more wild populations and assessing a wider range of morphological characters. Future research aimed at identifying a subset of the most promising individuals to develop *S. centrale* fruit of high quality and to uniquely adapt the species to different environments would be beneficial.

Genetic variation was compared to morphological data collected from the same *S. centrale* individuals and populations. Examination of the eight morphological characters from vegetative and floral features revealed a high degree of morphological variation. This variation corresponded with the genetic variation found, however there were some difference in individual groupings. Other studies have also observed this difference (Prentice, 1996; Heaton *et al.*, 1999). Two possible reasons for the difference between morphological and molecular data could firstly be that unlike DNA, morphology is susceptible to environmental conditions, such as a difference in habitat (Wolff *et al.*, 1995; Black-Samuelson, *et al.*, 1997; Brunell and Witkus, 1997; Fanizza *et al.*, 1999). Secondly, there may be differences in the mode and rate of change between morphology and genetics, indicating that change may proceed differently under similar selective pressure (Davis and Gilmartin, 1985; Quicke, 1993; Krauss, 1996).

While commercial plantations are often established using seedlings, propagation from seeds of selected superior plants can lead to undesirable variation in the progeny. Vegetative propagation is ideal for the clonal production of superior plants. Initial studies undertaken in this project indicate that this would be a viable and efficient method.

By adopting molecular markers for marker-assisted selection, breeding programs can be significantly improved with accelerated outcomes. This study focused on the identification of molecular markers linked to prickliness in *S. centrale* using two approaches, firstly using RAPD-PCR, and secondly developing a sequence-tagged site for prickliness. Individuals that showed high prickliness or no prickles in the Utopia Station population were selected, and one RAPD marker, using OPD-15 was

identified as a potential marker for non-prickliness. A marker for non-prickliness was detected in 100% of the non-prickly plants tested. Thus, the marker linked to non-prickliness has considerable potential to use in marker-assisted selection for breeding and improvement of *S. centrale.*

The RAPD marker for non-prickliness was converted to an STS to obtain a specific marker that could be easily and widely used in breeding programs. To achieve this the RAPD fragment was cloned and sequenced and specific primers were designed from the sequence. The STS marker was present in 96% of the plants assessed as non-prickly based on morphology.

For future *S. centrale* improvement programs in Australia, the STS marker linked to non-prickliness could be used as a selection tool. This tool provides an efficient way of identifying individuals possessing the prickliness trait. Other markers that may be the focus for future research include fruit colour, fruit aroma and flavour, powder yield, and total soluble solids in the fruit.

The improvement and production of *S. centrale* is important for the future success of this species in the horticultural industry. This research program has contributed to this development through addressing important issues linked to propagation, food safety, breeding mechanisms, diversity and a marker linked to a desired trait. There is now sufficient information to embark on a breeding and selection program for *S. centrale* with a high expectation of success. In addition, the research has indicated how current production protocols can be improved. Considerably more domestication work is needed to increase competitiveness, and continued improvement of this crop will determine its role as a food source in the future.

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Appendix

Observation of pollen tubes using fluorescence microscopy

1. Fixation:	Carnoy's fluid	for minimum of 2 hrs
	(Absolute alcohol	6
	Chloroform	3
	Acetic Acid	1)
2. Hydration:	70% alcohol	for minimum of 10 min
	30% alcohol	for minimum of 10 min
	Distilled water x 2	for minimum of 10 min
3. Softening:	0.8 N NaOH at 60°C	for approx. 1 hr
4. Staining:	0.1% W>S> aniline blue in 0.1 N K3PO4 (mix overnight and filter)	for minimum of 10 min
5. Mounting:	80% glycerol and squash	

Preparation of competent cells using CaCl method

1. Inoculate single colony onto 10 ml L-broth and incubate overnight at 37°C.
2. Dilute 2 ml overnight culture into 50 ml L-broth and grow until $OD_{600} = 0.6$.
3. Pellet cells at 4 000 rpm for 10 min in precooled Oakeridge tubes.
4. Resuspend cells in ice cold 0.1 M $MgCl_2$ (25ml/tube).
5. Spin 4 000 rpm for 10 min.
6. Resuspend cells in ice cold 0.1 M $CaCl_2$ (12.5 ml/tube) and combine into one tube.
7. Incubate on ice for 20 min.
8. Spin 4 000 rpm for 10 min
9. Resuspend cells in 1.6 ml ice cold 0.1 M $CaCl_2$ + 15% glycerol
10. Aliquot 200 μl lots into cold eppendorfs, store at -80°C.