Genetic variation within *Acacia karroo* Hayne

by

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Abstract

The assessment and sustainable management of biodiversity have recently become the subject of increasing global concern. Recent advances in biochemical and molecular genetics have provided improved techniques for rapid assessment of the level and the distribution of genetic diversity; knowledge of both is a prerequisite for genetic conservation and improvement. This study involved the use of cytological and isozyme techniques to characterize the genetic diversity and the mating system of *Acacia karroo* Hayne. *A. karroo* is the most widespread acacia in southern Africa, and is a source of many products, including fuelwood, timber, forage, fencing material, cordage, medicine, tannin, dye, sweet gum and honey. As a species adapted to harsh environments, *A. karroo* has an important role in soil enrichment and protection, and in microclimatic regulation. This study was based on populations sampled across the natural range of *A. karroo*.

Based on cytological evidence from 10 populations surveyed, *A. karroo* was found to be a tetraploid, consisting of only one cytotype, with a chromosome number of $2n = 52$. The tetraploid status was further supported by evidence from the isozyme inheritance patterns, which also indicated that the species is a segmental tetraploid.

All 12 populations surveyed for allozyme variation expressed a high level of genetic diversity. Ninety eight percent of the isozyme loci were polymorphic. The total gene diversity (H_T) was 88%, higher than that reported for other plant species; the mean number of alleles per locus was 3.7. The mean genetic identity was 90% within the expectations of conspecificity, and the coefficient of gene differentiation (G_{st}) was estimated at 5%, indicating low divergence between populations. Unweighted pair-group and rooted dendrogram analyses clustered the populations into three phylogenetic groups, which may be characterized as the northern, eastern and south-central-eastern groups. The distribution of some common alleles at the shikimate dehydrogenase and alcohol dehydrogenase loci were significantly correlated with some geographical factors, viz. latitude, longitude and rainfall.

Estimates of mating system parameters in one population indicated that the species has a mixed mating system. Multilocus outcrossing rate was estimated at 0.88; single-locus outcrossing estimates were heterogeneous, ranging from 0.53 to ≥ 1.00, confirming the variable characteristics of the loci. The mean outcrossing rate, estimated using fixation indices for all 12 populations, was 0.72; the gene flow rate (N_m) was estimated at 4.47, consistent with the entomophilous pollination mechanism reported for other tree species. Gene distribution within the population studied showed no specific spatial pattern.

The results of this study suggest that the gene pool of *A. karroo* can be sampled efficiently from relatively few trees within each of the three cluster groups, in conjunction with a systematic sampling for localized alleles. The high level of within-population variability indicates a high potential for genetic improvement, while the mixed mating system ensures exchange of genes between selected trees. However, due to lack of evidence of correlations between genetic distribution and phenotypic or ecological variants, additional sampling based on morphological and adaptive traits is recommended.

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CHAPTER ONE

INTRODUCTION

1.0. Introduction

Global concern for the conservation of biodiversity is manifested in the high political profile of the topic, and in the number of activities related to conservation strategies, e.g. the organization of conferences and meetings, the involvement of many national and international institutions, and the increased level of scientific research and documentation. Despite these efforts, only a small fraction of biodiversity, is, so far, under conservation. Many ecosystems are threatened by mismanagement and adverse environmental changes.

Forest ecosystems are amongst those which are the most threatened (e.g. Newman, 1990; National Research Council, 1991; Williams, 1991). The benefits of forests and trees, both in ecological terms and as a source of forest products, e.g. timber, food, fuelwood, medicine, and chemicals, to mankind, have been highlighted by, among others, Ledig (1986), Burley (1987) and Bawa and Krugman (1991); thus, the questions currently raised do not concern the rationale for conservation, but how conservation for specific objectives can be best effected.

Currently, about five hundred tree species occur in well managed forests; even fewer (approx. 60) are included in well established tree improvement programmes (National Research Council, 1991), and the remainder are exploited without explicit consideration for their conservation.

1.1. Genetic diversity and conservation

To conserve a given species, it is essential to explore its natural range, and define its taxonomic status, genetic pattern and mating system. Burley *et al.* (1986) and National Research Council (1991) indicated that it is the lack of sufficient information, for example, on genetic structure, reproductive biology and pollination ecology for most forest tree species, especially those in the tropics, that has constrained their conservation and inclusion in breeding programmes.

Plant species, especially perennials such as trees, depend on their genetic diversity for stability and survival under ever-changing environments (National Research Council, 1991). This variability forms the base on which natural selection may act (Harper, 1977), and has been exploited ever since the first domestication by man (Libby, 1973). Recent studies characterize genetic patterns of plant populations as dynamic and complex (Namkoong *et al.*, 1980; Namkoong, 1989; Namkoong, 1991; Epperson, 1992; Hamrick, *et al.*, 1992; Loveless, 1992), emphasizing the need for an informed approach when sampling to conserve and to utilize that variation. However, initially a more hit-and-miss approach to sampling is necessary so that baseline parameters can be established. In the past most of this information was either ignored (Libby, 1973), or has been gathered through limited inventory followed by establishment of provenance trials which were both laborious and slow (Cheliak and Rogers, 1990; National Research Council, 1991). The recent developments of biochemical and molecular techniques to answer some of these basic questions has supplemented and hastened the process of information gathering (Cheliak and Rogers, 1988), reducing the time taken in planning the best sampling strategies for specific purposes.

One tree species which has been rated highly for genetic conservation and improvement is *Acacia karroo* Hayne (Filer *et al.*, 1993). The species

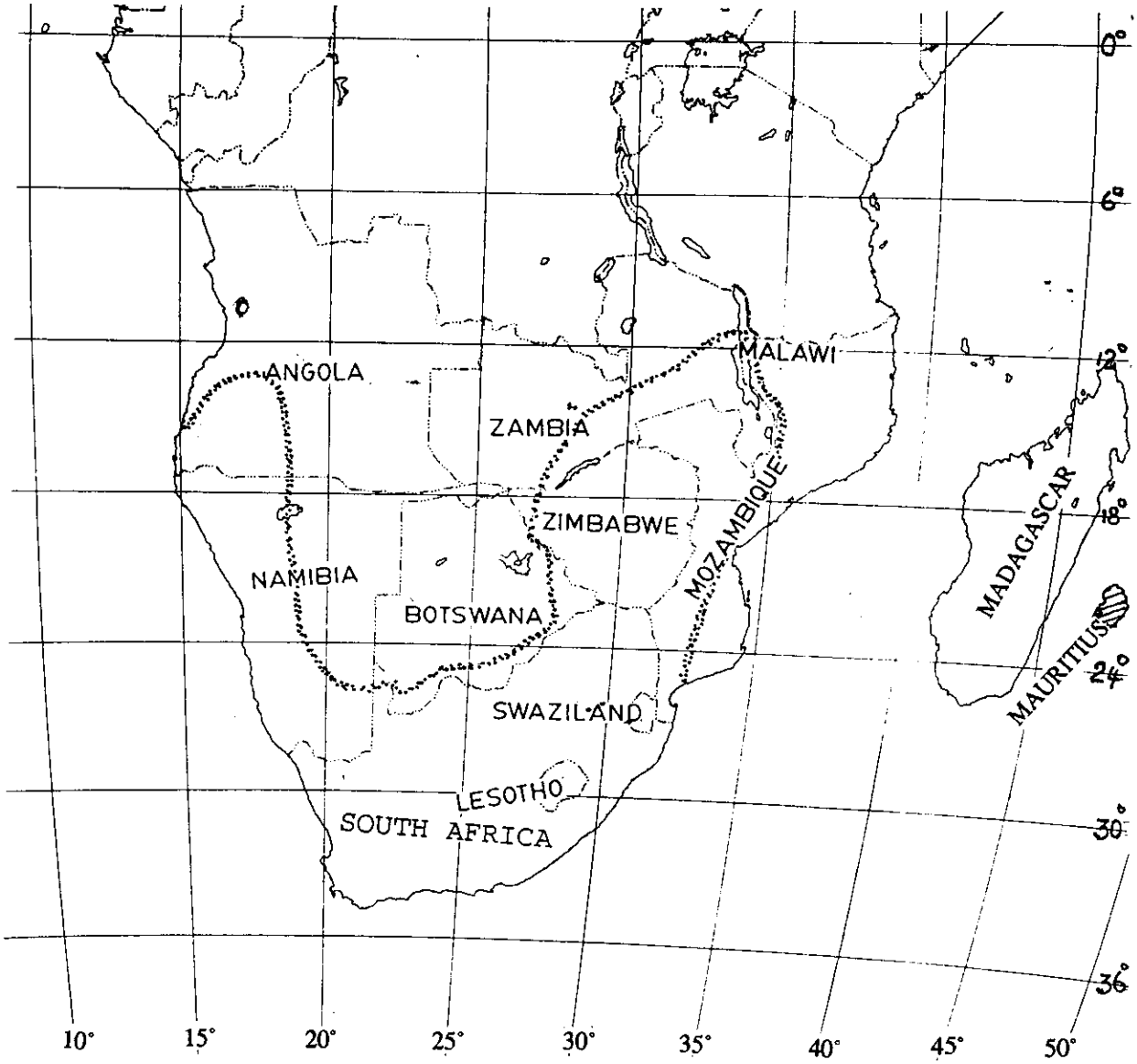
has a wide natural range throughout southern Africa over a variety of adverse conditions, providing multiple uses (Brenan, 1970; Ross, 1971a; 1979), together this implies considerable economic potential for rural development (Burley *et al.*, 1986; National Research Council, 1991). This chapter briefly describes *A. karroo* and its distribution, phenotypic variation and economic importance. Approaches to the understanding of its taxonomy and genetic diversity are summarized, and the objectives of this study are detailed.

1.2. Distribution and ecological range of *A. karroo*

According to Ross (1979) the taxon known as *A. karroo* is the most widespread acacia in southern Africa. It is distributed from latitude 12°S to 34°S, at the southern tip of Africa (Barnes, 1992). Within that area, it ranges from the eastern to the western coast, excluded only from sites which are very arid, cold and humid, or at high altitudes. Thus the species is indigenous to southern Angola, Namibia, Botswana, Zambia, Zimbabwe, Lesotho, Swaziland, Mozambique, Malawi, and South Africa, as shown in Fig. 1.1. Naturalized populations exist in the Island of Mauritius (Fig. 1.1) and in Western Australia (Scott, 1991). These populations in Mauritius and Western Australia could be some of the oldest recorded introductions in the species' history. *A. karroo* has also been introduced recently to other sites where it did not occur naturally, for example, Tietema and Merkesdal (1986) and Gwaze (1992) report of some species trials at Kgatleg District, Botswana and Matopos, Zimbabwe, respectively.

The diverse ecological conditions across the geographical range of *A. karroo* have resulted in the formation of several ecotypes (Ross, 1979). According to this study, all morphological variants of *A. karroo* could

Figure 1.1. The distribution of *A. karroo* by countries.



Northern boundary of the natural range of *A. karroo*.

Naturalized population of *A. karroo*.

only be classified as entities, of which the extreme forms are the fire-resistant shrubby type of the Nongoma district of Zululand, occasionally referred to as *A. incogflagrabilis* Gerstener, and the large trees with long spines found along the Zululand coast near Tugela Mouth, extending northwards into Mozambique. The remaining ecotypes are variants between these two extreme entities.

In its natural range, *A. karroo* occurs from sea level to an altitudinal limit of 1524 m in the Drakensburg Mountains, S. Africa (Gordon-Gray and Ward, 1975). The upper limit is determined by the frost-line. It occupies different habitats including dry thornveld, river valley scrub, bushveld, woodland, grassland, the banks of dry water courses, river banks, coastal dunes, and coastal scrub (Ross, 1979; Goldsmith and Carter, 1981; Acocks, 1988). The karroo biome as reported by Bosch and Taiton (1988) varies greatly along the temperature gradients, and the vegetation composition changes considerably within short distances depending on the topographical aspect. The species can occur in *A. karroo* savanna, or in dense, medium, short, evergreen thicket. Occasionally, it forms pure tree stands, but it is never found in dense high (tall) forests (Sim, 1906). Its ability to germinate after grass-fire and successfully develop as a pioneer or a secondary species (Weisser and Muller, 1983) has supported its existence on sand dunes, abandoned agricultural lands and grasslands.

The plasticity of *A. karroo* in response to climatic and soil conditions is well described by Acocks (1988). According to Hansley and Laker (1979, cited in Teague and Walker, 1988a), some trees of the species grow on shallow soils of very low water holding capacity, with weathering bedrock within 400 mm of the surface. A good growth performance on hot rocky banks is also documented by Sim (1906). The species is also listed among the plants which have high tolerance to arsenic soils (up to 10 000 ppm) and can be useful in revegetating mining sites (Wild, 1974).

The rainfall pattern in the vast geographic range of *A. karroo* is variable, as described by Acocks (1988). It ranges from Mediterranean type, with winter maximum rainfall, in southern and south-western coast of S. Africa, to tropical, with summer maximum in other parts where the species is distributed. The general mean annual rainfall within the *A. karroo* area ranges from 150 mm in the south-western parts of South Africa and along the fringes of Kalahari desert (Leistner, 1967) to 1500 mm in Inyanga, Zimbabwe (Barnes[1], pers. com.). The absolute diurnal temperature in its geographical range varies from 5-50°C.

1.3. Taxonomy and morphology of *A. karroo*

For a long time *A. karroo* Hayne was known by numerous confusing synonymous names, some of which were recognized as intraspecific taxa within the "*A. karroo* complex". However, Brenan (1970) concluded that the wide range of morphological variation exhibited by *A. karroo* was still not sufficient to warrant recognition of intraspecific taxa. Classical reviews of the historical changes in nomenclature of the species were presented by Ross (1971a; 1971b; 1979), in which he analyzed and reduced the numerous taxa within the complex to a single taxon. In these publications, he reported that the problems of nomenclature and taxonomy resulted mainly from phenotypic polymorphism of the species at different sites; however, the varieties could only be recognized as entities because they were linked to the "central *A. karroo* gene pool via numerous intermediate stages". Ross (1979) ultimately classified all the varieties into eight entities. The species is also known by many local names in different languages within its geographical range; the most common are those given by Sim (1906), Steadman (1925; 1933), Watt and Breyer-Brendwijk

[1] R.D.Barnes, Senior Research Officer, Oxford Forestry Institute, Oxford University, U.K.

(1962), and White (1962). These local names tend to compound the taxonomic confusion as they also recognize ecological varieties of the species by naming them differently (Gerstener, 1948, cited by von Breitenbach, 1989).

A. karroo grows variously as a multi-stemmed shrub, a slender and sparsely branched shrub, or a tree up to 22 m in height (Ross, 1979). Other morphological differences are encountered in bark characteristics, crown form, leaf size and number, pod and spine form. It is from these traits that von Breitenbach (1989) argues the error of classifying many variants, such as *A. natalitia* E. Mey, within *A. karroo*; he distinguishes *A. natalitia* from *A. karroo* by its distinctive bark patterns and colour, floral colour, pods, and spines.

However, the ranges of morphological variation, according to Ross (1979), did not warrant recognition neither as inter- nor intra-specific taxonomic categories. He subsequently grouped the variants of the species into eight entities as follows:

(1) The "typical *A. karroo*" is usually shrubby with dark rough bark and (1) 2-3 (5) pinnae pairs per leaf (for more detailed description of these variants and others that follow, see Ross (1971a; 1979) or Acocks (1988).

(2) The form referred to as *A. natalitia* is a small-to-medium sized tree with white bark, moniloform pods, 4-7 (13) pinnae pairs per leaf (von Breitenbach, 1989). It is this entity, according to Ross (1979) that extends into Zimbabwe.

(3) The small slender shrubs up to 1 m high found in the Kei River.

(4) The fire-resistant shrubby form that grows widely in Nongoma District of S. Africa (*A. inconflagrabilis*).

(5) The slender, sparingly branched trees up to 6m occuring in the Hluhluwe and Umfolozi Game Reserves ("spindle *A. karroo*").

The entities are known to posses bright reddish-brown minutely flaking bark, glaucous foliage, with or without large flattened petiolar glands.

(6) The large trees of *A. karroo* with greyish-white bark, spines up to 25 cm long, and long moniliform pods as described by Gordon-Gray and Ward (1975) distributed along the Zululand Coast, from Tugela River Mouth to Mozambique.

(7) The *A. karroo* from Pretoria eastwards characterized with sparse indumentum on the young branchlets, leaves, peduncles and pods (*A. karroo* var. *transvaalensis*).

(8) The shrubby form which resembles *A. tenuispina*, but they lack spinulose-mucronate leaflet apices and glandular pods.

Another prevailing confusion has been the differentiation of *A. karroo* from the seven glandular-podded *Acacia* species; *A. borleae* Verdoon, *A. exuvialis* Verdoon, *A. nebrownii* Burtt Davy, *A. permixta* Burtt Davy, *A. swazica* Burtt Davy, *A. tenuispina* Verdoon, and *A. torrei* Brenan. A study by Ross (1971b; 1979) revealed that these species have specific morphological features that can guide in their identification. Moreover, unlike *A. karroo*, the seven species have ecological preferences, and so their geographical locality usually assists in their identification. Despite its very variable characteristics, typical *A. karroo* retains some common morphological features such as the type of inflorescence and floral colour, which are conspicuous and can define it as a single taxon.

A. karroo is closely related to *A. seyal* Del. and *A. hockii* De Wild. but their ranges scarcely overlap. The three species are found in Zambia, Malawi and Mozambique, occupying different ecological zones (White, 1962). While the latter two species are distributed from northern Africa southwards, *A. karroo* spreads from the south northwards (White, 1962).

Hybridization is another factor which has been reported as prevalent between *A. karroo* and other adjacent *Acacia* species (Ross, 1971a; Barnes, 1992), resulting in "entities" which are closely related (Gordon-Gray and Ward, 1975; Ross, 1979; Robbertse *et al.*, 1981) and can not be easily classified as independent taxa nor as typical *A. karroo.* Unless properly identified, these hybrids can complicate the taxonomy with intermediate variable characters. Robbertse *et al.* (1981) categorized these phenotypic variants into sub-species, ecotypes, and varieties. Thus, for example, *A. tenuispina* was given varietal status as it interbreeds with *A. karroo.* Along the periphery of the species distribution, nine additional ecotypes were identified, which could be similar to those which Ross (1979) previously referred to as "entities".

Other characteristics of *A. karroo* have been used as taxonomic diagonistic features. For example, Anderson and Pinto (1980) and Anderson *et al.* (1984) observed that the composition of gum also varies among *Acacia* species, ecotypes and trees. Robbertse (1979) identified variation of wood anatomy within populations of *A. karroo.* In his work with enzyme peroxidases, Brain (1985; 1989) confirmed a geographical pattern of variation of *A. karroo* and clustered the species within the Republic of South Africa into three geographical races.

The complications surrounding the nomenclature of *A. karroo* and its related species will best be clarified by an integrated approach which is based on climatic, topographical, morphological and genetic data, analysed contemporaneously. Previous investigations have concentrated on phenotypic diversity (Swartz, 1982), with very little work examining the genetic variability (Brain, 1985; 1989).

1.4. Reproductive biology

1.4.1. Phenology

A. karroo is deciduous except in coastal and other frost-free areas where it continues to grow throughout the year (Sim, 1906; Teague, 1988a). The reported optimal day and night temperatures for growth are between 25°C and 35°C and 10°C and 15°C, respectively. The minimum threshold night temperature necessary to achieve any growth is between 3°C and 10°C (Teague, 1988a). Leaf growth is initiated at the beginning of the season (September-October) by the emergence of two to three leaves at each node as soon as there is subsoil moisture available to plants (Teague, 1988a). Shoot buds start growing when the leaves have reached half to three-quarter the full size. Each successive shoot continues to produce daughter shoots and leaves, provided that environmental conditions are favourable (Milton, 1987; Teague, 1988a). This opportunistic growth is an adaptation to take advantage of environmental changes. A fully grown branch relays growth opportunity to daughter branches by drooping down, thus cutting the apical dominance. Every growth phase is initiated using the food reserves in the form of non-structural carbohydrates (Teague, 1988b), which are quickly replenished. The subsequent growth thereafter depends on the level of available photosynthate.

Milton (1987) and Teague (1988a) concluded that the general growth pattern and the phenological cycle are not changed by the water stress, but are initiated either by temperature or day-length changes. The rate of growth initiation and development is determined by the suitability of environmental conditions. Growth initiation is usually restored and foliage production increased by defoliation, depending on the intensity of defoliation, phenophase, prevailing environmental conditions, and duration of recovery (Teague, 1988c; 1989b; Teague and Walker, 1988b).

Flowering starts in spring (October) and can be intermittently prolonged into summer (January-February), depending on the environmental conditions (Burtt-Davy, 1932; Steadman, 1933; Poynton, 1984; Teague, 1988a). Flowers are produced in the leaf axils of the newly grown shoots and are concentrated on top of the dominant and terminal half of the shoots (Coe and Coe, 1987; Teague, 1988a). According to Gordon-Gray and Ward (1975), the flowering season usually starts with involucellate flowers. These are sterile (Ross, 1971a; 1979) and different in structure from the flowers of the main capitulum. Their main function is to attract the pollinating agents and maintain them nearby to finalize pollination on the generative flowers (Fig. 1.2). The inflorescence consists of bisexual flowers, male flowers, and vestigial female flowers. Occasionally, male trees occur (e.g. Gordon-Gray and Ward, 1975), but those with only female flowers have not been reported.

Little vegetative growth continues on flowering shoots until the pods are well formed (Teague, 1988a). The trend of seed development in *Acacia* species is that the pods are not immediately filled (Doran *et al.*, 1983; Coe and Coe, 1987). The phase of development is probably delayed to protect the resources which could be consumed by animals eating low hanging pods filled with seeds, as suggested by Coe and Coe (1987). Once the pods are filled, they develop very quickly. The mature dry pods of *A. karroo* are dehiscent and have a thin leathery wall, as described by Robbertse (1975).

1.4.2. Breeding systems

Detailed information on specific breeding systems of *A. karroo* is still sparse or lacking. However, the general concept is that the species is zoomophilous. Like most other acacias with capitate inflorescences, *A. karroo* has pollen polyads consisting of 16 monads (Robbertse, 1974; Coetzee, 1955). Insects are suggested as the main pollinators (Gordon-Gray

Figure 1.2. Generative flowers and seeds of *A. karroo*.

A Generative flowers of *A. karroo*. Note the different stages of development. (Photo 3919. R.D. Barnes). **B** Mature seeds delicately attached to the pods before dispersal.

and Ward, 1975; Coe and Coe, 1987); this likelihood is strengthened by the coloration of the inflorescence and the nature of the pollen which is remarkably heavy and not well adapted for wind pollination. The presence of individual trees which are totally male indicates the tendency towards cross pollination, and the presence of more male flowers than females in a population ensures abundance of pollen. Guinet (1981) and Kenrick and Knox (1984) reported that the number of pollen grains per polyad in some acacias closely corresponds to the number of ovules per flower. The balance is maintained to ensure successful fertilization of all ovules by a single polyad (Kress, 1981). This observation was recently supported by a study of multiple paternity within pods of *A. melanoxylon* (Muona *et al.*, 1991). which reported that the probability of having full sib seeds in a pod was over 90% and about 46% in a raceme. The stigmas produce the post-pollination secretions to ensure that all pollen in a polyad develop pollen tubes. Muona (1989) notes that this type of correlated mating is possible in about 1000 species of *Acacia*.

The response to self-fertilization varies among acacia species (Arroyo, 1981). Moffett (1956) produced selfed seedlings of *A. mearnsii* which later expressed serious inbreeding depression from the early stages of growth. Recent studies in a number of Australian acacias, e.g., *A. retinodes, A. mangium, A. auriculiformis*, have also indicated variable response to self-fertilization (Kenrick and Knox, 1985; 1989; Moran *et al.*, 1992; Sedgley *et al.*, 1992). Results of isozyme studies suggest that most *Acacia* species which are self-incompatible have expressed high genetic diversity and high outcrossing rates (see Moran *et al.*, 1989a, 1992; Muona, 1989; Muona *et al.*, 1991). However, studies by Lande and Schemske (1985) indicated that most polyploid plants are self-compatible. Recently, Moran *et al.* (1992) reported a high rate of self-fertilization in tetraploids and hexaploids of *A. holosericea* A. Cunn. ex G. Don. *sensu lato* and *A. cowleana* Tate.

Elsewhere, successful selfing has been documented as a common phenomenon in the tetraploid *Leucaena diversifolia* (2n=4x=104, Brewbaker, 1987). No studies of controlled pollination have been undertaken with *A. karroo,* which is a tetraploid with 52 chromosomes (Atchinson, 1948; Darlington and Wylie, 1955; Harmant *et al.*, 1975). However, Burley *et al.* (1986) concluded that mainly self-incompatible, outcrossing species exhibit a combination of clinal variation, in response to broad climatic factors, and discontinuous variation, when distributed over disjunct environmental factors such as soils and relief. Such morphological variation is a pronounced characteristic of *A. karroo* across its range.

1.4.3. Seed dispersal and natural regeneration

The species regenerates in its natural range by coppicing and seed. Sim (1906) observed that felling of the trees at ground level encouraged coppice development, but that cutting at about 60 cm above the ground level did not favour coppicing. Natural regeneration from coppice has also been discussed by Teague (1988a). However, most regeneration of the species is by seed. On maturity, the pods dry and dehisce. After dehiscence, the seeds remain attached to the funicle until they are dispersed by wind (Fig. 1.2). Their discoid shape and size aid their transportation from the parents (New, 1984; Coe and Coe, 1987). Some of the seeds are dispersed by the animals that feed on them and on the pods (Lamprey, 1967; Milton, 1987). Seeds of most acacias are hard and when wetted with saliva, they become slippery which help them to survive crushing molars of herbivores, as reported by Hoffman *et al.* (1989) for *A. erioloba* and Gwynne (1969) for *A. nilotica.*

Seed germination of acacias is enhanced by any mechanism which breaks the dormancy caused by the hard seed coat (Doran *et al.*, 1983). In

nature, the dormancy is broken by micro-organisms, soil acids, and temperature fluctuations. This may take a long period, of up to 50-100 years for some species (Hoffman *et al.*, 1989). Bruchids make holes in seeds which can enhance imbibition of water, but they rarely help germination because they also attack the embryo. Consequently, bruchids lower germination percentage (Lamprey *et al.*, 1974; Hoffman *et al.*, 1989). Some herbivores that feed on the pods of acacias may not destroy most seeds inside by chewing or in their digestive system (see Miller, 1993). The ingested seed emerges in faeces after both mechanical and chemical scarification, encouraging germination whenever optimum conditions exist. Lamprey *et al.* (1974) found that seeds passed-out in faeces showed higher viability than those collected directly from, and under the trees, suggesting that those attacked by bruchids were destroyed in the guts of the herbivores. Grass-fires sometimes help in scarification of seeds of *A. karroo* (Sim, 1906; Coates-Palgrave, 1977); since controlled burning is part of the karroo-grassland management (Bosch and Taiton, 1988), the species is favoured under such regimes. Under optimum conditions, scarified seeds germinate in 2-8 days. Such a short germination period gives them the opportunity to utilize the limited moisture available after rain.

1.5. Utilization

The importance of *A. karroo* can be understood from Coates-Palgrave (1977), who described its presence in an area as an indicator of "a sweet veld, and an asset with unlimited uses on the farm". The uses of the species are detailed in several publications (Sim, 1906; Steadman, 1933; Watt and Breyer-Brandwijk, 1962; Coates-Palgrave, 1977; Poynton, 1984). The tree plays a major role in natural reforestation: it is usually found among the pioneer species on sand dunes, on rocky sub-soils, and on

Figure 1.3. Some of the benefits of *A. karroo*.

A Root nodules of *A. karroo*, known to enrich soil with nitrogen; B Rich palatable pasture of *A. karroo*-grassland vegetation on Umguza Valley Estate, Zimbabwe; C Gum from *A. karroo* (Photos 6574, 7645 & 6859, respectively, R.D. Barnes)

alluvial and saline soils. Where the trees are sparse, it enhances grass growth (Fig. 1.3) (Stuart-Hill *et al.*, 1987; Stuart-Hill and Taiton, 1989) by creating an equable micro-climate, and by enriching the soil with nitrogen, principally from falling leaves and nitrogen-fixing roots (Tolsma *et al.*, 1987). Although no record was found on nitrogen fixation level by *A. karroo,* Halliday and Somasegaran (1982) reported a quantity of 110 ± 30 Kg/ha per annum of nitrogen in a plantation of *Leucaena leucocephala,* a species within the same family. The nitrogen fixed by these species helps to rehabilitate degraded soils. Total removal or intensive defoliation of these trees is usually followed by a decrease in grass production (Aucamp, *et al.*, 1984). However, as the number of trees increases above 1000 per hectare, the grass community again declines (Friedel, 1987; Aucamp *et al.*, 1984). Aucamp and Danckwerts (1986) determined the adequate limit (tree equivalent limit) of *A. karroo* to be 297 trees/ha. At that density, the vegetation under *A. karroo* is fragile but favoured and more palatable to grazers, occasionally resulting in increased grazing pressure causing soil erosion (Acocks, 1988).

Detailed studies of the management of *A. karroo* rangelands were published by Bosch and Taiton (1988) and Hoffman (1988), who focused on grazing systems. Teague (1986; 1988b; 1989a) described the relationship between defoliation intensity, season, and phenophases. Aucamp *et al.* (1984) related tree density to stock quality, expressing the importance of the karroo biome in the development of livestock and wild life. In general, the species serves as food, shade and medicine to animals. The leaves, young shoots and fruits are good fodder for goats (Teague, 1989a; Ferrão and Ferrão, 1988). At times, goats are kept in areas with profuse regeneration as a measure of controlling unwanted growth. During dry winters, stock are fed on pollarded leafy trees. The gum is a favourite food for bush-babies and beetles (Anderson and Pinto, 1980). Ostriches eat

substantial quantities of the seeds and leaves, while the flowers support apiculture (Burtt-Davy, 1932; Poynton, 1984). The species is rated highly for propolis production (Konig, 1985) in southern Africa.

The wood is used principally for fuel and cattle shade fencing. In a recent survey of species favoured for fuelwood in Ciskei, S. Africa, Benbridge and Tarlton (1990) reported that *A. karroo* was the most favoured species. A partial explanation for that preference in terms of wood properties, i.e. the presence of dense heartwood and paranchyma embedded with crystals which keeps the fuelwood glowing on burning, was recently published by Prior and Cutler (1992; see also Gourlay and Kanowski, 1991). The trunk of *A. karroo* is used for making yoke-keys, polo mallets and, occasionally, water treated logs are used to produce timber for furniture (Sim, 1906; Goldsmith and Carter, 1981). The species also produces commercial gum (Fig. 1.3) which is used in medicine, confectionery, and adhesives. Analyses by Watt and Breyer-Brandwijk (1962) revealed that the gum does not contain poisonous hydrocyanic acid, and serves well as a cooking sugar. However, the gum of *A. karroo* and related species is inferior to the gum arabic of *A. senegal,* which itself suffers from over-production and competition with cheaper substitutes in world market (Anderson, 1987). Tannin and leather dyes extracted from the bark of *A. karroo* have suffered similarly because of the higher quality and quantity produced by introduced *A. mearnsii* (Sim, 1906; Moffett, 1956). Nevertheless, sweet gum and tannin from *A. karroo* have potential for exploitation for small scale industries and domestic utilities, reducing the cost on imports of similar products, while the astringent bark produced during tannin extraction is valuable for making ropes.

Other important uses of *A. karroo* include live fences and amenity plantings (Coates-Palgrave, 1977; Poynton, 1984). Steadman (1933) noted that the seeds are also eaten by people, though neither the time of

readiness nor preparation method were mentioned. A decoction from the bark cures diarrhoea and dysentery in human beings, and relieves cattle from tulp poisoning (Watt and Breyer-Brandwijk, 1962). However, the growth of the species in some places can be a nuisance, rather than a blessing, and it is included in the catalogue of problematic plants and weeds in South Africa and in Australia (Wells *et al.*, 1984; Scott, 1991).

1.6. Project background

The variety of utilization of *A. karroo* qualifies it as a multipurpose tree species and the variability prevalent in its wide geographical range offers the promise of genetic improvement. Any achievement in increasing the productivity of the species will raise the living standards of people who depend directly on its products and benefits. In addition, some products such as gum are of potentially high commercial value, and can be of national importance as an export commodity, as the world market changes towards natural products.

Most African *Acacia* species have not been studied beyond the taxonomic level; further, the taxonomy itself often requires a thorough revision as different forms are encountered. Proper identification is still a problem in a number of the sub-species proposed, including the variants of *A. karroo*. An integrated approach is required to correlate phenotypic variation with genetic diversity and environmental conditions. Genetic characterization, identification of ecological requirements, and estimation of productivity will form the basis for conservation, tree improvement, and utilization. The information is also of more general scientific interest, as it should contribute to the understanding of other dry-zone *Acacia* species.

1.7. Project objectives

1.7.1. General objectives

This study is part of a UK Overseas Development Administration (ODA)-funded research programme for evaluation and germplasm acquisition of the genetic resources of dry-zone African acacias. The main objectives of the study are to determine the genetic variation in *A. karroo* across its wide natural distribution and summarize the data in a form relevant to genetic conservation and breeding. The study also estimates mating system parameters for the species.

1.7.2. Specific objectives

The specific objectives of this study are:

(1) to develop appropriate laboratory methods for assaying a number of enzyme systems of *A. karroo*, as a tool to clarify genetic diversity;

(2) to characterize isozyme variation within and between populations sampled, across the likely range of genetic variation;

(3) to estimate mating system parameters;

(4) to recommend possible management strategies based on information gained.

1.8. Thesis organization

This thesis is presented in six chapters. To facilitate the understanding of every objective of the study; each chapter includes a review of relevant literature. Chapter Two presents an overview of isozyme techniques and related methods, and describes in detail the assay conditions established for this study. The findings of cytological study and genetic interpretations

of isozyme banding patterns are given in Chapter Three. Genetic diversity statistics are presented in Chapter Four, and mating system parameters in Chapter Five. The general conclusions, recommendations for management of the species and suggestions for further research are given in Chapter Six.

CHAPTER TWO

SAMPLING STRATEGIES AND ELECTROPHORETIC ASSAY TECHNIQUES FOR *A. KARROO* HAYNE

2.1 Introduction

In recent years, information pertaining to patterns of genetic variation in forest tree taxa has increased (Hamrick and Godt, 1989; Hamrick *et al.*, 1992). The upsurge can be attributed to several factors, among others, the growing awareness of the role of forestry in biodiversity conservation (e.g. Williams, 1991), the need to evaluate genetic diversity before and after management interventions (e.g. Bawa and Krugman, 1991; Savolainen and Kårkkåinen, 1992), the desire to understand genomic organization for tree improvement programmes (e.g. Neale and Williams, 1991), and the availability of refined and new techniques for gathering this information (e.g, Cheliak and Rogers, 1988). Although there has always been the need to define patterns of genetic variation of forest tree populations for better exploitation and management, it is the current refinement of the assay techniques that has revolutionized the investigations, reducing the time required for analyses and decision making. There is also the urge among forest researchers to keep forest science at a level of advancement comparable to that in other related fields.

The ultimate goal of these investigations is to characterize forest populations in terms of their genetic composition, hence the endeavours to access information at the level of DNA (deoxyribonucleic acid). A number of techniques, collectively forming the core of molecular genetics, have been developed to evaluate the genetic characteristics of plant

populations (Cheliak and Rogers, 1988; Hartl and Clark, 1989; Crawford, 1990; Murphy *et al.*, 1990; Neale and Williams, 1991). The choice of a technique depends on the aims of the study, the sensitivity and convenience of the technique, and the availability of resources. Currently, these evaluations are implemented using methodologies including DNA sequencing, gene mapping, restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs), and characterization of seed storage proteins and specific proteins, including isozymes.

DNA sequencing is the most informative of all the molecular techniques, as it determines all genetic variation (Crawford, 1990). However, the cost of DNA sequencing has limited its wider application, requiring the development of other techniques. The use of DNA fragments, for example, RFLPs and RAPDs, have been developed as methods of intermediate cost (Cheliak and Rogers, 1988; Dowling *et al.*, 1990; Neale and Williams, 1991; Bucci and Menozzi, 1992; Krutovskii and Wagner, 1992). However, even with these last methodologies, the high cost (compared to isozymes), the use of radioisotopes (RFLPs), the limited number of probes (RFLPs), the long processing time (RFLPs) and the problems associated with dominant bands hence the difficulty of scoring heterozygotes (RAPDs) make them less popular than isozymes (Dowling *et al.*, 1990; Neale and Williams, 1991). The isozyme technique is, therefore, the most cost-effective methodology presently available for many purposes, and is widely adopted for genetic analyses.

As with the other techniques above, analyses of the isozyme patterns of different taxa have proved to be of great value in, for example, establishing patterns of genetic variation, investigating evolutionary genetics, and in the verification of crossings, hybridization, introgression, polyploidy and paternity (Gottlieb, 1977a; Brown and Moran, 1981; Ayala,

1983; Adams, 1981; 1983; Murphy *et al.* 1990). One major problem not yet well resolved with most molecular techniques is their correlation with adaptive and phenotypic traits. Bush and Smouse (1992) reviewed studies correlating isozymes with adaptive characteristics and concluded that even with statistically significant results, a lot of questions remain unanswered, e.g. the linkage disequilibrium existing among allozyme loci. Isozyme studies also seem to offer low possibilities in these endevours, considering the limited number of markers that have been resolved using isozyme systems (Savolainen, 1992). DNA sequencing and RFLPs analyses are promising to solve some of these problems, assuming adequate single trait markers or quantitative trait loci can be identified (Cheliak and Rogers, 1988; Beavis *et al.*, 1991; Neale and Williams, 1991), Some traits have successfully been marked in some plant species using RFLPs, for example, height growth in maize (Beavis *et al.*, 1991), water-use efficiency and disease resistance in tomatoes (see Neale and Williams, 1991).

2.1.1. Advantages of isozymes in genetic studies

Some of the characteristics that make isozyme markers appropriate for genetic studies were summarized by Brown and Allard (1970) and Brown *et al.* (1989), and include:

(1) codominant inheritance;

(2) polymorphism and detectability at various loci;

(3) reduced influence of selective environmental forces and epistasis;

(4) resemblance of homologous loci in different taxa which makes their variation useful for comparative studies;

(5) applicability for studying natural populations;

(6) abundance of isozyme markers compared to morphological markers.

2.1.2. *Gel electrophoresis of isozymes*

Although gel electrophoresis techniques have been documented by many workers, including those mentioned above, there is no universally ideal single system of assay, even within one species. A set of optimum conditions has to be established empirically, depending on the organism, tissue, enzyme system and, at times, the locus (Brewer and Sing, 1970; Gottlieb, 1981; Wendel and Weeden, 1989). However, whatever the source of variation may be, the polymorphism is most useful when it reflects simple genetic control. For those amino acids that have either positive or negative charges, the variation in patterns is then assumed to reflect amino acid substitution in an enzyme which leads to difference in electrophoretic mobility on the supporting medium (Freifelder, 1976; Hartl and Clark, 1989).

Various supporting media have been employed for electrophoresis of isozymes, among them, paper, cellulose acetate, agarose, starch and polyacrylamide gels (Brewer and Sing, 1970; Feret and Bergmann, 1976; Freifelder, 1976; Wendel and Weeden, 1989; Murphy *et al.*, 1990). Given their relative advantages and disadvantages (see Freifelder, 1976), only the last two media have become widely established.

Advantages of starch and polyacrylamide media are based on: (1) capability to withstand high voltage assays; (2) good resolution of the patterns; (3) sieving effect due to variable concentration (9-18% for starch and 5-30% for polyacrylamide); (4) inertness to, and lack of diffusion of, enzymes with electrical charge. Starch has the specific advantage of being cheap and sliceable, giving several slab gels from a single run. Specific advantages of polyacrylamide are clearer resolution of bands, making them easier to record, and the possibility of a wider range of variable pore sizes on a single gel (e.g. disc electrophoresis). However, both starch and polyacrylamide also have specific disadvantages: while starch is difficult to

handle (see Brewer and Sing, 1970), polyacrylamide is neurotoxic, not sliceable, and more expensive. In this study, polyacrylamide gel electrophoresis was adopted because systems for its use were already established in the laboratory at the Oxford Forestry Institute.

The electrophoretic data are gathered as isozymes and allozymes (Murphy *et al.* 1990), which are expressed as bands of different mobilities. The bands reflect the allelic variation in the organism, and they can be scored and interpreted to express genetic diversity (Gottlieb, 1981). In some cases, instead of the usual alterations in mobility, the variation is expressed in the form of duplication or loss. This factor has been useful in establishing species phylogenies. For example, Wilson *et al.* (1983) found that closely related species belonging to Chenopodiaceae (a plant family) could be classified according to duplicated or missing alleles at the leucine aminopeptidase locus. Gene duplication has been noted also as a species diagonistic character, for example, in the genus *Clarkia* (Onagraceae) (e.g. Gottlieb, 1977b; Gottlieb and Weeden, 1979; Jones *et al.*, 1986) and *Eupatorium* (Asteraceae) (Yahara, 1989). Similar variation has been observed in the banding patterns of lactate dehydrogenase and creatine kinase of fishes and reptiles (Ferris and Whitt, 1977; 1978a; Sites *et al.*, 1986; Rao *et al.* 1989). Enzyme systems have been found also to vary according to tissue analysed; in fish and reptiles, different banding patterns were reported for the eye, brain, liver, and muscle tissues of the same organism (Ferris and Whitt, 1978b; Rao *et al.* 1989).

In plants, the number of isozymes exhibited by a particular enzyme for example, aspartate aminotransferase and phosphogluconate dehydrogenase, often reflects the number of subcellular compartments in which the same catalytic reaction is required, the ploidy level, or the presence of duplicated loci (Gottlieb, 1982; Weeden and Wendel, 1989). The amount of enzyme present in a compartment, and hence the quantity of

isozyme available, may be expressed by the staining intensity (Scandalios, 1969). Apart from the above-listed factors, variation of electrophoretic phenotypes may also be caused by physiological, ontogenetic, and environmental conditions. Marshall *et al.* (1974), Burdon and Marshall (1983) and Tanksley (1983) indicated that pathological status and environmental conditions, e.g. soil aeration and transplanting techniques, influence isozyme patterns of some enzyme systems such as peroxidase, alcohol dehydrogenase and esterases. The zymograms (electrophoretic banding patterns) may also vary in number and staining intensity when a plant is subjected to gaseous pollutants, as reported by Geburek *et al.* (1987) for *Pinus sylvestris*, Boyle and Morgenstern (1987) for *Picea mariana*, and Kargiolaki (1990) for some *Populus* species. Brewer and Sing (1970) and Anderson and Bowman (1979) related the variation of eletrophoretic patterns with ontogeny, and found that there is a notable change in patterns of some enzyme systems with age. More recently, Bapat *et al.* (1992) found remarkable variability of isozyme patterns in developing somatic embryos of wheat (*Triticum aestivum*).

These potential variations are minimized by growth of plant material under a standard set of conditions, and optimizing and standardizing electrophoretic conditions and tissue samples, respectively. Samples of each taxon and tissue are specifically prepared with regard to the problems they might pose. First consideration is given to the availability of sample material to meet the objectives of the study. Preparation methodology should consider the mechanical difficulties of tissue homogenization and associated problems caused by endogenous tannins, phenolic compounds, phenol oxidases and other unidentified cellular constituents (Feret and Bergmann, 1976; Soltis *et al.*, 1983; Wendel and Weeden, 1989). To reduce loss of enzyme activities and protein denaturation, the appropriate

method should adequately homogenize tissue within a minimum time in appropriate buffer.

This chapter describes the sampling of *A. karroo* trees according to morphological diversity, and the establishment of optimum electrophoretic assay conditions, including standardization of tissue homogenates, optimization of buffer systems and staining requirements, with an aim of achieving comparable isozyme resolutions. The resultant isozyme profiles are necessary to elucidate the genetic variation, ploidy level and genetic relationships, and to estimate mating system parameters for *A. karroo*.

2.2. Materials and Methods

2.2.1. *Sampling strategies*

According to Ross (1979, and as discussed in section 1.3 of Chapter One), there are eight major phenotypic varieties of *A. karroo* which have been classified only as entities. Most samples for this study were randomly selected within the distribution areas of those entities. The assumption was that if these entities represent mixed genepools, then a divergence at the molecular level, and hence in isozyme patterns, may be expected in them. Therefore, it was appropriate to sample randomly within several populations, with a likelihood that they included these entities. In total, 26 populations were sampled across the geographic range of the species (Table 2.1 and Fig. 2.1). Their relationships to the main morphological entities of Ross (1979) are discussed below. The classification of Ross (1979) though intensive, could not desribe all variants (entities) of *A. karroo* by sites, possibly due to the wide geographic range and the difficulties in distinguishing the entities unless they are extreme in form, especially

where they are mixed. Here, samples have been included that could not be identified definitely with those entities.

(1) The "typical *A. karroo*" is distributed mainly in the Karoo region and drier parts of the Cape Province in S. Africa. The entity was covered by sampling at Prince Albert, and possibly at Upington.

(2) The form referred to as *A. natalitia* is widely distributed in Eastern Cape Province, Natal, and Eastern Transvaal in S. Africa. The sampling for this form occurred along Cintsa River and at Queenstown. According to Gordon-Gray and Ward (1975), the same form is known to occur also around Tugela River, Babanango and Hluhluwe, where it is mixed with other forms. These populations were also sampled.

(3) The fire-resistant shrubby form grows widely in Nongoma District of S. Africa, and spreads to the north of Hluhluwe Game Reserve. Therefore, the germplasm could possibly be included in samples from Hluhluwe and Babanango.

(4) The "spindle *A. karroo*" occurs in the Hluhluwe and Umfolozi (near Babanango) Game Reserves, and around Tugela River. The same areas have been described by Ross (1971b; 1971c) as the sources of the "pubescent *A. karroo*". They might have been included in samples from Hluhluwe and Babanango.

(5) The large trees of *A. karroo* were included in the samples from Tugela River Mouth, Richard's Bay and Manzigwenya. Gordon-Gray and Ward (1975) reported that despite the variation in size and spines, *A. karroo* (*A. natalitia*) from around Durban (Natal) showed similarities in involucellate florets to those populations from Zululand Coast, around Kosi Lake, which are tall trees. This type is considered to extend to Ashburton, which was also sampled.

(6) *A. karroo* from the western part of Drakensberg Range, S. Africa, has been noted by Robbertse *et al.* (1981) to posses broader pinnules

Country	Population	District / Province	Latitude	Longitude	Altitude (m.a.s.l)	Rainfall (mm)	No. of Trees	Collector / . Number
1. Zimbabwe	Umguza Valley	Matebeleland	20° 10′S	28° 43′E	1200	600	120	R.D.B. & J.B.*
2. "	Mutorashanga	Makonde	17° 10′S	30° 42′E	1500	803	80	C.W.F. (1)
3. Malawi	Dedza	Dedza	14° 19′S	34° 16′E	1300	905	80	C.W.F. (448)
4. "	Zomba	Zomba	15° 36′S	35° 10′E	1030	1044	80	C.W.F. (447)
5. Zambia	Lusaka	Lusaka	15° 24′S	28° 18′E	1280	804	25	C.W.F. (483)
6. S. Africa	Kroonstad	Orange Free State	27° 29′S	27° 13′E	1348	606	31	R.D.B. (3176)
7. "	Babanango	Natal	28° 23′S	31° 17′E	1300	800	75	R.D.B. (3166)
8. "	Hluhluwe	Natal	28° 03′S	32° 03′E	550	900	80	R,D.B. (3162)
9. "	Queenstown	Cape	31° 52′S	26° 52′E	1077	559	65	R.D.B. (3208)
10 "	Manzingwenya	Zululand	27° 15′S	32° 46′E	5	1000	30	R.D.B. (3158)
11 "	Cintsa River	Cape	32° 52′S	28° 05′E	600	450	50	R.D.B. (3204)
12 "	Prince Albert	Cape	33° 12′S	22° 12′E	686	182	23	P.B.
13 "	East London	Cape	33° 01′S	27° 53′E	125	808	18	P.B.
14 "	Hartbeespoort	Transvaal	25° 44′S	27° 52′E	1326	740	17	P.B.
15 "	Talana	Transvaal	28° 09′S	30° 13′E	1247	835	14	P.B.
16 "	Kameiskroon	Cape	30° 12′S	17° 56′E	762	219	14	P.B.
17 "	Coega	Cape	33° 44′S	25° 35′E	58	572	13	P.B.
18 "	Cookhouse	Cape	32° 45′S	25° 47′E	754	603	12	P.B.
19 "	Weenen Tree	Natal	28° 50′S	30° 04′E	845	673	10	P.B.
20 "	Richard's Bay	Natal	28° 46′S	32° 06′E	20	1054	10	P.B.
21 "	Tugela Mouth	Natal	29° 20′S	31° 16′E	58	900	8	P.B.
22 "	Ashburton	Natal	29° 40′S	30° 21′E	684	400	8	P.B.
23 "	Venrhynsdorp	Cape	31° 35′S	18° 43′E	122	145	7	P.B.
24 "	Sardinia Bay	Cape	33° 52′S	25° 12′E	58	300	7	P.B.
25 "	Upington	Cape	28° 26′S	21° 41′E	805	197	6	P.B.
26 "	Cradock	Cape	32° 09′S	25° 36′E	872	339	5	P.B.

Table 2.1. Populations of *A. karroo* surveyed for electrophoresis analysis.
Samples were collected by Mr. C.W. Fagg (C.W.F.) and Dr. R.D. Barnes (R.D.B.) (O.F.I.), Dr. P. Brain (P.B.) (14 Richmond Avenue, Kloof 3610, Republic of South Africa). *Umgusa Valley (3095, the number not included in the Table) samples were collected by Dr. R.D. Barnes, and Janny Bickle (J.B.) (Umgusa Valley Estate, Zimbabwe). Note: Not all seeds collected were accompanied with vegetative samples.

Figure 2.1. The distribution of populations of *A. karroo* sampled for electrophoresis assays.

1. Umguza Valley; 2. Mutorashanga; 3. Dedza; 4. Zomba; 5. Lusaka; 6. Kroonstad; 7. Babanango; 8. Hluhluwe; 9. Queenstown; 10. Manzingwenya; 11. Cintsa River; 12. Prince Albert; 13. East London; 14. Hartbeespoort; 15. Talana; 16. Kameiskroon; 17. Coega; 18. Cookhouse; 19. Weenen Tree; 20. Richard's Bay; 21. Tugela Mouth; 22. Ashburton; 23. Venryhynsdorp; 24. Sardinia Bay; 25. Upington; 26. Cradock.

compared to those of trees on the eastern side. This variety was represented by sampling around Kroonstad and Kameiskroon.

(7) Most populations sampled in Malawi, Zambia and Zimbabwe appeared to represent different entities. Populations from Umguza Valley, Lusaka, and Dedza were similar in stem colour and crown form, as described by Fagg[2] (pers. comm.), but differed significantly in height from those at Zomba and Mutorashanga. The observed heights ranged from 4 m, on rocky sites at Dedza, to 20 m recorded at Zomba. Other characteristics also varied, for example, trees at Dedza have persistent large thorns, while at Zomba, the thorns were much reduced (pers. obser.).

The analytical material consisted of seeds from randomly selected trees of *A. karroo,* covering the major morphological variation from different parts of the geographic range (Table 2.1 & Fig. 2.1). A minimum of 5 and a maximum of up to 120 trees, were randomly sampled from each of the 26 populations. A distance of about 50 metres between the sampled trees was adopted to safeguard against sampling of closely related individuals. Seeds were sampled all over the crown, dried, cleaned and maintained separately for each tree selected. All seeds for the study were maintained under cold storage (+ 4°C) following field collection; they were removed from storage in lots sufficient for one week's work.

Genetic variation within and among populations was studied by sampling a single seed per sample tree, while estimates of mating system parameters was based on 60 seeds per tree. The appropriate sample size is still a debatable question in plant population analysis (Crawford, 1990; Bawa, 1992), but it is clear that the larger the sample size the more accurate the estimated parameters.

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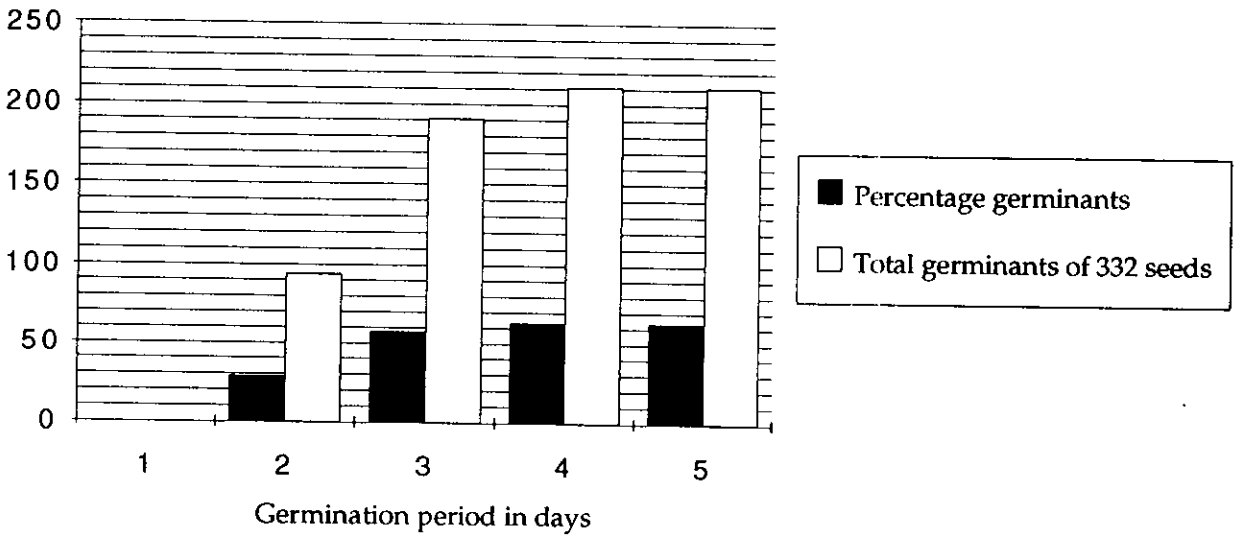
[2] C. W. Fagg, Research Officer, Oxford Forestry Institute, University of Oxford, UK.

2.2.2. *Laboratory methodology*

Prior to germination, the seed samples were rinsed with water to remove dust, and empty or rotten seeds were removed by flotation. Nicking was used as the germination pretreatment (Doran *et al.* 1983). Viable, single seeds from the sample trees were selected, nicked and raised in individual single germinating cells made in a polystyrene sheet, while seeds for progeny array analysis were germinated in petri-dishes. All seeds were germinated at room temperature (20°- 25°C), in 2-4 days (Fig. 2.2), with non-germinants showing either infection by fungi, internal destruction by bruchids, or both. The germinants were homogenized on the fifth day. The identity of every seed was maintained throughout the study time.

Figure 2.2. Cumulative germination rate of A. karroo.



2.2.3. *Homogenization*

Since *A. karroo* has been reported to contain enzyme inactivating compounds such as tannin (Watt and Breyer-Brandwijk, 1962), a complex extraction buffer of Tris-HCl (Soltis *et al.*, 1983; Wendel and Weeden, 1989)

was used, with modification. The composition per 100 ml of deionised distilled water was as follows: 0.1 M (1.21 g) Tris, 0.001 M (0.036 g) ethylenediamine tetraacetic acid disodium salt (EDTA (Na_2)), 0.01 M (0.08 g) potassium chloride (KCl), 0.01 M (0.20 g) magnesim chloride ($MgCl_2$), and 0.1 M (1.76 g) ascorbic acid. The solution was adjusted at pH 7.8, and maintained as stock in a freezer. Prior to usage, 2% (0.2 g) w/v of polyvinylpolypyrrolidone (PVPP), 0.5% (0.05 g) w/v polyvinylpyrrolidone-40 (PVP-40), 1% w/v of bovine serum albumin (BSA), and 0.1% (0.01 ml) 2-β-mercaptoethanol were added to every 10 ml of stock buffer. The roles of these buffer additives were explained by Kephart (1990).

To minimize variation due to physiological and ontogenetic conditions, only cotyledons at the same developmental stage were excised and homogenized. 0.5 ml of the extraction buffer was added to each sample and the extraction was performed in labelled microtubes over ice. Alternatively, microtubes with extraction buffer were frozen at -4°C freezer prior to addition of the samples. The frozen buffer not only cooled the tissue but helped in crushing before thawing. The cotyledons were mechanically crushed with a tapered brass rod device: grinding with chilled mortar and pestle was time-consuming, and the flat cotyledons were not easily digested by a power homogenizer without warming up. The grinder was rinsed with distilled water and cleaned with absorbent tissue paper between samples to avoid contamination. The homogenates were immediately transfered to an ultra-freezer (-70°C) to be stored for three or more days.

To ascertain the appropriate tissue for analysis, and the effect of extraction buffer additives used in the assay, seeds of different populations from two trees were germinated. The two cotyledons were severed from the radicle tissue of every seedling. Each cotyledon was further divided into two halves to make four samples. A total of five samples, including

the radicle tissue, were prepared from every seedling. The radicle tissue was homogenized in the standard buffer described above. The four cotyledon samples were homogenized in the same standard buffer but without the addition one of each, of the following components: ascorbic acid, PVP-40, PVPP, 2-β–mercaptoethanol. The samples of each extraction were electrophoresed in adjacent wells. Differences in resultant patterns are discussed.

2.2.4. *Gel preparation and electrophoresis*

A continuous buffer system of Tris-Borate-EDTA (see Wendel and Weeden, 1989) was used with modification. The pH was lowered to 8.4 and the molarity adjusted as follows: 0.09 M (10.89 g/l) Tris, 0.09 M (5.56 g/l) boric acid, and 0.025 M (0.93 g/l) EDTA. Polyacrylamide gels (16x18 cm) were prepared with the "Hoeffer S.E. 600" apparatus for vertical electrophoresis. Using the accessory divider plates, four club gel sandwiches were accommodated in every tank. The composition of the gels was as follows: 105 ml Tris-Borate-EDTA buffer of pH 8.4, 25.2 ml deionised distilled water, 10 ml (0.5%) solution of ammonium persulphate, 42.3 ml (27%) polyacrylamide, and 0.36 ml of tetramethylethylene diamine (TEMED). The final concentration of polyacrylamide used was 6.3%. Trials with a slightly lower concentration of 6.0% resulted in gels which were difficult to handle, with poorly defined wells.

Only homogenates sufficient for one day's work (20 samples assayed for 8 enyme systems) were removed from the ultrafreezer daily. The homogenates were centrifuged at 11 000 r.p.m (Camlab 4218) for 3 minutes at room temperature. Longer centrifuging time caused complete loss of activity by some enzymes, e.g. alcohol dehydrogenase and malic enzyme possibly because the homogenates warmed up in the process. The

supernatant was decanted and a 10 µl syringe used to load the gels. The 20 samples were loaded into the 20 wells of each gel which had been filled with the electrode buffer. As samples were denser than the buffer, they settled at the bottom of the wells. The apparatus was then assembled and each tank run for 4.5 hours (except glutamate dehydrogenase and β-esterase which required 5 and 4 hours, respectively) at 400 volts and 120 mA. The timing and a constant voltage or current were maintained by a power supply (Microcomputer electrophoretic power supply - model E455). The temperature in the tanks was maintained approximately 3°C by a multitemperature thermostatic circulator (LLK Bromma 2219). Usage of bromophenol blue to monitor the mobility was discontinued as it coagulated some samples. A running time of 4.5 hours duration was found appropriate for gel assembling, running, staining, and recording within the same day. Recording was done using data forms or photographs. Since only 8 enzymes could be assayed in a day, the samples were stored in a domestic freezer (-4°C) for one further day, without deterioration of the last-assayed enzyme systems in each lot.

2.2.5. *Staining procedures*

The basic staining procedures and recipes for various enzymes for different taxa and tissues have been reviewed by Brewer and Sing (1970), Conkle *et al.* (1982), Vallejos (1983), Soltis *et al.* (1983), and Wendel and Weeden (1989). From these basic recipes, optimum staining conditions were established for *A. karroo* by manipulation of pH, substrate concentration, and buffer ionic strength. Tris-HCl staining buffer were used throughout the study. Staining reagents other than enzyme-specific substrates were: tetrazolium thiazolyl blue (MTT), diazonium salts (i.e. fast

blue BB salt, and fast blue RR salt), cofactors [nicotinamide dinucleotide (NAD), β-nicotinamide dinucleotide phosphate (β-NADP), nicotinamide dinucleotide reduced (NADH], phenazine methosulfate (PMS), a catalyst for most assays with tetrazolium stains. Other than NADH, these frequently-used reagents were retained refrigerated as aqueous solutions of 10 mg/ml, and dispensed in 1-2 ml volumes.

In order to mark the position of every sample during the staining procedure, a notch was cut at the top right hand corner of every gel next to the last specimen. A total of 16 enzyme systems were stained initially, but only 12 showed consistently good resolution (Table 2.2). Leucine aminopeptidase, alanine aminopeptidase, and isocitrate dehydrogenase did not give consistently good resolution at the initial stages. Fluorescent esterase had good resolution but was difficult to record, as the bands diffused, thus making them unscorable. Similar observations had been reported previously by Soltis *et al.* (1983). These four enzyme systems were therefore not assayed further in the studies (see Appendix I for recipes).

2.3. Results and Discussion

The 12 enzyme systems which gave scorable banding patterns are listed in Table 2.2, while their staining schedules are summarized in alphabetical order below. All the staining was performed in stainless steel trays with lids to keep out of the light. Photographs resulting from the assayed enzyme systems are presented in Chapter Three.

Table 2.2. Enzyme systems assayed in *A. karroo.*

Enzyme system		Enzyme Commission (EC). No.
1 Alcohol dehydrogenase	(ADH)	1.1.1.1
2. Aspartate aminotrasferase	(AAT)	2.6.1.1
3. Diaphorase	(DIA)	1.6.99.-
4. αlpha-esterase	(α-EST)	3.1.1.-
5. βeta-esterase	(β-EST)	3.1.1.-
6. Glucose-6-phosphate dehydrogenase	(G-6-PD)	1.1.1.49
7. Glutamate dehydrogenase	(GDH)	1.4.1.2
8. Malate dehydrogenase	(MDH)	1.1.1.37
9. Malate dehydrogenase (Malic enzyme)	(MDHP)	1.1.1.40
10. Menadione reductase	(MR)	1.6.99.-
11. 6-Phosphogluconate dehydrogenase	(6-PGD)	1.1.1.44
12. Shikimate dehydrogenase	(SDH)	1.1.1.25

2.3.1 Alcohol dehydrogenase (ADH, E.C. 1.1.1.1)

0.1 M Tris-HCl	pH 8	100 ml
95% Ethanol		1.0 ml
NAD		1.0 ml/10 mg
MTT		1.0 ml/10 mg
PMS		0.2 ml/ 2 mg

The enzyme was assayed on the first day as it deteriorated on the second. Incubation was at 37°C and slight overstaining was better for clear photographs since the gel background was transparent. When rinsed with cold water, the bands remained clear for a longer period. There were lower band numbers observed in the radicle compared to the cotyledons. Omission of any of the buffer additives caused no noticeable difference in the resolution.

2.3.2. Aspartic aminotransferase (AAT, E.C. 2.6.1.1)

0.1 M Tris-HCl pH 7.25	100 ml
L-Aspartic acid	100 mg
α-Ketoglutaric acid	33 mg
Pyridoxal-5-phosphate	4 mg
BSA	50 mg
Fast blue BB salt	100 mg

Since aspartic and α-ketoglutaric acids have low solubility in the buffer, the mixture was incubated for some minutes before the pH could be corrected to 7.25. The other components were added when the gels were ready for staining. The enzyme was stable and could be stained on the second day of the sample without any loss of intensity. The enzyme could be assayed within a pH range of 7.0-8.0, but the bands were much sharper at pH 7.25. Extracts from the radicle had lower number of active loci compared to the cotyledons. Omission of one of the additives from the standard extraction buffer did not change the resolution.

2.3.3. Diaphorase (DIA, E.C. 1.6.99.-)

0.05 M Tris-HCl pH 8	100 m
2,6-Dichlorophenol-indophenol	0.2 mg
NADH	10 mg
MTT	0.5 ml/ 5 mg

Using of 2,6-dichlorophenol-indophenol in quantities more than 0.2 mg gave dark blue gels with robust bands, but they soon spreaded out into each other, making them difficult to score. The gel was incubated at 37°C but overstaining was avoided. The bands were clearer when excess stain

was rinsed off with cold water. The number of bands observed for the radicle tissue were higher than for the cotyledons, and their staining was more intensive but less distinctive than the latter.

2.3.4 α-Esterase (α-EST, E.C. 3.1.1.-)

0.1 M Tris-HCl	pH 7.25	100 ml
α-Napthyl acetate		100 mg
Fast blue RR salt		100 mg

α-Napthyl acetate was dissolved in 1 ml of acetone before mixing in the stain buffer. The enzyme stained faster than others, requiring appropriate care to avoid overstaining. When stained at room temperature, the staining duration was long and proper monitoring could be effected. When there were a number of gels to be stained, esterases were among the last stained, to avoid the loss of banding patterns. The *α-Est* bands were black on a slight greenish background and the first locus appeared next to the sample origin. There were a reduced, but deeply stained, number of bands in the radicle tissue compared to cotyledons. Poor resolution was observed when either PVP-40 or ascorbic acid was missing from the extraction buffer.

2.3.5 β-Esterase (β-EST, E.C. 3.1.1.-)

0.1 M Tris-HCl	pH 7.25	100 ml
β-Napthyl acetate		100 mg
Fast blue RR salt		100 mg

The optimum conditions were similar to those for α-esterase, but staining of the two enzymes together did not give clear resolution. It was necessary to stain on the first day for esterases to have good resolution.

The bands formed were reddish and migrated more to the anode than with other enzymes assayed; therefore, the gels were run for 4 hours to avoid loss of the anodal bands. The bands were stained more deeply in the radicle tissue compared with the cotyledons. Omission of PVPP or 2-β-mercaptoethanol in the standard buffer resulted in poor resolution.

2.3.6 Glucose-6-phosphate dehydrogenase (G-6-PD, E.C. 1.1.1.49)

0.1 M Tris-HCl	pH 7.25	100 ml
D-Glucose-6-phosphate (disodium salt)		50 mg
NADP		1.5 ml/15 mg
MTT		1.5 ml/15 mg
PMS		0.3 ml/3 mg

Although the bands were recordable, they were not very consistent in all populations. Addition of $MgCl_2$, $CaCl_2$, BSA (Conkle *et al.*, 1982; Wendel and Weeden, 1989), or staining at different pHs and buffer concentrations, did not impove the resolution. The enzyme from the radicle sample showed very weak activity. Omission of 2-β-mercaptoethanol or PVPP in the standard buffer impaired resolution of the isozymes.

2.3.7 Glutamate dehydrogenase (GDH, E.C. 1.4.1.2)

0.1 M Tris-HCl	pH 8.0	100 ml
L-Glutamic acid		1 g
NAD		1.0 ml/10 mg
MTT		1.0 ml/10 mg
PMS		0.5 ml/5 mg

L-Glutamic acid (monosodim salt) dissolves slowly; thus, after mixing with the buffer, it required warming in the oven to hasten the process, after which the pH was corrected to 8.0. The enzyme had to be incubated much longer than the other enzyme systems at 37°C before the bands were recordable. Deeper staining of the bands was observed on the radicle tissue, and omission of any buffer additive was not reflected in the resolution. Only after running the gels for 5 hours were the bands separated; before that, all samples were monomorphic. There was no apparent increase in the number of bands for other enzyme systems due to the longer running time for *Gdh* resolution.

2.3.8 Malate dehydrogenase (MDH, E.C. 1.1.1.37)

0.1 M Tris- HCl	pH 8.0	100 ml
1.0 M DL-Malic acid	pH 7.0	3.0 ml
NAD		10 mg/1.0 ml
MTT		10 mg/1.0 ml
PMS		3 mg/0.3 ml

One molar malic acid solution was prepared and adjusted to pH 7.0 with saturated then 1.0 M solution of sodium hydroxide. The malic acid was added to the buffer and the pH corrected to 8.0, before the addition of other recipes. Better resolution was obtained when the three last components were added in powder form. However, resolution between the bands was not sufficiently clear for photographic recording. Extracts from the radicle tissue had fewer bands than those from the cotyledons, and those without 2-β-mercaptoethanol or ascorbic acid in the extraction buffer showed poor resolution. The case of elimination of cytosolic forms of *Mdh* by ascorbic acid (Goodman *et al.*, 1980) could not be confirmed or dismissed, since the bands remained constant, but of poor resolution.

2.3.9 Malate dehydrogenase (MDHP) or Malic enzyme (ME, E.C. 1.1.1.40)

0.1 M Tris-HCl	pH 8.0	100 ml
1.0 M DL-Malic acid	pH 7.0	3 ml
NADP		10 mg/1.0 ml
MTT		10 mg/1.0 ml
PMS		3 mg/0.3 ml
$MgCl_2$		100 mg

The procedure for *Mdhp* followed that for malate dehydrogenase, except in the usage of NADP and magnesium chloride. However, staining of the two enzymes together was not feasible because some loci overlapped, and *Me* takes a longer time to stain. Malic enzyme could be stained between pH 7.0 and 8.0. Only one zone showed clear activity at pH 7.0, increasing to three, in some populations, at pH 8.0. Malic enzyme and malate dehydrogenase were stable and could be assayed on the second day. The banding patterns of the radicle tissue corresponded to that of the cotyledons, but the staining intensity was reduced when 2-β-mercaptoethanol or PVPP was omitted in the standard buffer.

2.3.10 Menadione reductase (MR, E.C. 1.6.99.-)

0.1 M Tris-HCl	pH 7.25	100 ml
Menadione (sodium bisulphite)		20 mg
NADH		10 mg
MTT		2.0 ml/20 mg

The enzyme resolution varied markedly among the samples and between the loci. The enzyme had low activity on the second day, and was therefore among the first assayed in every set of samples. Poorly resolved, but deeply staining, bands were obtained from the radicle tissue (Figure

2.3b). The effect of missing additives in the extraction buffer was not conspicuous.

2.3.11 6-Phosphogluconate dehydrogenase (6-PGD, E.C. 1.1.1.44)

0.1 M Tris- HCl	pH 8.0	100 ml
6-Phosphogluconic acid (trisodium salt)		25 mg
NADP		1.0 ml/10 mg
MTT		1.0 ml/10 mg
PMS		0.3 ml/ 3 mg

6-Phosphogluconic acid was first dissolved in the buffer and the pH corrected to 8.0. The enzyme was stable and could be assayed on the second day. Slight overstaining was useful for clear photographs. Homogenates from the radicle tissue had variable numbers of bands compared to those from cotyledons; omission of any of the above mentioned extraction recipes had no noticeable effect on band resolution.

2.3.12 Shikimate dehydrogenase (SDH, E.C. 1.1.1.25)

0.1 M Tris-HCl	pH 8.0	100 ml
Shikimic acid		50 mg
NADP		1.5 ml/15 mg
MTT		1.5 ml/15 mg
PMS		0.4 ml/4 mg

Shikimic acid was dissolved in the staining buffer and pH corrected to 8.0 before mixing with other components. The enzyme was stable and was among the last stained in every sample. The clear background of the gel allowed photographing, after slight overexposure. Omission of 2-β-mercaptoethanol from the buffer additives reduced the resolution, but the

Figure 2.3. Variation in resolution of isozyme patterns of cotyledon and radicle tissues.

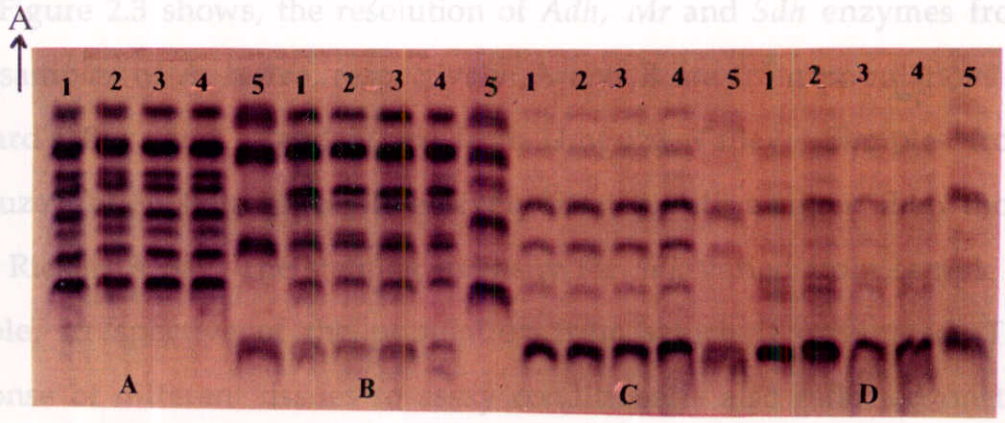


Figure 2.3a. Isozyme patterns of *Adh* loci of samples of *A. karroo* (A,B,C,D): A and B are from Richard's Bay population, and C and D are from Umguza Valley. The treatments within the samples are: 1-4 are cotyledon tissue extracted without 2-β-mercaptoethanol, PVPP , PVP-40 and ascorbic acid respectively, and 5 is radicle tissue. Note that A5 and B5 have exchanged positions. Arrow indicates anodal migration. (Photo 2996).

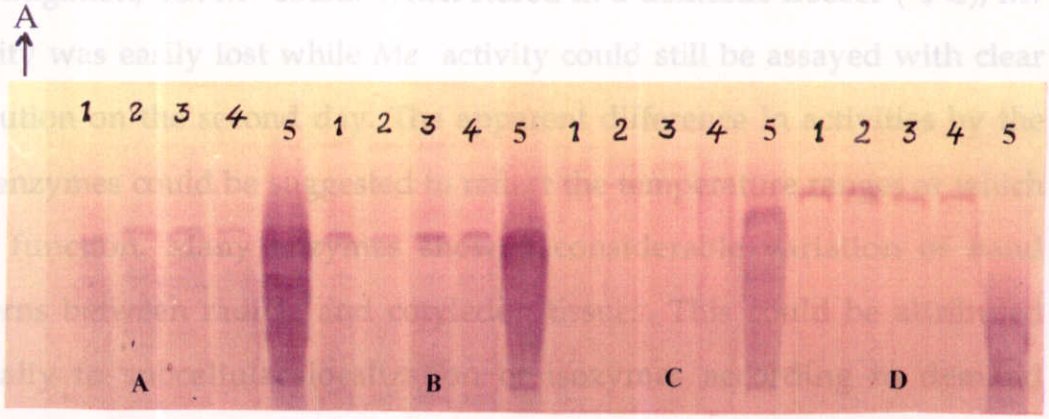


Figure 2.3b. Resolutions of radicle and cotyledon tissues according to *Mr* isozymes. (Photo 2996).

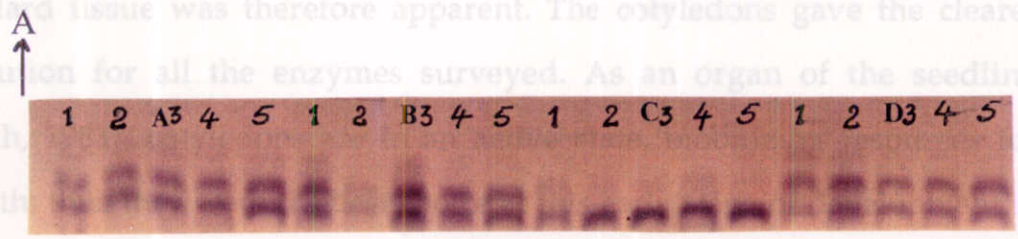


Figure 2.3c. The isozyme patterns of *Sdh* for the same samples and treatments. (Photo 2996)

same number of loci and alleles were observed in the radicle and cotyledon tissues.

Figure 2.3 shows, the resolution of *Adh, Mr* and *Sdh* enzymes from four samples of *A. karroo,* respectively: A and B are from populations of Richard's Bay, and C and D are from Umguza Valley. Samples from Umguza Valley stained less intensively for the *Adh* enzyme than those from Richard's Bay. The staining intensity for *Sdh* varied from sample to sample, irrespective of the population from which it originated. The response of different tissues to assay conditions is also reflected on the resolutions of *Mr* isozymes.

Another example could be drawn in the case of *Mr* and *Me* enzymes (not illustrated). During tissue preparation, *Me* could not withstand long centrifugation, but *Mr* could. When stored in a domestic freezer (-4°C), *Mr* activity was easily lost while *Me* activity could still be assayed with clear resolution on the second day. The apparent difference in activities by the two enzymes could be suggested to reflect the temperature ranges at which they function. Many enzymes showed considerable variation of band patterns between radicle and cotyledon tissues. This could be attributed partially to subcellular localization of isozymes according to demand (Gottlieb, 1981), and partially to optimization of extraction conditions for genotypes, tissues and enzymes which could cause artifacts. The need for a standard tissue was therefore apparent. The cotyledons gave the clearer resolution for all the enzymes surveyed. As an organ of the seedling (Smith, 1981), cotyledons are in an active state, mobilizing resources for growth; thus they were the best choice at this early stage of development.

2.4. Conclusion

Observations in this study were consistent with the general conclusion that the range of variables for good electrophoretic resolution are enormous, and one can only search for the optimum conditions empirically. The analysable variation is represented by the isozyme patterns that account for the number of bands, their distribution and the staining intensity, but the probability of experimental artifacts is also high until sufficient samples are assayed and analysed genetically. Chapter Three presents the genetic interpretation of the isozyme patterns observed in the gels run under the conditions discussed above.

CHAPTER THREE

EVIDENCE OF POLYPLOIDY IN *A. KARROO*

3.1. Introduction

A polyploid plant is one with more than two complete sets of chromosomes. Depending on the pairs of genomes involved, a polyploid may be a triploid, tetraploid, pentaploid, hexaploid, or an octaploid, and so on, but tetraploidy is the most common (Wright, 1976). Stebbins (1950) described four types of polyploids: autopolyploids, segmental polyploids, true allopolyploids and autoallopolyploids. Wright (1976) noted that autopolyploids and segmental polyploids exist as triploids or tetraploids; true allopolyploids occur from tetraploid and above, while autoallopolyploids are confined to hexaploidy and higher levels only. However, polyploidy should be understood as a continuum where allopolyploids are on one end and autopolyploids are on the other, with segmental polyploids providing every intermediate situation between the extremes (Stace, 1989).

The type of a polyploid is determined by the parents that were involved in its formation, e.g. a single self-fertilizing individual, different individuals within a population, different ecotypes of a species, congeneric species or species of different genera (deWet, 1980). The first three categories form autopolyploids, possibly with minor divergence in the genomes (Stebbins, 1980). Examples of autotetraploids include *Medicago sativa* (Quiros, 1982), *Solanum tuberosum* (Quiros and McHale, 1985) and *Tolmiea menziesii* (Soltis and Soltis, 1988). Occasionally, congeneric plants, or plants from different genera, do breed, forming allotetraploids. Segmental polyploids are formed when the crossings involve pairs of

genomes that have chromosomes or segments which are similar. True allopolyploids are derived from hybridization between two or more distantly related species, of which chromosomes are so different thus if they form a hybrid there is no bivalent pairing, or only a small number of loosely associated bivalents are formed. Examples of allopolyploids include *Nicotiana tabacum, Spartina townsendii* and *Primula kewensis* (Riley, 1949). Autoallopolyploids are usually formed when polyploid species with three or more pairs of distant genomes do cross-breed. The three types - segmental allopolyploids, true allopolyploids and autoallopolyploids - are generally referred to as amphidiploids, since their chromosomes that originated from similar genomes, form bivalents at meiosis, imitating a diploid species (Riley, 1949). In autopolyploids, homologous chromosomes show various configurations at metaphase, i.e univalents, bivalents, trivalents, quadrivalents, rings, and so on depending on the chiasma formation and ploidy levels (Riley, 1949; Stace, 1989).

3.1.1. Formation of polyploids in natural populations

Several postulations about the formation of polyploids have been reviewed and discussed (see Riley, 1949; Stebbins, 1950; Wright, 1969; deWet, 1980; Lewis, 1980a). Originally, it was hypothesized that polyploids are formed spontaneously through somatic and zygotic chromosome doubling. A few examples of polyploids believed to have been formed this way were discussed by Stebbins (1950) and deWet (1980), but the most widely documented case is that of *P. kewensis*. The important factor for spontaneous polyploidization to occur is the initial crossing of two species with dissimilar genomes and the formation of an infertile hybrid. Following hybridization, chromosomal segregation might fail to occur during mitotic division in some tissues of the resultant plant. Such organs

might asexually reproduce polyploid genets. Occassionally, plants may express endopolyploidy (chromosomal chimeras) in that certain tissue parts will be diploid whilst others will be polyploid (Riley, 1949; Stace, 1989). Chromosomal chimera may be induced by environmental conditions and has been used in the development of polyploid variants of *Solanum* species (Riley, 1949; Stace, 1989; Grant, 1981). Somatic chromosome doubling has been observed more often in injured tissues of plants (D'Amato, 1975) and it is one of the factors enlisted as of potential threat to cryopreservation of plant tissues.

Formation of polyploids through fertilization of unreduced gametes is an accepted possibility, since such occurrences are found in nature (Lewis, 1980a). Another widely accepted model of polyploid formation is through the triploid stage: here, an unreduced diploid gamete from one parent is fertilized to triploid status. The resultant triploid plant will also produce another unreduced triploid gamete due to nondisjuction of chromosomes. The unreduced gametes can further be fertilized by another gamete to a more stable and fertile polyploid stage (Riley, 1949; Wolf *et al.*, 1990). In nature, these stages can take place adjacently in areas of introgression, where there are possibilities of hybridization and back-crossing. There is evidence that there can be the multiple origin of a polyploid in different sites leading to some genetic variation. Such evidence has been documented for allopolyploids *Tragopogon mirus, T. miscellus* (Roose and Gottlieb, 1976; Soltis and Soltis, 1989), *Senecio cambrensis* (Ashton and Abbott, 1992; Harris and Ingram, 1992), using analyses of isozymes and cpDNA. Multiple origin of autopolyploids has been reported also for *Heuchera micrantha* (Ness *et al.*, 1989) and *H. grossulariifolia* (Wolf *et al.*, 1990) using similar molecular techniques. In these studies, it has become clear that isozymes can detect recurrent

polyploidization, but cpDNA is more explicit since it is maternally inherited in most angiosperms (Soltis and Soltis, 1989).

3.1.2. Biological significance of polyploidy

Polyploidy affects a number of characteristics in plant life, for example, the morphology, physiology and mating systems (Stebbins, 1950). Generally, polyploids are known to have larger cells, which occasionally lead to an increase in size of a plant compared to the diploid conspecies. Distinguishing differences have also been noted on the morphology of pollen and guard cells of polyploids compared to their diploid counterparts (Lewis, 1980a).

Polyploids are generally less fertile than diploids (Riley, 1949; Stebbins, 1950; 1980; Wright, 1949), a factor which is usually attributed to unequal segregation, heterogenetic association of chromosomes, or segments, leading to lethal combinations. Triploids and pentaploids are the least fertile due to an unbalanced chromosome number (Ford, 1976; Richards, 1986). However, to compensate for the low fertility, polyploidy in most cases restore self-compatibility, even if the progenitors were self-incompatible (Lande and Schemske, 1985; Richards,1986; Barrett and Shore, 1987). Some of the polyploids develop apomictic or other asexual reproduction means, e.g. the odd polyploids in *Crepis, Malus, Rubus* and *Taraxacum* species (see Stebbins, 1950; Grant, 1981). However, the most important role of polyploidy in evolution of plants is the creation of a breeding barrier between the newly formed species and its diploid progenitors, leading to speciation (Ford, 1976; Grant, 1981). This kind of speciation is known to have occurred in many polyploids including *Medicago sativa* L., *M. falcata* L. (Quiros, 1982), *Solanum tuberosum* L. (Quiros and McHale, 1985) *T. menziesii* (Soltis and Soltis, 1988), *A.*

cowleana and *A. holosericea* (Moran *et al.*, 1992) and other polyploid species above mentioned.

A comparison of growth characteristics between polyploids and their diploid counterparts has always given mixed results (Wright, 1976). In most cases, triploids have been more impressive, surpassing the conspecific diploids and higher polyploids in growth rate. Higher polyploids often perform more poorly than diploids (Wright, 1976). However, recently Moran *et al.* (1992; but see Thomson, 1992) studying growth rate in *A. cowleana* and *A. holosericea*, observed a higher growth rate in hexaploids compared to tetraploids of both species.

3.1.3. Polyploidy in plant improvement

It is interesting to note that a number of agricultural and horticultural crops are polyploids, for example, cotton, bananas, wheat, tobacco, apples, pears, strawberries, potatoes, coffee, peanuts and sugarcane (Stebbins, 1950). Thus, some of their properties, including unbalanced segregation of chromosomes, have been used to develop new varieties.

In forestry, most polyploid tree species are found among the angiosperms, but their status is still to be exploited. One reason for this is that very few forest tree species have been studied and bred through many generations. This situation is even more pronounced for tropical tree species (Burley *et al.*, 1986). Exceptionally higher productivity has been realized with natural triploids of European poplar and a few other related artificial hybrids (Wright, 1976). Conversely, only a few natural polyploids have been found among the gymnosperms and artificial ones are often retarded in growth, making them suitable mainly for ornamental plantings, an exception being *Sequoia sempervirens* 2n=6x=66, which is a natural polyploid that attains great size (Wright, 1976; Grant, 1981).

Seemingly, polyploids have no specific geographic pattern in their distribution (see Stebbins, 1950; Wright, 1976; Lewis, 1980b), but are believed to be more versatile and to occupy more adverse environments than their diploid progenitors (Fincham, 1972; Ford, 1976; Stebbins, 1980; Grant, 1981). Some of them are frequent colonizers employing asexual, apomictic and self- fertilization as regenerating strategies. In Africa, where *Acacia* species are almost equally divided into diploids and polyploids, no advantageous distribution pattern has been established in terms of the ploidal levels (Ross, 1979), and comparative studies of polyploids versus their diploid progenitors are still awaited.

Evidence of polyploidy has been obtained from chromosome counts, chromosome pairing patterns and morphological variation, but more recently, isozyme and other molecular techniques have become useful in determining ploidal levels (Harris and Ingram, 1992; Soltis and Soltis, 1993). This chapter presents the cytological and isozymic evidence of the level and the nature of ploidy of *A. karroo.* Techniques for cytological studies are presented in the second part of this Chapter, while the isozyme studies are reported in part 3. The distribution of the cytotypes and genotypes is also discussed.

3.2. CHROMOSOME NUMBER IN *A. KARROO*

3.2.1. Introduction

Phenotypic variations of *A. karroo* Hayne have been reported in several publications (Brenan, 1970; Ross, 1971a; 1971b; 1973; 1975; 1979; Robbertse *et al.,* 1981; and von Breitenbach, 1989), have been summarized in Chapters One and Two of this study. In some of the above publications, the authors have called for the establishment of intra-specific taxonomic

categories of varieties, subspecies, and even separate species from the "normal *A. karroo*" (Brenan, 1970), a term which is indicative of ambiguity within the taxon. Detailed investigation of this taxonomic problem from morphological features has simply compounded the situation as new varieties come to light (Robbertse *et al.*, 1981), and suggests high genetic differentiation within the taxon.

These morphological diversities have been implicated to suggest that most populations of *A. karroo* are a complex of a number of closely related *Acacia* species (von Breitenbach, 1989). According to Crawford (1985; 1989), congeneric species are often (but not always) divergent at the genes specifying soluble enzymes. Thus, from the allozymic data, it can be ascertained whether two different morphological types represent one or more gene pools. However, interpretation of allozyme data is affected by ploidy level, and thus requires some basic understanding of the cytotypes within a taxon. Cytological studies on *Heuchera grossulariifolia* (Wolf *et al.*, 1990), *Polystichum talamancanum* (Barrington, 1990), *Cystopteris tennesseensis* (Haufler *et al.*, 1990), and *Acacia tortilis* (Olng'otie, 1992) demonstrated that prior knowledge of cytotype distribution within the study population is essential in the interpretation of the complex electrophoretic patterns that arise with increasing ploidy level.

The chromosome number of *A. karroo*, under its original name of *A. horrida* (L.) Willd., was initially reported by Grimpu (1929, cited by Atchinson, 1948; see Darlington and Wylie, 1955) as 2n=4x=52, locating the species among the polyploids of the genus. It is important to note that the true *A. horrida* (L) Willd. is only distributed in eastern Africa and Asia (Lock, 1989), and is not sympatric with *A. karroo*. Subsequently, the chromosome number of *A. karroo* was confirmed by Vassal (1974) and Harmant *et al.* (1975).

In some *Acacia* species, the accepted subspecies have variable chromosome numbers: e.g. *A. tortilis* subspecies *tortilis* (Forssk.) Brenan, ssp. *spirocarpa* (A. Rich.) Brenan, ssp. *heteracantha* (Burchell) Brenan and ssp. *raddiana* (Savi) Brenan have both 2n=4x=52 and 2n=8x=104 (Darlington and Wylie, 1955; Olng'otie, 1992). Hybrid swarms can also deviate from the expected chromosome number: e.g. the triploid 2n=3x=39 reported for *A. laeta* by Khan (1951) and confirmed by Elamin (1976). Although sterile trees of *A. karroo* have been observed in nature (Ross, 1971c), their chromosome numbers have not been reported, and they were not investigated in this study, as seeds were used to produce roots for chromosome assay. No cytological survey to determine the distribution of cytotypes among the existing ecotypes and proposed varieties had been undertaken for *A. karroo.*

The aims of this study were to ascertain whether there are cytotypes other than 2n=4x=52 among the variable ecotypes of *A. karroo*, and to determine the distribution of cytotypes within the populations, so as to be able to interpret any complexity of allozyme data caused by variability in the ploidy level.

3.2.2. Materials and Methods

Chromosome numbers were examined using the root-tip squash method on actively growing roots, with modifications for the species. Seeds of *A. karroo* were collected from the various populations as listed in Table 3.1, which are a sub-set of those listed in Table 2.1. This sampling was mainly based on variation on seed size, colour and geographical locations within the species' range. It is worth noting here that seeds from some populations had consistent distinctive morphological features including size and thickness of testas, which influenced germination

period and growth rate. Ten randomly selected seeds from each population were germinated in petri dishes at a temperature of about 30°C. The larger seeds took longer (3-4 days) to germinate, and their root growth to the required length was more rapid than that from small seeds, which germinated within 24 hours, but were not ready until the third or fourth day.

The rapidly growing roots were severed when they were about 2-3 cm long and pretreated in either saturated aqueous solution of paradichlorobenzene (PDB) at 4°C or 2 mM 8-hydroxyquinoline at room temperature (18°C) for 4-7 hours. Pretreated roots were then fixed in freshly prepared acetic alcohol (1:3 parts v/v glacial acetic acid to absolute ethanol), for at least one hour, or until required. Roots were hydrolysed in a Normal solution of hydrochloric acid in a water-bath at 60°C for 8-12 minutes, then stained in Feulgen (85.9 ml commercial Schiff's reagent in 1 l 1 M HCl) at room temperature in the dark for at least one hour. The stained portion of the root was then chopped into smaller pieces under a microscope. A drop of 2% aceto-orcein [2 ml 1M HCl, 9 ml stock solution (4.4 g orcein powder in 100 ml glacial acetic acid) and 11 ml H_2O] was added to aid staining, definition, and spreading of individual cells. Squashing was done under a clean coverslip overlaid with chromatography paper to remove excess stain. Slides were temporarily ringed with rubber solution to stop desiccation before permanizing using carbon dioxide under pressure (Bowen, 1956 cf. Johnson and Taylor, 1989) and euparol. Slides were frozen, then dehydrated in absolute ethanol before euparol was applied. Mounted slides were maintained in an incubator at a temperature of about 43°C. for 2 weeks for euparol to harden.

Chromosomes were counted, and photographs taken with a Zeiss Photomicroscope III with a X 100 oil immersion objective. Permanent

slides made from the present survey, and negatives of the photographs were stored in the Jodrell Laboratory, Royal Botanic Gardens, Kew, UK.

3.2.3. Results and Discussion

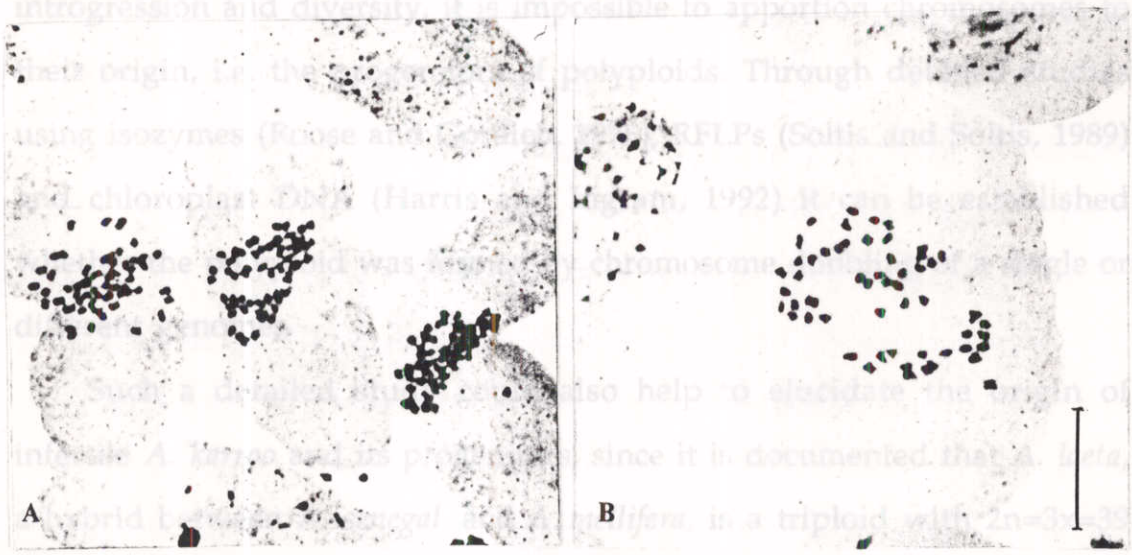
The most easily counted chromosomes were obtained from pretreatment with paradichlorobenzene for 7 hours and the a hydrolysis time of 12 minutes. Trials with a shorter pretreatment time or 8-hydroxyquinoline did not render the material suitable for adequate chromosome staining. Difficulties of staining the root tips of other acacias with Feulgen or aceto-carmine have been reported by Khan (1951) and Olng'otie (1992). The former attributed the problem to tannin content, while the latter observed similar problems on slightly older material and concluded that it was due to woodiness. This study also faced the same problems, while the tendency of the assay tissue to stick together makes even the spreading of cells more difficult; but with an empirical approach on tender roots, a satisfactory stain and spread can be obtained.

The survey revealed that all populations studied had only one cytotype with 2n=4x=52 chromosomes, as indicated in Table 3.1. Chromosomes were too tiny to observe variations in karyotypes, but on well squashed cells, with every chromosome separated, some chromosomes appeared lager than others and could easily confuse the total number (Fig. 3.1). Variation in chromosome sizes within the nuclei of some *Acacia* species has been documented by Khan (1951) for allopolyploids of *A. arabica* and *A. farnesiana,* with chromosome lengths ranging from 0.9-1.55 µm and 1-1.7 µm respectively. Each species was reported to exhibit a total of 52 chromosomes.

Table 3.1. Chromosome number in *A. karroo* Hayne.

		Origin			Jodrell cyto.	2n
Country	Population	Long.	Lat.	Alt.	No.	
Zambia	Lusaka	15°24'S	28°18'E	1280m	929	52
Zimbabwe	Umguza Valley	20°10'S	28°43'E	1200"	9210	52
Malawi	Dedza	14°19'S	34°16'E	1300 "	9210-2	52
Malawi	Zomba	15°36'S	35°10'E	1030 "	9210-1	52
S. Africa	Manzingwenya	27°15'S	32°46'E	100 "	9213	52
S. Africa	Hartbeespoort	25°44'S	27°52'E	1326 "	9214	52
S. Africa	Tugela Mouth	29°20'S	31°16'E	58 "	9215	52
S. Africa	Vanrhynsdorp	31°35'S	18°43'E	122 "	9216	52
S. Africa	Hluhluwe	28°03'S	32°03'E	500"	9212	52
S. Africa	Queenstown	31°52'S	26°52'E	1077"	9211	52
S. Africa	Babanango	28°23'S	31°17'E	1300 "	9212-1	52
S. Africa	Sardinia Bay	33°52'S	25°12'E	58 "	9215-1	52

Figure 3.1. Chromosomes of *A. karroo*.



A *A. karroo* (9214), adjacent dividing cells; **B** *A. karroo* (9214) 2n=52. Scale bar = 10µm.

The growth rate had direct influence on the mitotic index of the material. Fast growing material had a greater number of dividing cells,

and hence a better chance of providing good results. The high growth rate was obtained when seeds were raised at 30°C as opposed to room temperature.

Despite the reported morphological variability in the ecological zones where *A. karroo* is distributed (Ross, 1979), the species has retained a similar chromosome number within its range. There are possibilities that hybrids occasionally occur in areas of introgression with other *Acacia* species, for example with *A. tenuispina*, but such cases should be sampled specifically (Robbertse *et al.*, 1981). Since *A. karroo* is a polyploid, if it cross-breeds with other *Acacia* species with similar genome, the hybrids formed will also have the same complement of chromosomes and identification will not be possible without a thorough study. Brenan (1983), reporting on *A. tortilis*, noted that hybridization even at sub-species level can cause taxonomic uncertainities unless sufficient evidence is studied. Until a detailed examination of karyotypes is undertaken in these areas of introgression and diversity, it is impossible to apportion chromosomes to their origin, i.e. the progenitors of polyploids. Through detailed studies using isozymes (Roose and Gottlieb, 1976), RFLPs (Soltis and Soltis, 1989) and chloroplast DNA (Harris and Ingram, 1992) it can be established whether the polyploid was formed by chromosome doubling of a single or different genomes.

Such a detailed study could also help to elucidate the origin of infertile *A. karroo* and its progenitors, since it is documented that *A. laeta*, a hybrid between *A. senegal* and *A. mellifera*, is a triploid with 2n=3x=39 chromosome number, but remains fertile (Khan, 1951; Elamin, 1976). The confirmation that all ecotypes sampled are tetraploids is useful in allozyme analysis, which in turn will reveal the nature of the polyploid (Chapter Three, Part 3): autotetraploid, allotetraploid, or segmental

tetraploid. Olng'otie (1992) found, using isozyme techniques, that all sampled populations of *A. tortilis* were autopolyploids.

3.3. ELECTROPHORETIC EVIDENCE FOR POLYPLOIDY AND ALLOZYME POLYMORPHISM IN *A. KARROO.*

3.3.1. Introduction

Application of enzyme phenotypes to ascertain ploidy level has been demonstrated for many plant species, among them *Tragopogon* species (Roose and Gottlieb, 1976), *Coreopsis grandiflora* (Crawford and Smith, 1984), *T. menziesii* (Soltis and Rieseberg, 1986), and *Cystopteris tennesseensis* (Haufler *et al.*, 1990). In these cases, polyploids were shown to display isozyme multiplicity relative to diploids. Through the allozyme data, the nature of polyploidy can be determined, since multisomic inheritance is associated with autopolyploids, while fixed heterozygosity is a character of allopolyploids (Roose and Gottlieb, 1976; Waples, 1988; Krebs and Hancock, 1989; Cai and Chinnappa, 1989).

Cytologically, *A. karroo* is grouped among the polyploids of African *Acacia* species, with a chromosome set of 2n=4x=52 (Atchinson, 1948; Khan 1951; Darlington and Wylie, 1955; Vassal, 1974; Ross, 1979; New, 1984; Chapter 3.2). However, the exact ploidy level of *Acacia* species, but more generally, the members of the family Leguminosae, to date still remains a matter of scientific interest (see Goldblatt, 1981). Whilst some studies in acacias (Grimpu, 1929; Atchinson, 1948; Darlington and Wylie, 1955; Vassal, 1974; Harmant, 1975) indicated that x=13 is the base chromosome number, others, for example, Khan (1951) and Goldblatt (1981) do consider the base number to be x=7, followed by aneuploidy reduction to x=13 in a tetraploid state. In that case, most *Acacia* species

currently accepted as diploids were of ancient polyploid origin. Khan (1951) provided a detailed evidence based on the observations on nucleoli and chromosome counts in *A. decurrens, A. dealbata, A. mollisima, A. senegal, A. mellifera x A. senegal, A. arabica, A. modesta* and *A. farnesiana* to confirm that acacias were ancient polyploids. Elsewhere, studies of Oncidiinae with 2n=10-60 chromosomes (Chase and Olmstead, 1988), *Psilotum nudum* with 2n=104 (Soltis and Soltis, 1988), and homosporus pteridophytes (Haufler, 1989) indicate that higher chromosome number alone was not a guarantee for polyploidy. It is most likely that studies based on molecular techniques will settle the question of ploidy level in acacias as they are accomplished. The level of ploidy can be verified by techniques based on DNA (Wolf *et al.*, 1990), but inheritance patterns of allozymes will also identify the status (Hart, 1970; Odrzykoski and Gottlieb, 1984). Electrophoretic characteristics associated with duplicated genes include the number of loci displayed, asymmetrical banding patterns, fixed heterozygosity, and multiple alleles within the same locus (Barrett and Shore, 1989; Gottlieb, 1981; Weeden and Wendel, 1989; Kephart, 1990). In polyploids, such duplication is expressed at many loci rather than at the one or two which may also be found in diploid species (Gottlieb, 1982).

Brain (1986; 1989) carried out an initial analysis of electrophoretic polymorphism of *A. karroo*, based on the enzyme peroxidase. The work revealed that some genotypes expressed multiple bands that were not consistent with the normal disomic inheritance pattern of diploid organisms. The work, however, covered only a small region of the species' geographical range, and sampled only one enzyme system. Ayala and Kiger (1984) recommended that unbiased genetic analysis be based on a minimum of 20 isozyme loci, while Baverstock and Moritz (1990) reduced this number to a minimum of six gene-markers. Generally, the number of isozyme-markers used should depend on the objectives of the research.

This chapter examines the possible genetic interpretation of 12 enzyme systems encoded by 19 gene loci, sampled from 26 populations, covering the geographical range of the species. Inferences are drawn about the possibility of a higher level of ploidy, its nature, and genotypic polymorphism.

3.3.2. Materials and Methods

3.3.2.1. *Plant material*

Seed samples from individual trees were collected from the 26 populations listed in Table 2.1 and identified in Figure 2.1. Other relevant details were summarized in section 1.3 and 2.2.1. In total, seeds for analyses were collected from 888 trees.

3.3.2.2. *Electrophoretic phenotypes*

In order to determine the electrophoretic relationship within and among populations, all the 12 enzymes assayed (Chapter Two) were scrutinized for variation in patterns. 19 loci expressed banding patterns that could reflect the ploidy level of the study species. The enzymes were designated according to the international nomenclature followed by arabic numbers, with the most anodal designated as the first locus. All the assayed enzymes migrated anodally, and the number and patterns of bands which constitute a locus were inferred from the subunit structure of the enzyme as described by Gottlieb (1981), Wendel and Weeden (1989), Kephart (1990), Crawford (1990), and Murphy *et al.* (1990). Alleles at a locus were specified by lower case letters following Cai and Chinnapa (1989) and Shore (1991). Where two loci migrating to same position on a gel, the possible homoeologous alleles were assigned similar letters, but the most

anodal was differentiated with an apostrophe, or two in the case of cathodal homoeolog.

To confirm that the migration of allozymes at a locus was similar, samples from various populations were run adjacent to each other on the same gel and, in several combinations, for all the enzymes. Thereafter, 20 samples of progeny arrays of individual trees, from different populations, were run separately to assist in understanding allelic segregation (see Fig. 3.2). The phenotypes were thereafter scored into genotypes, assuming that the variation in band patterns reflected allelic polymorphism, described as "the occurrence together in the same habitat of two or more discontinuous forms of a species (in this case, a trait or an allele) in such proportions that the rarest of them cannot be maintained by recurrent mutations" (see Ford, 1976). The ratios of the various genotypes at every locus, for all polymorphic loci, to the total sample size were then expressed as genotypic frequencies. Only the 12 populations which had sample sizes greater than 20 were entered for comparative analyses. Smaller populations were referred to principally when they displayed rare genotypes.

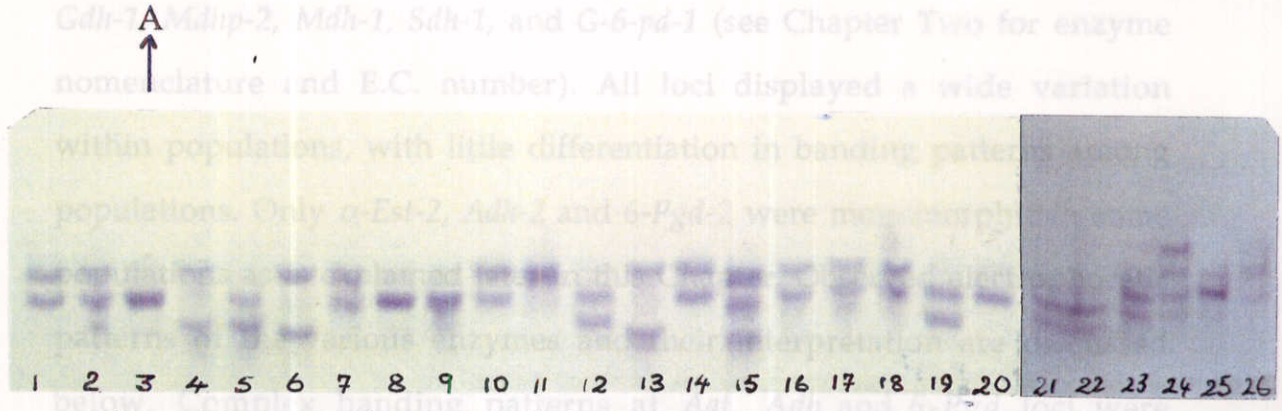


Figure 3.2. Segregation of alleles in 20 progenies of a maternal tree at the shikimate dehydrogenase locus. Genotypes with single bands were scored as homozygotes, and with more than one band - heterozygotes. Heterozygotes were scored as balanced when the bands stained evenly, and unbalanced when the staining intensity of the bands differed. Homozygotes are represented in columns 3, 8, 9, 11 and 20; triallelic genotypes in column 2 &15 the rest are balanced heterozygotes. (Photo 291659). The arrow indicates anodal migration. No. 21-26 are from another maternal tree showing tetraallelic (24) and unbalanced heterozygote (25) (Photo 994241).

According to Moran and Hopper (1987), alleles can be classified as widespread when they occur in two or more populations, and rare if they occur in only one population. Those alleles occurring in one population with a frequency greater than 10% are common, while those with a frequency of less than 10% in any population are rare. Here, a parallel classification, with modifications, has been applied to genotype frequencies. Genotypes from single loci were categorized as widespread, regional, or rare. Arbitrarily, genotypes that appeared at frequencies greater than 10% in 5 to 12 populations were classified as widespread, those with similar frequency in 1 to 4 populations were designated as regional, while those that occurred in lower frequencies in one or more populations were identified as rare.

3.3.3. Results and Discussion

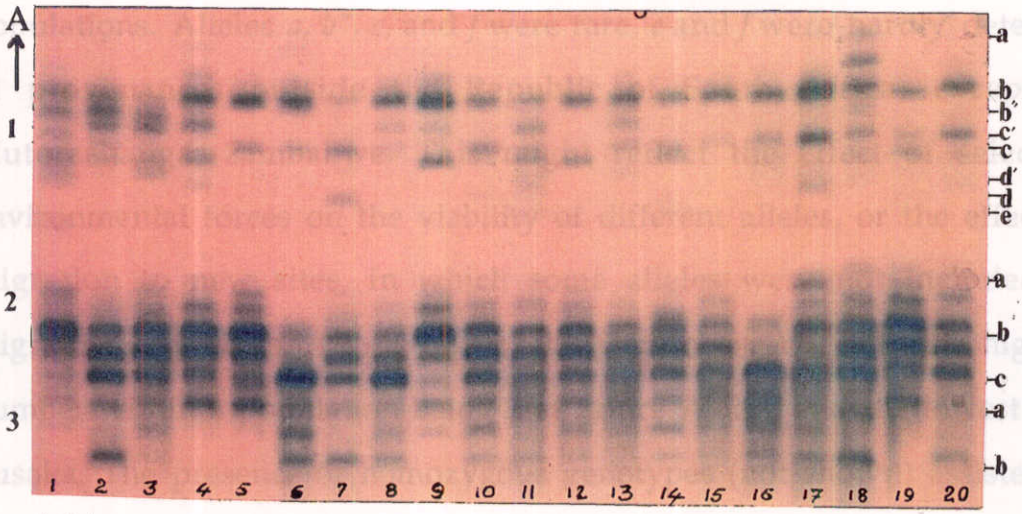
The 19 loci included in the analysis were *Aat-1, Aat-2, Aat-3, Adh-1, Adh-2, Dia-1, α-Est-2, β-Est-1, Mr-1, Mr-2, Mr-3, Mr-4, 6-Pgd-1, 6-Pgd-2, Gdh-1, Mdhp-2, Mdh-1, Sdh-1,* and *G-6-pd-1* (see Chapter Two for enzyme nomenclature and E.C. number). All loci displayed a wide variation within populations, with little differentiation in banding patterns among populations. Only *α-Est-2, Adh-2* and *6-Pgd-2* were monomorphic in some populations as is explained later in this Chapter. Observed electrophoretic patterns of the various enzymes and their interpretation are discussed below. Complex banding patterns at *Aat, Adh* and *6-Pgd* loci were interpreted assuming that alleles were expressed by homoeologous chromosomes, such interpretation will definately alter if these bands reflect different overlapping loci and any other migration pattern.

3.3.3.1. Aspartate aminotransferase (Aat)

All populations exhibited three isozymes of *Aat* (Fig. 3.3a). Locus *Aat-1* was the most complex, with some genotypes having a maximum of four alleles. In total, six alleles were recorded, as *a, b, c, d, e,* and *f* (*f* not shown in Fig. 3.3a). Inheritance mode was mixed tetrasomic-disomic in all populations; thus, there were two alleles which could appear either as a pair, or independently. This feature was noted with alleles *b, c,* and *d*. Their counter-parts were named as alleles *b"*, *c'*, and *d'*, respectively. It was observed that these alleles affected the position of heteromeric bands, but there were no heterodimer bands formed between the original and the counterparts (Fig. 3.3a). Another notable phenomenon at this locus was the failure of some alleles to form interallelic heterodimers in some heterozygous genotypes. *Aat* is a dimeric enzyme and different alleles within a locus in a heterozygote should form heterodimers (Gottlieb, 1973c; Roose and Gottlieb, 1976; Weeden and Wendel, 1989). Here, the most likely explanation for the failure of the the alleles to form heterodimers is the difference in the homology of the allozymes. These alleles might have originated from homoeologous chromosomes.

Another characteristic indicating such a likelihood of difference in origin of these alleles was their expression either as single bands or as pairs in different genotypes. A similar inheritance pattern has been observed in *Stellaria longipes* (Cai and Chinnappa, 1989). The locus showed signs of fixed heterozygosity in some samples with multiple alleles, which suggested the possibility of an allopolyploid. In an allopolyploid, alleles from the genomes are inherited additively and might be structurally different to prevent any interaction (Gottlieb, 1981). Alternatively, such a complex banding pattern may reflect an overlap of two loci comigrating on the gels. In such a case, the interpretation of the banding patterns presented here will change.

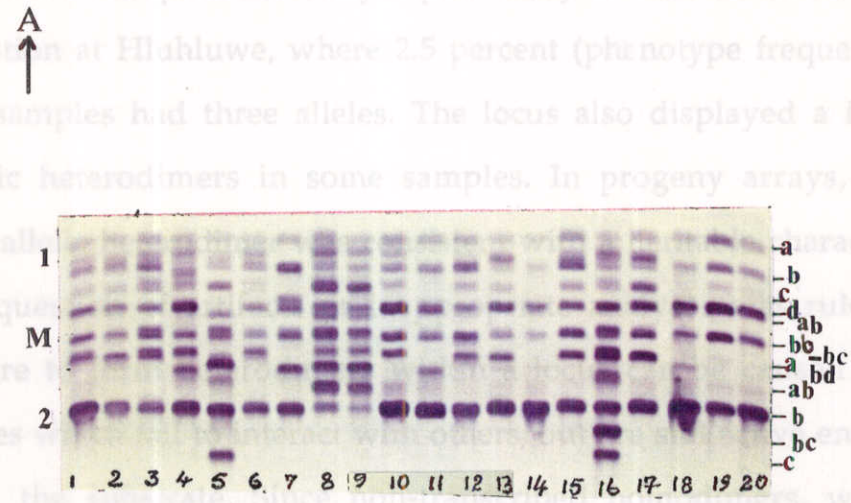
Figure 3.3a. Electrophoretic phenotypes of *Aat* isozyme loci of *A. karroo*.



Aat-1:

Left to right: bb"c'c, bb"b"c', b"b"cc, abcd", bbbc'[8], abbc', bddd, bbbc', bbcc[1], bbbd', bc'ce, bbbe, bbc'c[3], bbdd, bbbn, bbcd', abbd, abc'd, bbce & bbcd. Note the absence of heterodimers and low dosage effect in some phenotypes (see text for discussion). The superscripts refer to genotype distribution and frequency on Table 3..2. ***Aat-2:*** bbbc[3], bccc[2], bbcc, bbbc, bbbc, cccc4, bbcc[1], bccc, bbbc, bbcc, bbcc, bbcc, bccc, bbcc, bccc, cccc, aacc, abcc, abbc & abcc. : ***Aat-3:*** aaan, aabb[1], aaab, aaaa[2], aaaa, abbb, abbb, abbb, aabb,aaan, aabb, aabb, aaab[3],aaab, aabb, aabb, aabb, abbb[4], aaaa & abbb. Note the absence of homodimer in number 1 from left, and heterodimer on number 2, 7 & 18. (Photo 303194). n = null allele.

Figure 3.3b. Electrophoretic phenotypes of *Adh* isozyme loci of *A. karroo*.



Adh-1: aabc[2], aabc, abcc[3], abcd[4], abcc, abbc[1], abdd[9], abbc, abbc, aabc, aabd[8], abbc, aabc, aabb[5], abbc, abbc, abcc, aabd, aabc, & aabb. Note the double alleles in No. 3 from left, comigration with allele *d* in 4, and comigration due to double heterozygosity in 5, 8, 9 &16. ***Adh-2*** : bbbb[1], bbbb, bbbb, bbbb, aacc[6], bbbb, bbbb, aabb[2], aaab[10], bbbb, bbbb, bbbb, bbbb, bbbb, bbbb, bbcc[4], bbbb, bbbb, bbbb & bbbb. (Photo 127599).

The most frequent band was *b* which approached fixation level in all populations. Alleles *a*, *b"*, *e*, and *f* were rare, *e* and *f* were hardly detected in provenances outside the Republic of South Africa, except at Mutorashanga, Zimbabwe. This might reflect the effect of selective environmental forces on the viability of different alleles, or the effect of migration to new sites, in which some alleles were not included in migrating genotypes. A total of 9 alleles were observed, with the highest number within a population being 7 at Prince Albert, and the lowest 4 at Lusaka. The presence of homozygous genotypes (not shown) assisted in maintaining the interpretation on the possibility that this locus was a reflection of multiple alleles within a locus of an allotetraploid.

Aat-2 exhibited three alleles displaying a tetrasomic mode of inheritance. Some samples expressed all three alleles together, with symmetrical staining.

Aat-3 was predominantly expressed by two alleles with an exceptional situation at Hluhluwe, where 2.5 percent (phenotype frequency 0.025) of the samples had three alleles. The locus also displayed a lack of inter-allelic heterodimers in some samples. In progeny arrays, the missing intraallelic heterodimer was consistent with a heritable characteristic, thus the question of artifacts or inappropriate analyses was ruled out. Such failure to form heterodimers within a locus can be caused by defective alleles which fail to interact with others, but are still active enough to react with the substrate. Since non-transcribed homodimers, which formed heterodimers were also encountered, it could also be possible that there were null alleles. Similar observations were reported for phosphoglucose isomerase in artificial mutants of *Clarkia xantiana* (Jones *et al.*, 1986), who demonstrated that such defects were heritable. Similarly, such failure to interact could indicate that these alleles were homoeologous. Inter-locus heterodimers were consistently absent in all populations, which suggested

that the *Aat* isozymes in *A. karroo* are variable in homology and are possibly localized in different cell compartments (Weeden and Wendel 1989).

A secondary band of activity anodal to loci one and two was noticed, but its genetic role could not be established as it was very variable in intensity and was not consistent with any subunit structure. This phenomenon was consistent with activities that had been reported for *Stephanomeria exigua* (Gottlieb, 1973c), which were termed "ghost bands". Kephart (1990) suggested that these bands are characteristic of some enzyme systems. Here, it can be suggested that these bands seems to represent silenced alleles. Their activities varied among individuals and, in family progeny arrays, the characteristic inheritance patterns could be observed in the bands.

3.3.3.2. *Alcohol dehydrogenase (Adh)*

Three zones of activity were recognized in this enzyme. The most anodal was designated as *Adh-1*. The middle part was interpreted as a zone of interaction, while the cathodal zone was named *Adh-2* (Fig. 3.3b). *Adh-1* showed very little variation within and between populations.

Three bands were common, but up to five bands were obtained in some samples of some populations. These bands were interpreted as three to four alleles. The fourth allele, *d*, was not found in some populations (see Table 4.1). At the locus *Adh-1* only allele *a* and *c* formed heterodimer band which comigrated with allele *b*. No heterodimers were found formed between other putative alleles at this first locus.

Alcohol dehydrogenase is a dimeric enzyme and is expected to form intragenic heterodimers in heterozygous situation (Hart, 1969, 1970; Efron *et al.*, 1973; Marshall *et al.*, 1974; Brown *et al.*, 1974; Roose and Gottlieb, 1980). According to Gottlieb (1982), the ability of two alleles to form

heterodimers is a sign of structural similarity. Thus the most likely explanation for alleles *a,b, c* & *d* at *Adh-1* locus selectively forming heterodimer bands could be based on their structural relationship: firstly, it is possible that alleles *a* and *c* originated from one genome, while others have various homology; secondly, alleles *b* and *d* might have undergone mutational changes, and may therefore have deviated from the other two.

The fact that *Adh-1* showed fixed hetorozygosity (alleles *a* and *b*) in most samples assayed supports the possibility of an allotetraploid, hence the divergence in homology of various alleles. In certain cases, instead of an allele being represented by a single band, two bands appeared close together, stressing the possibility that homoeologous loci existed. The second hypothesis might not hold because the frequency of mutations is usually low (see Ayala and Kiger, 1984), and that without deleterious effects is even lower. Most development of new genetic material, and hence speciation, is more easily explained by hybridization (Barber, 1970). It is most unlikely that two alleles would mutate at the same time, with higher deviations from the remainder, and still remain positively functional. The observation that some alleles never formed heterodimers at this locus corroborates with results of *Aat*. In general, *Adh-1* was less intensely staining than the other two zones, but dosage effects were sufficiently well expressed that co-migration and double alleles could be scored (Birchler, 1983).

Adh-2 was composed of four alleles, *a, b, c,* and *d,* which showed a tetrasomic mode of inheritance. No sample expressed more than two alleles. Over 66% of all samples were homozygous for allele *b* (see Fig. 3.3b), and, in Manzingwenya, all samples were monomorphic for this allele. Allele *d* was very rare, and was only recorded at Dedza and Babanango. Cases of null alleles were also observed at this locus, in the following percentages of their total sample sizes: Cintsa River, 16%; Dedza,

3.8%; Mutorashanga, 2.5%; Queenstown, 1.5%. Bands at this locus stained intensely, assisted in detecting null alleles, homozygotes, balanced and unbalanced heterozygotes. The null alleles possibly were of two types: those which were totally inactive, and a second group which formed heterodimers with the first locus, but were themselves not transcribed to form bands with the substrate.

The middle zone was formed by inter-locus heterodimers and was also intensely stained (marked M in Fig. 3.3b). The number of bands and the staining depth depended on the activity of the putative homodimers in *Adh-1* and *Adh-2*. Therefore, these bands were useful for detecting cryptic alleles and their status (double or single) in the *Adh-1* complex. Interaction of *Adh-1* and *Adh-2* was even more spectacular in that some cryptic alleles ended up being blended and migrating separately, while other alleles migrating closely ultimately formed comigrating heterodimers. Some of the inter-locus heterodimers formed between null alleles at the second locus and putative alleles at first locus showed higher activity by staining more intensely. Jones *et al.* (1986) found that, in *Clarkia* species, genotypes heterozygous for null alleles showed normal growth, but homozygous genotypes were never recovered, leading to the conclusion that the state was lethal.

In a separate study with *Clarkia* species, Gottlieb and Higgins (1984) found that approximately the same level of enzyme is maintained in organisms with and without duplicated loci. Variable regulation of enzyme activity has also been reported in *Zea mays, Lupinus angustifolius* and *Lycopersicon* species subjected to waterlogging and various culturing techniques in transplanting (Marshall *et al.*, 1974; Tanksley, 1983). These observations indicate that an organism always tends to acclimate to internal and external enviroments in order to maintain an optimum fuctional level of an enzyme for survival. It is therefore possible that the

available resources are always mobilized to functional alleles; thus, for genotypes with duplicated alleles the activity is reduced, but in those with missing alleles, it is increased to compensate for null alleles. This could have caused the deeper staining dosage visualized in some heterodimers formed with null alleles (see Fig. 3.5 in this Chapter).

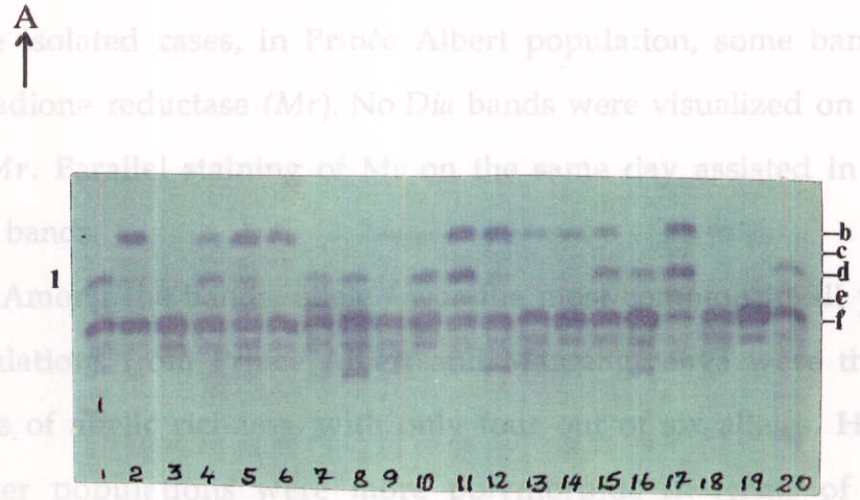
Given the available experimental evidence, one can only speculate on the reason why *Adh-2* formed heterodimers with all alleles in *Adh-1*. It is probable that this second locus, *Adh-2* was the original, and would therefore have been common to progenitors of *A. karroo*. *Adh-1* may have differed in the two progenitors, but retained more similarities to *Adh-2* than to each other. Both loci of *Adh* expressed characteristic ghost bands.

3.3.3.3. *Diaphorase (Dia)*

Dia had three zones of activity which were interpreted as three encoding loci. The cathodal and the middle zones were not evenly resolved in many samples and were therefore omitted from the pattern analyses. The anodal locus, *Dia-1*, was clear, and formed the basis of polymorphism study in all populations. The enzyme displayed a total of six bands (*a, b, c, d, e, f*), of which one sample could express between one and four bands but none expressed more than four together (Fig. 3.3c). Allele *a* was expressed at Kroonstad.

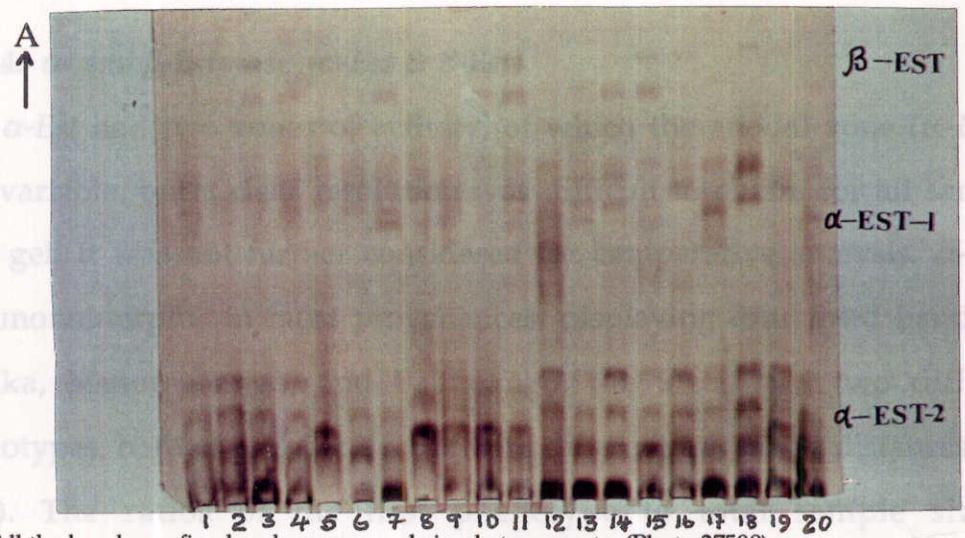
This enzyme is known to display considerable variation from species to species in terms of isozymes expressed, and in quaternary structure. In the reviews by Weeden and Wendel (1989) and Kephart (1990), the enzyme is presented as a monomer, a dimer, or a tetramer. In *A. karroo*, the expression of single, double, triple and quadruple bands could best fit a monomer. There was also unbalanced staining, a factor which indicated tetrasomic inheritance. Diaphorase is one of reductase enzymes, which are

Figure 3.3c. Electrophoretic phenotypes of *Dia-1* isozyme locus of *A. karroo*.



Dia-1: ddff, bbff^5, ffff^1, bdfff^8, bdff, bbff, deff^2, dfff, bfff, ddff^4, bddf, bbff, bfff, bfff, bdff, dfff, bbdf, bfff, ffff, & deff. (Photo 27598).

Figure 3.3d. Electrophoretic phenotypes of α- *Est* isozyme locus of *A. karroo*.



All the bands are fixed and every sample is a heterozygote. (Photo 27598).

not substrate specific (Wendel and Weeden, 1989) and may stain with other reductase enzymes. This was not a common phenomenon, but in some isolated cases, in Prince Albert population, some bands imitated menadione reductase (*Mr*). No *Dia* bands were visualized on gels stained for *Mr*. Parallel staining of Mr on the same day assisted in eliminating stray bands.

Among the bands, allele *f* was the most common in all populations. Populations from Prince Albert and Manzingwenya were the lowest in terms of allelic richness, with only four out of six alleles. However, the former populations were more polymorphic in terms of phenotypic patterns, with eight phenotypes: the latter had only four.

Accessible data show that diaphorase enzyme has been examined in three other acacias (Moran *et al.*, 1989a; 1989b), but these were diploids with only one active locus reported. All had three alleles at the reported locus. *A. karroo* displays more alleles and more loci, consistent with the polyploid state.

3.3.3.4. α- and β-Esterase (α-Est & β-Est)

α-Est had two zones of activity, of which the anodal zone (*α-Est-1*) was variable, but a clear resolution was difficult to obtain for all samples on a gel. It was not further considered for comparative analysis. *α-Est-2* was monomorphic in most provenances, displaying four fixed bands. At Lusaka, Mutorashanga, and Babanango, the locus had two different phenotypes, both of four bands, but with different migration distances (Fig. 3.3d). The ratios of the first phenotype to total sample size at Mutorashanga, Babanango and Lusaka were 75%, 25% and 10%, respectively. Two samples at Umguza Valley also displayed a pattern which seemed to exhibit heteromeric bands. This was an interesting observation within this species, but not unusual for the enzyme. Gottlieb

(1981) cites barley as an example of a species with a complex inheritance mode of *Est*. Although the form of *Est* often reported is monomeric, other forms with a dimeric subunit structure have been found (*see* Gottlieb, 1981; Murphy *et al.*, 1990).

β-Est also exhibited two loci: the most anodal was scored as *β–Est-1*, and the cathodal as *β-Est-2*. *β-Est-2* displayed variable resolution in different provenances. In some, e.g. Umguza Valley, it consisted of a single monomorphic band which was fast fading out before others were stained. At Kroonstad, two bands were evident, one fading and the other one normally stained, but both were fixed. Only at Manzingwenya were the two bands segregating and did not fade out. *β-Est-1* expressed five bands which were scored as alleles *a, b, c, d,* and *e* respectively, in a monomeric enzyme system. Allele *b* was predominant in most populations, except at Mutorashanga and Cintsa River, where *c* was more common, and Lusaka where *d* showed a higher frequency. Prince Albert had the highest percentage of samples with four alleles expressed (4.3%), and the highest frequency of homozygotes (34.8%) of total samples. Most samples expressed two to three bands and were highly polymorphic (Fig. 3.4).

Esterases are known to be highly polymorphic in plant species, e.g. in *Hordeum spontaneum*, 27 alleles were assayed from four loci. They are also known to harbour cryptic alleles; thus, by varying the electrophoretic assay conditions, 8 *Est-1* alleles of *Peromyscus maniculatus* were increased to 35 alleles (Aquadro and Avise, 1982; cf. Murphy *et al.*, 1990), while other enzymes such as *Mdh* and *Aat* remained unchanged. Tanksley (1983) reported that esterase may be environmentally induced. The enzyme may expess dominance inheritance also (Abbott *et al.*, 1992). All these uncertainities, and the fact that they are non-specific to substrates (Wendel and Weeden, 1989), tend to limit wider utilization of esterases.

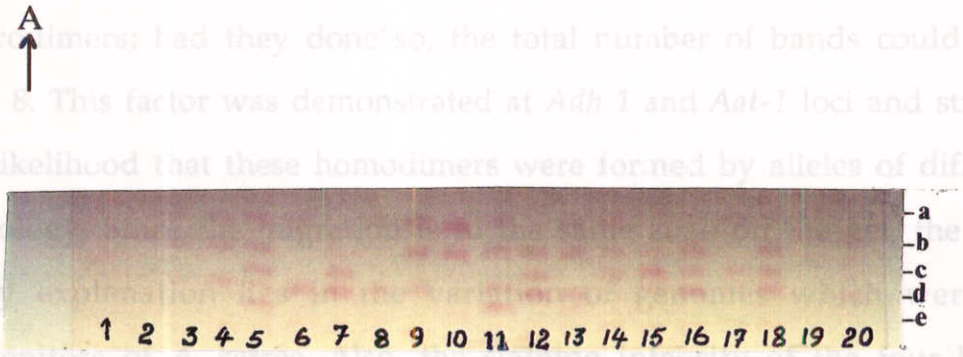
However, whenever assay conditions of an *Est* locus is perfected , and its genetics is determined to eliminate tissue specificity and dominance inheritance (Abbott *et al.*, 1992), useful inferences can be made. The inheritance mode of *β–Est-1* in *A. karroo* was typically tetrasomic, with homozygous, balanced and unbalanced heterozygous patterns. The availability of samples with two, three, four and five alleles (Fig. 3.3e) show that there is polymorphism and higher ploidal level.

3.3.3.5. 6-Phosphogluconate dehydrogenase (6-Pgd)

6-Pgd is one of the enzymes of carbohydrate metabolism which is frequently assayed in plant species. In *A. karroo,* two isozyme loci of 6-phosphogluconate dehydrogenase were found. The anodal locus was designated *6-Pgd*-1, and the cathodal *6-Pgd-2*. Of the two loci, *6-Pgd-1* was the most complicated and differentiated *A. karroo* population into two groups; identified as northern and southern. The northern populations including Lusaka, Mutorashanga, Umguza Valley, Dedza, Zomba, Kroonstad, Hluhluwe and Babanango displayed a pattern where the first two anodal alleles had very low variation, and were near fixation. The third and the fourth bands played a significant role in sample variation because they segregated independently. The remaining populations including Manzingwenya, Queenstown, Prince Albert and Cintsa River also had four bands, but all segregated as pairs, the first two being independent from the last two (Fig. 3.3f).

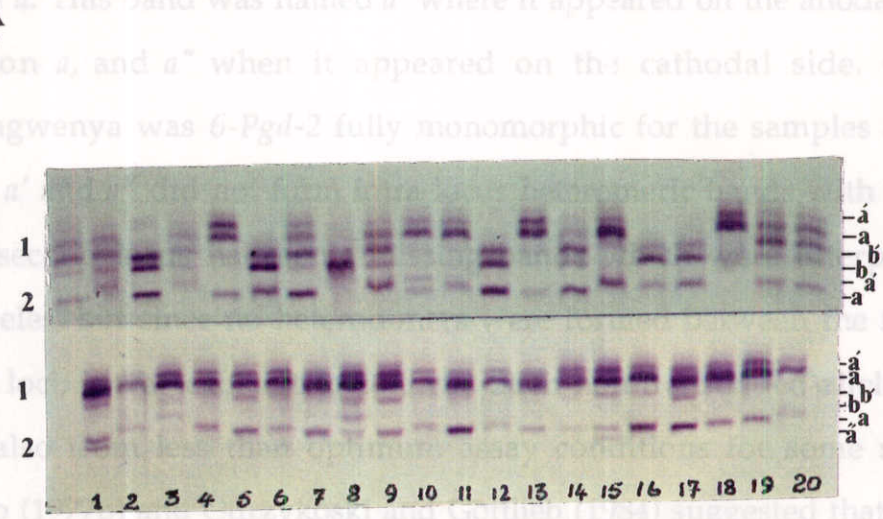
The bands were designated as *a', a, b', b,* with *a'* being the most anodal. The two middle alleles *a* and *b'* stained darker, especially when the *b* allele was present. This could be expected since the enzyme is a dimer (Weeden and Wendel, 1989; Kephart, 1990; Murphy *et al.*, 1990), and heterodimeric bands formed comigrated with *a* and *b'* homodimers. In phenotypes with three bands, deep staining of band *b'* could only reflect

Figure 3.3e. Electrophoretic phenotypes of *β–Est-1* isozyme locus of *A. karroo*.



Note the polysomic inheritance pattern expressed by five and four bands (column 5 &11) (Photo 185943).

Figure 3.3f. Electrophoretic phenotypes of *6-Pgd* isozyme loci of *A. karroo*.



6-Pgd-1: **(southern populations, see text);** a'ab'b^{2}, a'ab'b, b'b'bb, a'ab'b, a'a'aa, b'bbb^{9}, a'ab'b, b'bbb, a'ab'b, a'aaa, a'aaa, b'b'bb^{10}, a'a'aa4, a'ab'b, a'aaa^{1}, b'bbb, b'bbb, a'aaa, a'ab'b & a'ab'b. *6-Pgd-2:* aaaa, a'a'a'a', aaaa, a'a'a'a', aaaa, aaaa, aaaa, n, a'a'a'a', aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, n, aaaa & aaaa. *6-Pgd-1* **(northern populations):** aab'b', aaaa, a'ab'b, a'aaa, aab'b' a'aaa, a'ab'b, a'ab'b^{3}, a'aaa, a'aaa, a'aaa, a'aaa, a'ab'b, a'ab'b, a'aaa, a'aaa, & a'aaa. *6-Pgd-2*: aaa"a", aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaaa, aaa"a", aaaa, aaaa, aaaa, aaaa & aaaa. (Photo 127602 & 127599). n is null allele.

unbalanced heterozygosity. The most notable observation was that the four homodimers - *a'*, *a*, *b'*, and *b* - were not randomly forming heterodimers; had they done so, the total number of bands could have been 8. This factor was demonstrated at *Adh-1* and *Aat-1* loci and stresses the likelihood that these homodimers were formed by alleles of different homology. Since the migration is to the same zone on the gel, the most likely explanation lies in the variation of genomes which were the progenitors of *A. karroo*. Also, the staining intensity of the four bands varied; this was interpreted as dosage effect and further differentiated the samples. The frequency of all the electromorphs varied between populations.

6-Pgd-2 was predominantly monomorphic with one band expressed. However some phenotypes expressed a second band which migrated close to band *a*. This band was named *a'* where it appeared on the anodal side to common *a*, and *a"* when it appeared on the cathodal side. Only in Manzingwenya was *6-Pgd-2* fully monomorphic for the samples assayed. Alleles *a'* and *a"* did not form intra-locus heteromeric bands with putative *a*. The second locus had some missing bands which were interpreted as null alleles, but since no heterodimers were formed between the first and second loci, it was difficult to confirm. Such non-transcribed alleles could result also from less than optimum assay conditions for some samples. Gottlieb (1977b) and Odrzykoski and Gottlieb (1984) suggested that the two isozymes of *6-Pgd* do not form inter-locus heteromeric bands because they have different homology.

Since null alleles had been observed at other loci in *A. karroo* where they either formed heteromers or failed to be transcribed, there is a likelihood of their presence. Gottlieb (1981) described the presence of null alleles within a species as a character of ancient polyploid in which diploidization is being reestablished by silencing redundant genes

(pseudogenes). This view tallied with previous observations (Li, 1980) on fish, which suggested that normal functions can be maintained by a single locus of some enzymes, while redundant alleles are lost gradually.

6-Pgd-1 is usually (not always) located in cytosol, while *6-Pgd-2* is the plastid allozyme. The enzyme is highly conserved and is usually found as two isozymes, except in cases of duplication (Gottlieb, 1977b; 1981; 1982). A similar number of *6-Pgd* loci have been found in other acacias: *A. anomala* (Coates, 1988), *A. crassicarpa and A. auriculiformis* (Moran *et al.*, 1989a), *A. melanoxylon* (Muona *et al.*, 1991), and *A. tortilis* (Olng'otie, 1992). The fact that the two loci expressed patterns of fixed heterozygotes suggests a possibility of polyploidy rather than gene duplication in a diploid (Gottlieb and Weeden, 1979). These bands also exhibited asymmetrical staining of 1:3 in fixed heterozygotes (double homozygotes) and unbalanced heterozygotes.

There is also a possibility that the species had undergone polyploidization more than once, or it was formed between two separate allopolyploids, as evidenced by the appearance of fixed alleles which interchange positions at *6-Pgd-2* locus, and the variable pattern of northern versus southern populations at *6-Pgd-1*. Multiple origin of polyploids has been reported for allopolyploids of *T. mirus* and *T. miscellus* (Roose and Gottlieb, 1976; Soltis and Soltis, 1989) and *S. cambrensis* (Ashton and Abbott, 1992), in autopolyploids, *H. micrantha* and *H. grossularifolia* (Ness *et al.*, 1989; Wolf *et al.*, 1990). The low number of genotypes with two bands in *6-Pgd-2* probably indicate that the process of gene silencing of the duplicate alleles must be nearing completion at that locus. However, the existence of null alleles is contrary to the nature of isozyme conservation, in that there will be no active enzyme in one cell compartment. Although some investigations have shown that some genotypes can survive complete loss of some enzymes, e.g. leucine

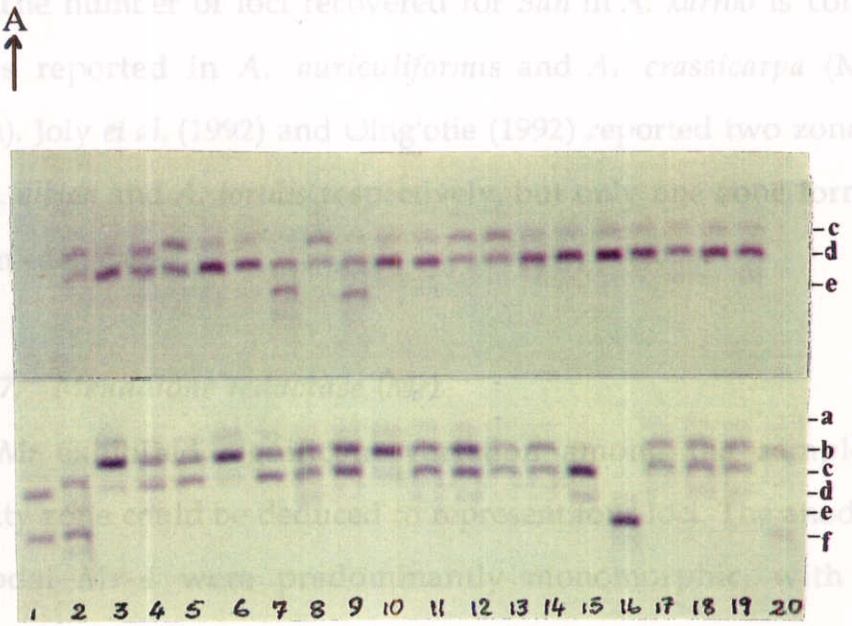
aminopeptidase in *Chenopodium* species (Wilson *et al.*, 1983) and glutamate dehydrogenase in lines of *Zea mays* (see Newton, 1983), it may vary with each enzyme. Genotypes of *A. karroo* with null alleles in one of the cell compartments perhaps face elimination at a later stage of development.

3.3.3.6. Shikimate dehydrogenase (Sdh)

Sdh exhibited one zone of activity which had up to six bands, representing six alleles, designated from *a-f*. The distribution of these bands followed a geographic pattern, as was the case with *6-Pgd-1*. Populations from Zambia, Zimbabwe (excluding Mutorashanga) and Malawi had only three ubiquitous alleles; Mazingwenya (S. Africa) expressed four, Mutorashanga (Zimbabwe), Hluhluwe and Babanango (S. African) populations expressed five alleles, while Prince Albert, Coega and Cintsa River, displayed six alleles.

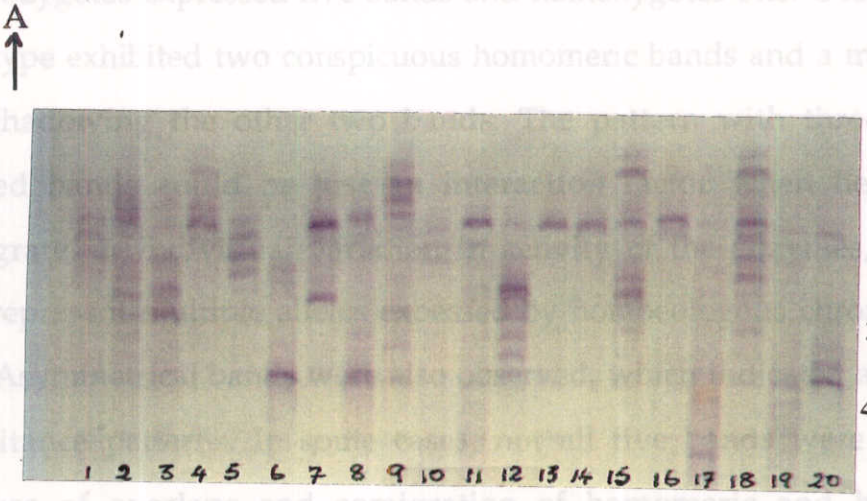
Sdh is a monomer (Weeden and Wendel, 1989; Kephart, 1990) displaying single bands for homozygotes, and double bands for heterozygotes in diploid species. In *A. karroo,* samples exhibiting homozygotes (i.e. *aaaa*) and heterotetramers (*i.e abcd, abbc, abbb*) were assayed. The genotypes were well reflected by the staining dosage effect (Fig. 3.3g). At Babanango, some genotypes exhibited four alleles which migrated closely together and, in Prince Albert, these alleles were separated slightly, but the pattern was maintained. Thus, the two most anodally migrating alleles were close together, separed by a gap from the cathodal alleles, which also migrated closely. However, the six alleles segregated independently whenever a genotype was not showing four bands. Apart from the sites above, genotypes with four bands were found at Hluhluwe, Cockhouse, and Sardinia Bay, but constituted only 2 percent of the genotype frequency (Fig. 3.4). That pattern with four bands provided

Figure 3.3g. Electrophoretic phenotypes of *Sdh-1* isozyme locus of *A. karroo*.



***Sdh-1* (northern population):** ccdd^{3}, cddd^{2}, ccdd, ccdd, cddd, cddd, ddee^{6}, ccdd, ddee, cddd, cddd, ccdd, ccdd, cddd, cddd, cddd, cddd, cddd, cddd & cddd ***Sdh-1* (southern population);** ccee, ccee^{9}, abbc, bbcc^{4}, bbcc, abbb^{10}, bbcc, bbcc, bbbb^{5}, bbcc, bbcc, bbcc, cccd^{7}, eeee, bbcc, bbcc, bbcc, bbcc, & bbff. (Photo 27598 & 127602).

Figure 3.3h. Electrophoretic phenotypes of *Mr* loci of *A. karroo*.



Note the variability in every sample and the number of loci designated 1-4 in menadione reductase above. (Photo 127602).

more evidence for polymorphism and tetrasomic inheritance in the species, hence confirming the polyploid nature of *A. karroo.*

The number of loci recovered for *Sdh* in *A. karroo* is consistent with others reported in *A. auriculiformis* and *A. crassicarpa* (Moran *et al.*, 1989a). Joly *et al.* (1992) and Olng'otie (1992) reported two zones of activity for *A. albida* and *A. tortilis* respectively, but only one zone formed scorable loci in each case.

3.3.3.7. Menadione reductase (Mr)

Mr exhibited significant variation among the samples, and the activity zone could be deduced to represent four loci. The anodal *Mr-1* and cathodal *Mr-4* were predominantly monomorphic, with the former having an estimated 5% and the latter 2% heterozygotes. The middle zone with *Mr-2* and *Mr-3* was very variable, with the bands confirming the existence of homozygotes and heterozygotes (Fig. 3.3h). The enzyme has been reported by Huenneke (1985) and Bousquet *et al.* (1988) to express tetrameric patterns, and this was confirmed in the present work. Heterozygotes expressed five bands and homozygotes one. Occasionally, a genotype exhibited two conspicuous homomeric bands and a middle band overshadowing the other two bands. The pattern with three intensely stained bands could be just an interaction factor when heterodimers comigrate, or individual variation in activity of the enzymes, but could also represent multiple alleles expessed by homoeologous chromosomes.

Asymmetrical bands were also observed, which indicated a tetrasomic inheritance patterns. In some cases, not all five bands were visualized because of overlaps and comigration of homomeric and heteromeric bands. The major difficulty with this enzyme was its sensitivity to assay conditions because of immense variability. It was difficult to find optimum conditions for the potential 20 allozymes on a four-loci

heterozygote, and for all 20 samples on a gel. Some samples on a gel did not give good resolutions for some loci, leading to paucity in data which hindered determination of the exact distribution of genotypes. Otherwise, the inheritance of *Mr* was typically tetrasomic and polymorphic, portraying the nature of ploidy and the genetic diversity in *A. karroo.* There were no interlocus heteromers formed, indicating that the isozymes are different in homology and, possibly, are localized in different subcellular compartments.

3.3.3.8. Glucose-6 phosphoglucose dehydrogenase (G-6-pd)

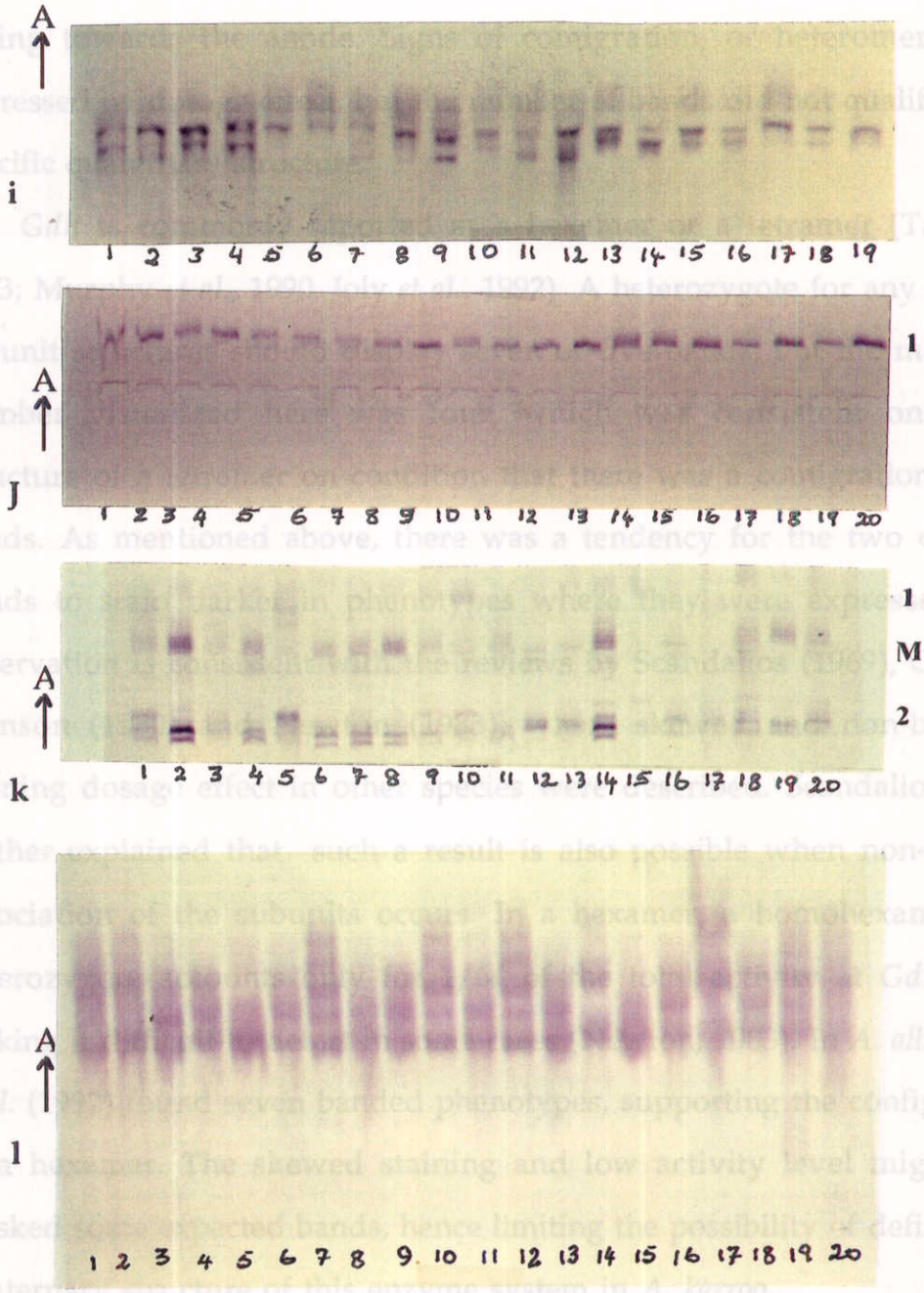
G-6-pd had two zones of activity. The anodal activity zone was smeared and could not be interpreted, while the cathodal zone exhibited three bands. Phenotypes had either three bands or a single band, but a few cases were noted where only one homodimer and a heterodimer were expressed. This may be interpreted as either a sign of unsuitable conditions of assay, non-transcribed alleles or double homozygosity at the locus. The dosage effect was demonstrated by some samples in the form of unbalanced and balanced heterozygotes. The enzyme is a dimer (Murphy *et al.* 1990) and asymmetrical staining indicates a tetrasomic form of inheritance.

The enzyme did not, however, give good resolution for all the samples assayed, and the presence of an unresolved zone above the observed bands made the scoring more difficult. It was discontinued before all populations were run, but there was evidence of polymorphism in the assayed samples (Fig. 3.3i).

3.3.3.9. Glutamate dehydrogenase (Gdh)

Gdh expressed one zone of activity only (Fig. 3.3j). The migration distance of this enzyme from the origin was the shortest of all studied. The

Figure 3.3i-l. Electrophoretic phenotypes of *G-6-pd, Gdh, Mdhp* and *Mdh* isozyme loci of *A. karroo*.



i: *G-6-pd*, j: *Gdh*, k: *Mdhp*, l: *Mdh*. Note: what appear as unresolved bands in *Gdh* locus photograph are fine bands closely migrating. (Photo 8691, 755545, 127599 & 394688, respectively).

locus was polymorphic with samples exhibiting from one to four fine bands. Most electromorphs with multiple bands had a staining intensity fading towards the anode. Signs of comigration, or heteromers, were expressed by dosage effect, but the number of bands did not qualify it to a specific quaternary structure.

Gdh is commonly reported as a hexamer or a tetramer (Tanksley, 1983; Murphy *et al.*, 1990, Joly *et al.*, 1992). A heterozygote for any of these subunit structures should display seven or five bands, but the maximum number visualized here was four, which was consistent only with structure of a tetramer on condition that there was a comigration of two bands. As mentioned above, there was a tendency for the two cathodal bands to stain darker in phenotypes where they were expressed. This observation is consistent with the reviews by Scandalios (1969), Carr and Johnson (1980) and Newton (1983), where skewed and non-binomial staining dosage effect in other species were described. Scandalios (1969) further explained that such a result is also possible when non-random association of the subunits occurs. In a hexamer, a homohexamer of a heterozygote accounts only for 1/64 of the total activity at *Gdh* locus, making it difficult to detect in some cases (Newton, 1983). In *A. albida*, Joly *et al.* (1992) found seven banded phenotypes, supporting the configuration of a hexamer. The skewed staining and low activity level might have masked some expected bands, hence limiting the possibility of defining the quaternary structure of this enzyme system in *A. karroo.*

In most plant species previously assayed, only one active locus of *Gdh* was found (Gottlieb, 1982; Guries and Ledig, 1982; Haufler, 1985, Moran *et al.*, 1989a; Joly *et al.*, 1992; Olng'otie, 1992). However, in a few species mentioned by Newton (1983) and *Eucalyptus pulverulenta* (Peters *et al.*, 1990), two loci have been reported. It is therefore possible that only one polymorphic locus actually exists in *A. karroo.* If the locus is tetrameric or

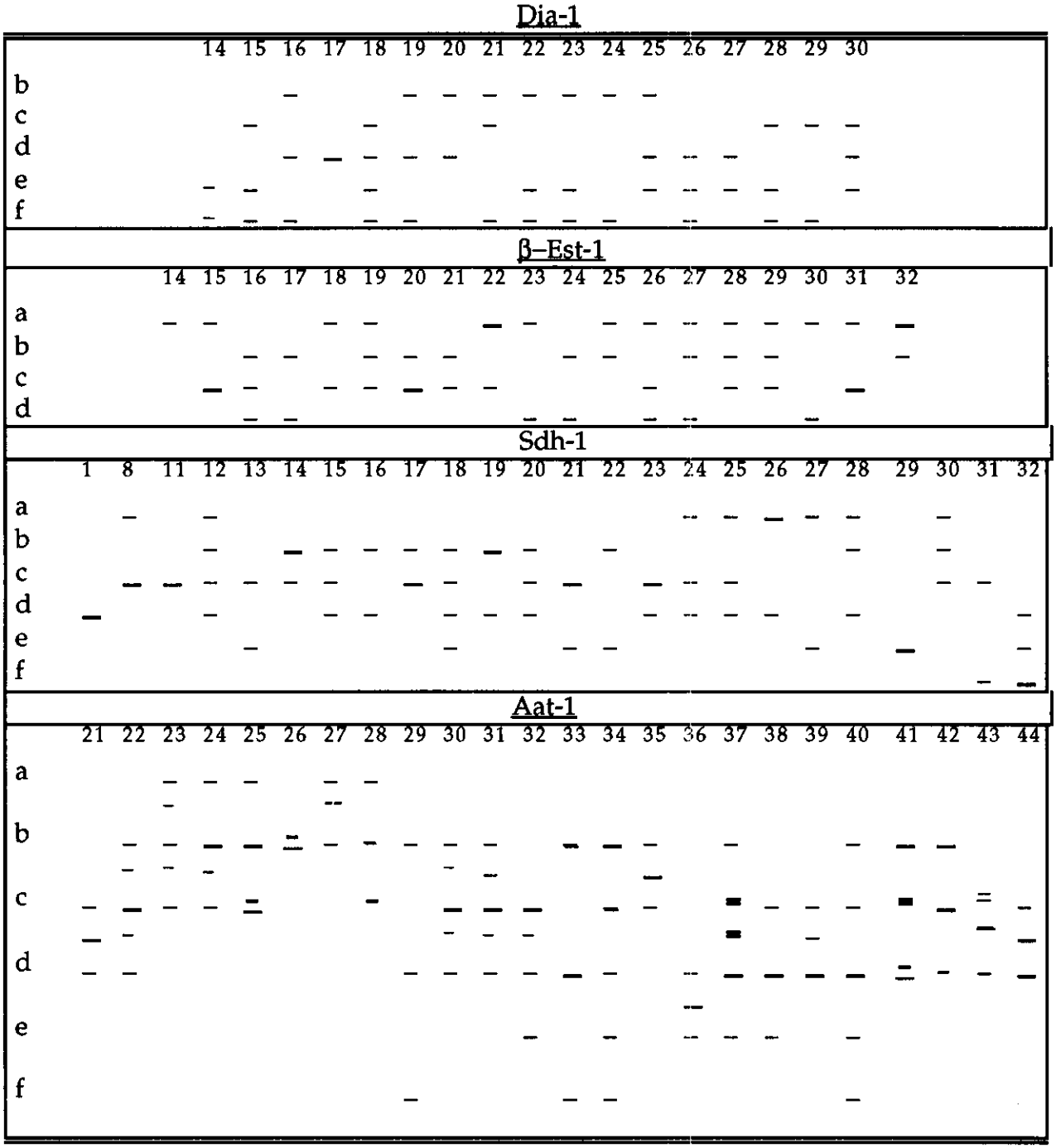
hexameric, then homozygotes should show single bands, while herozygotes exhibit seven or five bands, respectively. Thus, the two banded phenotypes observed here may represent fixed heterozygosity. This evidence suggests the status of *A. karroo* as an allopolyploid.

3.3.3.10. Malate dehydrogenase (Mdhp)

Mdhp, originally known as Malic enzyme (Aebersold *et al.*, 1987 cf. Murphy *et al.*, 1990), displayed three zones of activity, of which only the cathodal locus was consistently clear (Fig. 3.3k). In some provenances such as Ashburton and Cockhouse, the middle zone (m) also clearly stained and expressed up to 11 fine closely spaced bands, but their origin could not be determined with much certainty because the anodal zone (*Mdhp-1*) was smeared. These bands were not used further.

The cathodal zone was designated as locus *Mdhp-2* on the basis that the middle zone (m) could be interlocus heterodimers. The *Mdhp-2* locus displayed four bands which were polymorphic. Observed phenotypes had variable bands, ranging in number from one to four. The one-banded phenotypes were noted as homozygotes, and the remainder with two, three, or four bands were considered heterozygotes. The two banded heterozygotes were considered as double homozygotes and the three to four banded phenotypes classified as balanced and unbalanced heterozygotes depending on the gene dosage effect and the patterns. Murphy *et al.* (1990) and Kephart (1990) described *Mdhp* as a tetramer; therefore, up to five bands could be expected for heterozygotes. Five banded genotypes were not visualized at this locus but, because of frequent staining dosage effect on some bands, comigration of bands could be proposed.

Figure 3.4. Isozyme patterns of various genotypes at 4 isozyme loci. Other genotypes are indicated on photographs 3.3a-h. The numbers correspond with genotype numbers in Table 3.2. Alleles are numbered from a-f.



Presented genotypes:
1. Dia-1 from 14-30. eeff, ccef, bdff, dddd, cdef, bddf, bbdd, bcff, bbef, beff, bbff, bbde deef, ddee, ccef, ccff and cdde **2. β–Est-1 from14-32**. aaaa, accc, bbcd, bbdd, aacc, abcc, bccc, bbcc, aaac, aadd, bbdd, aabb, accd, aacd, abcc, aabc, aaad, accc, and aaab; **3. Sdh-1:** 1 dddd, 8 accc, 11 cccc, 12 deee,**13-32** abcd, ccee, bbbc, bccd, bbdd, bccc, bcde, bbbd, bcdd, ccce, bbee, cccd, acdd, accd, aaad, aaff, aabd, eeee, abcc, ccff, and deefe; **4. Aat-1 from 21-44**. ccdd, bccd, abbc, ac'cc, aac'c, b'bbb, abbb, ac'c'c', bbff, bbcd, bcdd, ccce, bbbf, bddf, bbcc, ddee, bc'ce, ccee, cddd, bbdf, bbd'd, bbbc', c'cdd, cddd.

The presence of asymmetrical staining and fixed heterozygotes, however, indicated that the patterns were influenced by ploidal level. However, the observed dosage ratios of 2:2, 1:1:1:1, 1:1:2:1, 1:1:2:2, 1:3, and other combinations, were not consistent with the banding patterns of a tetramer. Joly *et al.* (1992) obtained two bands only in the same locus of *A. albida* (*Faidherbia albida*), a diploid species, and suggested that the enzyme behaved like a monomer. In the case of a monomer, anything up to four bands are possible in a tetraploid, and signs of comigration and variable dosage effect can be expected. In *A. anomala,* Coates (1988) found a single locus but it was monomorphic, so no quaternary structure could be deduced. Moran *et al.* (1989a) reported one locus for *A. crassicarpa* and *A. auriculiformis,* both of three alleles, but the subunit structure was not discussed. In *A. tortilis* (Olng'otie, 1992), the enzyme expressed two dimeric loci. Thus it can be suggested that the subunit structure of this enzyme varies from species to species. In this study of *A. karroo,* however, complications still occured with phenotypes of four variably staining bands, and those with two bands but without heteromers. It is therefore possible that the enzyme is a monomer exhibiting mixed inheritance mode. However, genotypes could not be scored from this locus because of unclear subunit structure.

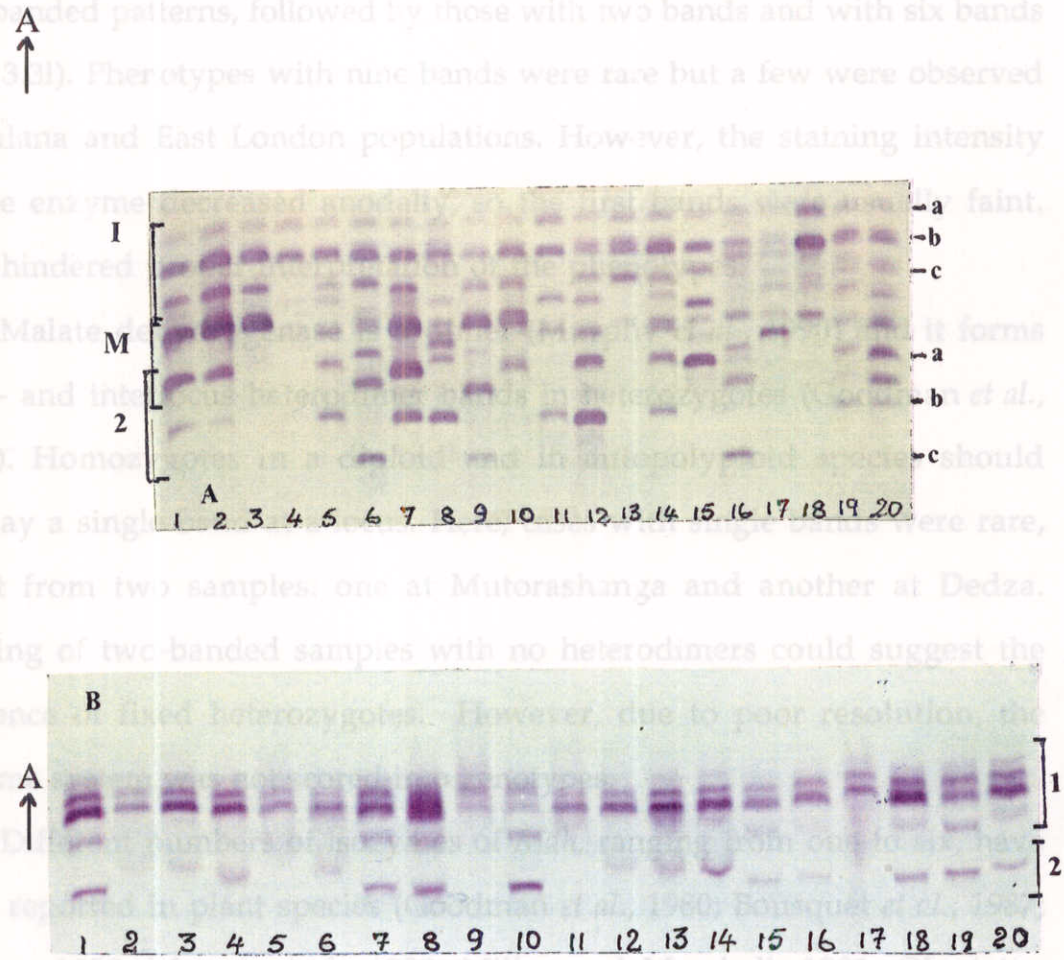
Although the genotypes could not be scored because of unclarified banding patterns and subunit structure of this enzyme in this species, it again demonstrates that allozyme polymorphism and tetrasomic inheritance are expressed because of the multiple bands and variable dosage ratios which are only possible in polyploids.

Table 3.2. The frequencies of observed isozyme genotypes for 10 isozyme loci expressed as percentage of 839 individuals distributed in 12 populations of *A. karroo*. The number of populations where a particular genotype was scored are in parentheses.

Gen. No.	Aat3-	6-Pgd-2	Aat-2	Adh-1	Adh-2	6-Pgd-1	Dia-1	β–Est-1	Sdh-1	Aat-1
44										<1[1]
43										<1[1]
42										<1[1]
41										<1[1]
40										<1[1]
39										<1[1]
38										<1[1]
37										<1[1]
36										<1[1]
35										<1[1]
34									<1[1]	<1[1]
33									<1[1]	<1[1]
32								<1[1]	<1[1]	<1[1]
31								<1[1]	<1[1]	<1[1]
30							<1[1]	<1[1]	<1[1]	<1[1]
29							<1[1]	<1[1]	<1[1]	<1[1]
28							<1[1]	<1[1]	<1[1]	<1[1]
27							<1[1]	<1[3]	<1[1]	<1[1]
26							<1[2]	<1[3]	<1[1]	<1[1]
25							<1[2]	<1[4]	<1[1]	<1[1]
24							<1[2]	1.1[4]	<1[1]	<1[1]
23							<1[2]	1.2[4]	<1[1]	<1[2]
22							<1[2]	2.2[4]	<1[1]	<1[2]
21							1.3[2]	1.4[5]	<1[1]	<1[2]
20							3.8[2]	3.0[5]	<1[2]	<1[2]
19							<1[3]	1.4[6]	<1[3]	<1[2]
18							<1[3]	1.5[6]	<1[3]	1.3[2]
17							<1[3]	2.1[6]	2.0[3]	<1[3]
16							<1[3]	2.7[6]	<1[4]	1.2[3]
15							<1[3]	2.9[6]	1.2[4]	<1[4]
14						<1[1]	<1[3]	3.6[6]	2.0[4]	<1[4]
13						<1[1]	<1[4]	3.7[6]	1.7[4]	1.2[4]
12			<1[1]	<1[1]	<1[1]	<1[1]	6.2[4]	3.0[7]	3.0[4]	1.6[4]
11			<1[3]	<1[1]	<1[2]	<1[1]	2.8[5]	4.9[7]	<1[5]	2.7[6]
10			<1[4]	<1[2]	<1[2]	<1[1]	7.1[5]	5.4[7]	1.5[5]	2.3[7]
9			<1[4]	<1[3]	<1[3]	1.5[1]	4.9[6]	2.3[8]	1.9[5]	3.6[7]
8			1.7[6]	<1[3]	1.1[3]	2.3[1]	4.8[7]	2.3[8]	3.2[5]	2.6[8]
7		<1[2]	1.8[7]	3.8[5]	<1[4]	<1[2]	3.1[8]	2.7[8]	4.0[5]	4.7[8]
6	<1[1]	1.4[3]	5.3[8]	1.3[7]	1.9[4]	2.6[3]	3.4[8]	3.0[8]	4.1[8]	5.8[8]
5	5.9[11]	<1[4]	5.2[11]	7.2[7]	1.1[5]	<1[4]	5.8[8]	3.3[8]	6.9[8]	9.1[8]
4	10.4[11]	6.8[7]	10.2[12]	4.6[10]	3.3[7]	12.0[11]	13.4[9]	2.7[9]	16.0[10]	9.1[8]
3	14.7[11]	4.7[8]	19.1[12]	22.5[11]	7.1[11]	23.5[11]	10.6[10]	15.7[9]	10.7[11]	13.3[9]
2	22.8[12]	4.1[11]	23.7[12]	23.1[12]	17.4[11]	26.8[12]	14.0[11]	12.5[11]	21.1[11]	9.4[12]
1	45.9[12]	78.4[12]	31.0[12]	34.1[12]	66.4[12]	27.8[12]	16.8[11]	14.6[11]	11.1[12]	13.5[12]

NB: The genotypic pattern is not identical for a corresponding number in a different locus. The genotypes were arbitrarily divided into three categories of distribution: rare (bold print), rare-regional (underlined print) and widespread genotypes(plain print). Frequencies range from less than 1% in a single population (<1[1]) to 78.4% in all populations (78.4 [12]).

Figure 3.5. Isozyme patterns of genotypes with null alleles at *Adh*-2 and *6-Pgd*-2 loci in *A. karroo*.



A *Adh-1 & 2.* Active null alleles expressed at *Adh*-2 in columns No. 3, 10, 13, 17 & 18 form active *1b2b* heterodimers, but do not form any with *1a* or *1c*. Allele *1a* at *Adh-1* is weakly stained in all samples but *1b* is duplicated, as can be seen in staining intensities and double bands of heterodimers. In column No. 4 no heterodimers are formed, indicating a null allele, that is non-prescribed and non-active. B *6-Pgd-1 & 2*. Null alleles are possibilities at *6-Pgd*-2 in columns No. 5, 9, 11 & 17 (Photo 127602).

3.3.3.11. Malate dehydrogenase (Mdh)

The enzyme *Mdh* expressed a single zone of activity, in which up to nine bands were observed. The most frequent phenotypes were three and five banded patterns, followed by those with two bands and with six bands (Fig. 3.3l). Phenotypes with nine bands were rare but a few were observed in Talana and East London populations. However, the staining intensity of the enzyme decreased anodally, so the first bands were usually faint. This hindered proper interpretation of the phenotypes.

Malate dehydrogenase is a dimer (Murphy *et al.*, 1990) and it forms intra- and interlocus heterodimer bands in heterozygotes (Goodman *et al.*, 1980). Homozygotes in a diploid and in autopolyploid species should display a single band at a locus. Here, cases with single bands were rare, apart from two samples: one at Mutorashanga and another at Dedza. Finding of two-banded samples with no heterodimers could suggest the presence of fixed heterozygotes. However, due to poor resolution, the enzyme system was not scored into genotypes.

Different numbers of isozymes of *Mdh,* ranging from one to six, have been reported in plant species (Goodman *et al.*, 1980; Bousquet *et al.*, 1987; Coates, 1988; Muona *et al.*, 1989; Millar and Marshall, 1991; Olng'otie, 1992).

3.3.3.12. Distribution of isozyme phenotypes

Table 3.2. shows the genotypic distribution and polymorphism for the 10 assayed loci in 12 of 26 populations. The polymorphism varies from locus to locus, but the loci that exhibited high number of alleles also showed high number of genotypes. For example *Aat-1* showed the highest number of alleles compared to other loci, and this was reflected in the 44 polymorphic genotypes counted. Some enzymes, for example *Adh-1* and

Dia-1, had four and six alleles, respectively but these alleles were either fixed (*a* and *b* at *Adh-1*, *f* at *Dia-1*) or very rare (alleles *d* at *Adh-2* and *a* and *e* at *Dia-1*), thus limiting their contribution to genotypes formed. It was apparent that approximately 67% of the genotypes were widespread, 20% were rare, and only 13% could be classified as regional (Table 3.2). The observed genetic variability in regional genotypes (13%) could contribute to some distinctive morphological features in different ecological zones, reflecting site-specific selective forces.

3.3. 13. Evidence of polyploidy

The important criteria classifying *A. karroo* as a polyploid are the presence of 2n=4x=52 chromosomes, the expression of multiple alleles, asymmetrical banding intensities, and fixed heterozygosity, as discussed above. Display of triallelic and tetraallelic heterozygotes at a single locus has been identified as a sign of gene duplication in diploids (Gottlieb, 1977b; Gottlieb, 1981; Odrzykoski and Gottlieb, 1984; Jones *et al.*, 1986; Cai and Chinnappa, 1989). Polyploidy is more pronounced as the number of such duplications increases at different loci (Gottlieb, 1981; 1982). In *A. karroo*, multiple-allelic genotypes were observed at all loci identified in Table 3.2 other than *6-Pgd-2*.

Asymmetrical banding intensities have been of much use in identifying polyploid plants in most electrophoretic assays (Quiros, 1982; Martinez-Zapater and Oliver, 1984; Soltis and Rieseberg, 1986; Krebs and Hancock, 1988; Wolf *et al.*, 1989; Schnabel *et al.*, 1991; Olng'otie, 1992). They are therefore associated with polysomic inheritance, which is only possible in autopolyploids. They also provide evidence for the presence of two or more copies of homologous chromosomes which show random pairing among homologues, and an unequal segregation of chromosomes at meiosis. Here, all the assayed isozymes other than *6-Pgd-2* expressed

asymmetrical banding. Such a banding pattern can also occur in allopolyploids when allozymes from different homoeologous alleles have coincidental mobility, as has been demonstrated in wheat (Hart, 1970; Stankovics and Naggy, 1992). Asins and Carbonell (1987) and Soltis and Soltis (1990) have cautioned that these patterns do vary from species to species and with the developmental stages of polyploids. Clear polysomic inheritance was seen when monomeric enzymes, such as *β-Est-1* and *Sdh-1*, exhibited 4 bands.

Fixed heterozygosity is considered as strong evidence of polyploidy in electrophoretic studies and is a pattern formed only in allopolyploids (Roose and Gottlieb, 1976; Werth *et al.*, 1985; Cai and Chinnappa, 1989; Haufler *et al.*, 1990). According to Gottlieb (1981), two homoeologous alleles are expressed in a fixed heterozygote, representing homoeologous chromosomes, each of which may originate from a diploid species. These homoeologous chromosomes do not pair at meiosis, so the genes derived from both parents are passed on to all gametes. At fertilization, the zygote formed has the additive genotype of the parents. Under electrophoretic assays, these allopolyploids exhibit preferential pairing and show disomic inheritance at some loci. Fixed patterns were observed at *Aat-1*, *Adh-1*, *6-Pgd-1*, and *6-Pgd-2*, while *α-Est-2* was fixed with four alleles in every sample. Such pairing could also be hidden in some of the monomeric enzymes which expressed triallelic and tetraallelic genotypes, especially with *Sdh-1* at Babanango.

Although the presence of null alleles can be verified through controlled breeding experiments (Goodman *et al.*, 1980; Jones *et al.*, 1986), it is possible to infer them where an enzyme has good resolution and repeatability, as was the case here with *Aat-3*, and *Adh-2*. Null alleles are characteristic of duplicated loci, especially in allopolyploids, where their presence is buffered by the homoeologous genes (Werth *et al.*, 1985). Only

in rare cases, genotypes have been reported with null alleles with no alternative buffering genes (see Wilson *et al.*, 1983; Newton, 1983). Null alleles were also observed at *6-Pgd-2* loci in *A. karroo*, but their presence poses a technical question. *6-Pgd-2* is an isozyme in either cytosol or plastid (not determined in this study), depending on a plant species. If true null alleles exist, as is possible during crossing of parents with reciprocally silenced genes (Werth and Windham, 1987), it will be interesting to know how these genotypes develop further without active enzyme in one cell compartment; perhaps these genotypes face post-zygotic elimination at another stage in their development.

On the other hand, the presence of null alleles or silenced genes signifies antiquity of a polyploid (Gottlieb, 1981). It has been established that most ancient polyploids undergo diploidization by silencing redundant genes (pseudogenes), a factor which is responsible for diploids with very variable or high chromosome numbers (Ferris and Whitt, 1977; Li, 1980; Gastony, 1986; Haufler, 1989; Soltis and Soltis, 1990). The electrophoretic evidence here suggests existing possibilities of formation through recurring polyploidization and an unequal segregation of alleles, a factor which has most likely contributed to difference in banding patterns of *6-Pgd-1* locus, the presence of three homoeologous alleles in *6-Pgd-2*, and up to 4 such alleles in *Adh-1* and *β–Est-1*. Recurrent polyploidization is a phenomenon not unique to this species as other examples were discussed in section 3.1 of this chapter.

3.3.4. Conclusion

Based on the above evidence, it is proposed that *A. karroo* is a segmental polyploid which has speciated as a result of inter-specific hybridization. According to Riley (1949) and Wright (1969), segmental

allotetraploids are formed between species that are sufficiently closely related to permit considerable pairing of some chromosomes or segments. A full description of a segmental polyploid was given by Stebbins (1950) as "a polyploid containing two or more pairs of genomes which possess in common a considerable number of homologous chromosomal segments or even whole chromosomes, but differ from each other in respect to a sufficiently large number of genes or chromosomal segments, so that the different genomes produce sterility when present together at the diploid level". The major properties of segmental polyploidy are: (1) the ability for genetic segregation, displaying morphological and chromosomal difference between the parental species, and (2) the tendency to backcross with autopolyploid derivatives from either of their parental species, forming fertile hybrids. Through this hybridization and introgression, they may obliterate all the barriers that existed. Thus, segmental allopolyploidy represents an unstable status in species evolution. Natural selection in this type of polyploidy acts to eliminate the barriers delaying the establishment of either autopolyploidy or true allotetraploidy; but often works to restore the nearest status.

The evidence of genotypic polymorphism confirmed above is possibly a reflection of wide genetic variation and versatility due to hybridization, introgression and polyploidization. It is possible that the above factors together are responsible for the general adaptability and phenotypic plasticity which has characterized the widespread distribution of *A. karroo,* on one hand, but on the other have created difficulty in spontaneous recognition of the species at every site. How far the factors have contributed to the genetic differentiation at various sites forms the topic of the next chapter.

CHAPTER FOUR

GENETIC DIVERSITY WITHIN AND AMONG POPULATIONS OF *A. KARROO*

4.1. Introduction

The discovery that genes code for proteins, combined with the development of the techniques of protein electrophoresis, provided means of estimating genetic variation in natural populations (Adams, 1983; Ayala and Kiger, 1984; Cheliak and Rogers, 1988; Hartl and Clark, 1989; Crawford; 1990). To date, these analyses have investigated various species, among them some forest tree species (e.g. Hamrick *et al.*, 1981; Brown and Moran, 1981; Loveless and Hamrick; 1984; Hamrick and Loveless, 1986; Muona, 1989; Hamrick and Godt, 1989; Bawa and Krugman, 1991; Hamrick *et al.*, 1992; Moran, 1992). Observations from these studies indicate that trees maintain high levels of genetic diversity within populations, and are far less geographically differentiated than herbaceous plants (Hamrick *et al.*, 1992).

Among tree species, conifers were reported to display higher variability at isozyme loci, but lower differentiation between populations due to efficient gene flow through pollen and seed dispersal mechanisms (Hamrick *et al.*, 1981; Muona, 1989, Mitton, 1992). Only in a few specific cases, where populations have gone through bottlenecks or have developed tolerance to inbreeding, has the variability been substantially reduced, as reported for *Pinus resinosa* (Fowler and Morris, 1977), *Pinus torreyana* (Ledig and Conkle, 1983) and *Thuja plicata* (Copes, 1981).

However, as more information on genetic diversity and differentiation becomes available, it has become clear that angiosperms are

also highly variable (Hamrick *et al.*, 1992), including tropical tree species (Loveless, 1992; Hamrick, 1992). Some angiosperms expressing high levels of genetic diversity are: *Eucalyptus* species (Moran and Bell, 1983; Moran and Hopper, 1987; Moran, 1992), *Acacia* species (Coates, 1988; Moran *et al.*, 1989a; 1989b; Muona *et al.*, 1991; Joly *et al.*, 1992; Olng'otie, 1992), *Metrosideros* species (Aradhya *et al.*, 1991), *Robinia pseudoacacia* (Surles *et al.*, 1989), *Gleditsia triacanthos* (Schnabel and Hamrick, 1990), *Maclura pomifera* (Schnabel *et al.* 1991), and *Cavenillesia platanifolia* and *Platypodium elegans* (Hamrick and Murawski, 1991) from tropical South America. However, these studies indicate that each species is faced with specific ecological and biological factors that determine the distribution of genes. The angiosperms which have gone through bottlenecks similarly to conifers express low variability, for example, *E. caesia, E. johnsoniana* (Moran and Hopper, 1987) and *Acacia mangium* (Moran *et al.*, 1989b).

Although new genes are created only through mutations (Ayala and Kiger, 1984), they are hardly incorporated in a population unless they are advantageous or buffered by an alternative allele in a heterozygote (see Kimura, 1968). Thus, biological traits and ecological factors largely determine the distribution, recombination and survival of the genes. The role of some of these factors, most notably breeding system, reproductive mode, species distribution patterns, population size, longevity, fecundity, and anthropological interventions are briefly outlined below in the case of *A. karroo*. Further detailed discussions on the influence of these factors on genetic diversity are presented by Brown and Moran (1981), Hamrick *et al.* (1979), Loveless and Hamrick (1984), and Hamrick and Godt (1989).

4.1.1. Breeding Systems

The breeding system of plants is one of the major factors that influences their population structures, and hence their genetic diversity

(Loveless and Hamrick, 1984; Hamrick, 1992). The breeding system comprises the mode of fertilization, the method of reproduction and the mechanism of seed dispersal (Burley *et al.*, 1986; Richards, 1986).

4.1.1.1. Mode of Fertilization

Outcrossing species are characterized by large effective population sizes that contribute to gene exchange, reducing intrapopulational genetic differentiation (Mitton, 1992). On the contrary, inbreeding reduces gene flow and population groups from similar ancestry tend to grow together, increasing the possibility of genetic differentiation and gene fixation within a population. High rates of inbreeding have also been associated with polyploids because they may mask a heavy genetic load (Barber, 1970; Bawa and Krugman, 1991; Cai and Chinnappa, 1991). In general, inbreeding alone does not lead to reduction in genetic diversity, but more often it is associated with inbreeding depression, lethal genes and environmental selective forces for specific fitness (Charlesworth, 1985; Lande and Schemske, 1985).

The breeding systems of *A. karroo* are not fully documented, but studies on floral morphology and phenology (Gordon-Gray and Ward, 1975) indicate the species is likely to be outcrossing. The species is androdioecious and flowering is asynchronous (Gordon-Gray and Ward, 1975; Ross, 1979). Such characteristics have been suggested to favour outbreeding (Loveless and Hamrick, 1984, Hamrick and Godt, 1989; Murawski and Hamrick, 1991; Bawa and Krugman, 1991). Studies of other *Acacia* species, e.g. *A. retinodes, A. mearnsii, A. melanoxylon* and *A. auriculiformis* (Kenrick and Knox, 1989; Muona *et al.*, 1991; Sedgley *et al.*, 1992) have reported mixed mating, with cross breeding favoured. However, studies by Olng'otie (1992) and Moran *et al.* (1992) for polyploid acacias reported high selfing rates.

Inbreeding can at times occur in the form of consangineous mating (mating between relatives) (Loveless and Hamrick, 1984; Silander, 1984; Ritland, 1989), but this is mainly determined by the tolerance level of the species to inbreeding (Kenrick and Knox, 1986; 1989; Les *et al.*, 1991). Asynchronous flowering and low tree density may enhance selfing in a self-compatible species (Murawski and Hamrick, 1991; Adams, 1992). Some species have developed extraordinary associations to avoid the consequences of inbreeding; for example, some *Ficus* species have adopted asynchronous flowering and allofusion (several genotypes growing as one tree) as a means of ensuring availability of pollinators and cross pollination (Thomson *et al.*, 1991).

4.1.1.2. Method of reproduction

Sexual reproduction in plant species enhances intrapopulational genetic diversity due to random exchange of genes. However, as discussed above, sexual reproduction must be associated with efficient long distance pollen transfer mechanisms to combat any form of related mating. Evidence from some acacias such as *A. mearnsii, A. retinodes, A. mangium,* and *A. auriculiformis* (Moffett, 1956; Kenrick and Knox, 1985; 1989; Sedgley *et al.*, 1992) show that they are entomophilous and self-incompatible; the pollinators are also genus specific, but the foraging distances for these pollinators have not been determined. The pollinators identified by Sedgley *et al.* (1992) on *A. mangium* and *A. auriculiformis* included insects of orders Hymenoptera (Halictidae and Apidae), Diptera (Syphidae and Bibionidae) and Coleoptera. *A. karroo* is sexually reproducing and entomophilous (Gordon-Gray and Ward, 1975), but the level of self-compatibility and distance of pollen transmission have not been studied.

Tree species which reproduce asexually can exhibit highly genetically subdivided populations on one hand, and low genetic differentiation on the other (Parker and Hamrick, 1992). Species which have stumps and roots which could resprout after several years of dormancy, or repeatedly, usually contribute to the formation of new generations with high genetic diversity, especially when coupled with seed recruitment, or efficient means of vegetative propagule dispersal (Les *et al.*, 1991; Jelinski and Cheliak, 1992; Parker and Hamrick, 1992). Exclusively asexual reproduction increases the chances of genetic differentiation in a population (Hamrick and Godt, 1989). Although specific studies of asexual reproduction in *A. karroo* have not been undertaken, cases of regenerating stumps have been documented (Sim, 1906; Teague, 1988b). Since the species is known to reproduce sexually (Chapter One), some populations are likely to be composed of both coppice and new generations recruited from seeds within and those trasferred from other areas, influencing the genetic diversity.

4.1.2. Seed dormancy

Loveless and Hamrick (1984) noted that long seed dormancy reduces intrapopulational genetic subdivision because recruitment of new generations continues after the death of mother plants. *Acacia* species are known for long seed dormancy (Doran *et al.*, 1986), and germinations from dormant seeds may be expected after several years. Thus, a population could be a mixture of individual trees of different origin: those originating from long time occupants and those introduced by migration, influencing the genetic diversity. The case of dormant seeds can be likened to the case of coppicing stumps except, in the former, the latent stage is from the earlier occupants.

4.1.3. Life cycle

Species with longer generation time tend to have larger effective population sizes (Hamrick and Godt, 1989). The life cycle affects the population genetic structure similarly to asexual reproduction and seed dormancy. A longer life cycle is usually associated with high fecundity; thus, a single genotype is capable of mating with members of several generations of a population. *A. karroo* is perennial which is likely to increase its fecundity. The species is also known to be weedy (see Chapter One), which explains the possibility of seeds being dispersed into areas already occupied by scattered older trees, reducing interpopulational differentiation and increasing intrapopulational genetic diversity. As a colonizing species, it is only in locations where further migration is curtailed that the populations of *A. karroo* are likely to express genetic differentiation, resulting from small population sizes and genetic drift. Possible sites of such interpopulational differentiation, followed by genetic fixation of the species, are Mauritius and Australia which are separated from the other populations and gene transfer mechanisms might not be effective. However, with time, such populations could also gain some genetic diversity following either accumulation and fixation of mutations, hybridization between introduced and native species, introgression or combined effects.

4.1.4. Geographic range

Tree species that are widely distributed express a relatively low level of genetic differentiation (Hamrick *et al.*, 1981; Moran and Hopper, 1987; Hamrick and Godt, 1989; Hamrick *et al.*, 1992). Small populations are most likely to exhibit high genetic differentiation, enhanced by genetic drift (Moran and Hopper, 1987; Murawski and Hamrick, 1991), but the differentiation is influenced by the breeding system, pollination

mechanisms and the distance between adjacent populations. *A. karroo* is widely distributed (see Fig. 1.1) and is most likely outbreeding (Gordon-Gray and Ward, 1975). The other factor that influences the pattern of genetic diversity is the density of trees within a population. Murawski and Hamrick (1991) and Bawa and Krugman (1991) have indicated that low densities can encourage genetic differentiation in a populations in cases where self-incompatibility and pollination mechanisms are not efficient.

4.1.5. Population spatial structure

Wright's model of isolation by distance (Wright, 1969) explains the spatial structure of genetic differentiation. Members of a species grouped together are most likely to have high genetic similarity (Parker and Hamrick, 1992) and populations distributed along a linear gradient are likely to display higher genetic differentiation than those with wider distribution patterns (Loveless and Hamrick, 1984). Over its wide geographical range, *A. karroo* displays spatial structure related to the environment; the species is excluded from dry areas, humid regions with high forests, and frosty altitudes (Sim, 1906; Acocks, 1988; Brain, 1989). Such patterns are likely to influence the genetic structure of the species unless counteracted by efficient means of gene flow. Results of recent studies in *Populus tremuloides* (Jelinski and Cheliak, 1992) were consistent with the proposal that spatial structure alone may not result in high interpopulational genetic differentiation in the presence of efficient gene flow systems, or a gene bank in the form of dormant seeds or sprouting stumps. A gene bank in this form is characteristic of *A. karroo*, but conclusions about the effectiveness of gene flow in the species can only be speculative, based on its colonizing ability.

4.1.6. Anthropological interventions

One common consequence of human interventions in plant populations is fragmentation during exploitation, or clearing for other land uses. Depending on the scale and duration, these interventions could reduce the genetic diversity. However, recent fragmentation of an originally widespread population might not immediately cause a loss of genetic diversity, especially in situations where gene exchange can still occur (Young and Merriam, 1992; Hamrick, 1992). Conversely, through human interventions, it has been possible also to bring within mating proximity individuals which would not, in nature, be so united, e.g. those from extreme ends of a species range. Such interventions can help to restore genetic diversity by jointly conserving samples from different fragmented populations. The method has been used effectively for domesticated plants as reported by Hamrick *et al.* (1981), Torres *et al.* (1978, cited by Hamrick *et al.* 1981) and Godt and Hamrick (1991). However, some species have lost most of their genetic variability due to high selection intensity in their few original sources (for example, barley, tomatoes, *Phlox*, radiata pine) (Libby, 1973; Brown and Moran, 1981),

A. karroo mainly exists in its natural populations, but some loss or gain in genetic variability and change in population structure must have resulted from human activities across the species range. Some populations of the species have been sited in Mauritius and Australia, and will have come under different selective pressures in those exotic locations. Together with the genetic isolation, this may lead over time to genetic differentiation from the original population. Within the species natural range, transfer of seeds from one area to another could widen the genetic diversity in those areas, and raise the chances of hybridization with other acacias nearby, for example. *A. tenuispina*, whilst clearing for other land

uses e.g. agriculture and settlements could more often than not reduce the variability.

4.1.7. Genetic diversity and ploidy level

Several investigations of the level of genetic diversity among congeneric species involving diploids and their polyploid counterparts have confirmed that polyploids exhibit higher heterozygosity than diploids (Bayer, 1989; Ness *et al.*, 1989; Soltis and Soltis, 1989b; Wolf *et al.*, 1990; Schnabel *et al.*, 1991; Soltis and Soltis, 1993). About 40% of plant species are known to be polyploids, and most of these are angiosperms (Stebbins, 1980). It seems likely, therefore, that the general mean expected genetic diversity in plant populations will rise to a level higher than the current 14% (Hamrick and Godt, 1989) as more studies of genetic variation in angiosperms are completed. Evidence from cytological and isozyme patterns (Chapter Three) shows that *A. karroo* is a polyploid, thus, raising the expectations of observing a high level of genetic variation.

Many previous studies have indicated that metric traits of *A. karroo* are variable across populations. This differentiation was not, however, reflected in the ploidy level of the species which cytological studies (Chapter Three) revealed to be constant. Quantitative traits are liable to be more strongly influenced by environment than are isozyme loci (Gottlieb, 1982; Muona, 1989); thus, they cannot be good indicators of genetic diversity in natural populations. Isozyme gene markers have been found to be stable and reliable as indicators of genetic variation and population structure (Nei, 1972; 1973; 1978; Brown and Moran, 1981; Gottlieb, 1981; Adams, 1983; Hillis and Moritz, 1990) because of their direct link to genes and co-dominant inheritance patterns. As with other species of economic importance, it is imperative to elucidate the genetic diversity of *A. karroo,* in order to determine strategies for conservation and breeding

programmes. Other information about genetic resources, evolutionary trend and colonizing ability may also be evaluated through the determination of allozyme pattern and diversity (Crawford, 1983; 1985; 1989; 1990; Barrett and Shore, 1989).

This Chapter addresses itself to the genetic variability within and among populations of *A. karroo*. Ten polymorphic isozyme loci of 839 sample trees from 12 populations which had more than 20 samples were used to determine the variation of genetic parameters. The possible evolutionary trend of the populations of the species were illustrated using dendrograms. The applications of observed genetic variation in conservation strategies and breeding programmes are discussed.

4.2. Materials and Methods

4.2.1. Enzyme loci

Six enzyme systems of the 12 previously assayed (Chapter Two) had patterns that could be interpreted in terms of loci and alleles. Banding patterns were only scored for the first 12 populations (Table 2.1) which had more than 20 samples (Umguza Valley, Mutorashanga, Dedza, Zomba, Lusaka, Kroonstad, Babanango, Hluhluwe, Queenstown, Manzingwenya, Cintsa River and Prince Albert). These six enzymes were encoded by 10 structural genes. The ten loci scored were: *Aat-1, Aat-2, Aat-3, Adh-1, Adh-2, Dia-1, β-Est-1, 6-Pgd-1, 6-Pgd-2* and *Sdh-1*. The banding patterns of these loci were interpreted as genotypes and alleles on the basis of enzyme quaternary structure, enzyme conservancy and inheritance pattern, as explained in Chapter Three.

Most of the enzymes fitted tetrasomic inheritance pattern, although there were some loci which expressed complex patterns occasionally, with fixed heterozygosity and disomic inheritance patterns (e.g. *Aat,-1 Adh-1* and *6-Pgd-1*). However, this observation did not affect the final results

since all alleles at these loci were segregating, tetrasomic inheritance was assumed in the estimates of genetic parameters. An expression of disomic or tetrasomic inheritance of the isoloci were assumed to indicate difference in the loci of homoeologous chromosomes forming *A. karroo* and was used mainly to identify the alleles (e.g. Fig. 3.3b & 3.3f).

4.2.2. *Statistical analysis*

The banding patterns (phenotypes) were scored into genotypes, which were further transformed into genotypic frequencies by counting individuals of similar genotypic categories and calculating their ratio to the total sample size. According to the Hardy-Weinberg principle, the frequencies of all the genotypic categories in a sample population must sum up to unity (Ayala and Kiger, 1984); that is :

$$\sum_{i=1}^{n} Gi = 1 \qquad (1)$$

where Gi is the genotypic frequency, and; n is the number of genotypes observed at the locus.

The ratio of genotypes which expressed heterozygosity was calculated for every locus, and mean observed heterozygosity within a population was estimated following Weir (1990) as:

$$\bar{H}o = \frac{\sum_{i=1}^{m} \frac{\sum_{i=1}^{n} Gij}{N}}{m} \qquad (2)$$

where N is the sample size in a population;

Gij is the number of heterozygous genotypes observed and;

m is the number of loci scored, including those that are monomorphic.

For diploid species with two alleles at a locus, only three genotypic categories can be expected, but the number increases in the case of diploids with multiple alleles and for higher ploidy levels. The expected number of genotypic categories in an autotetraploid was calculated according to Waples (1988) as:

$$\left[\frac{k(k+1)}{2}\right]^2 \qquad (3)$$

where k is the number of alleles observed at the locus (see appendix II).

However, in most cases the number of these theoretical genotypes was too great to be observed; for example, a locus with three alleles should exhibit 36 genotypes whereas some sample sizes were less than that number.

Parameters expressing genetic variation were calculated both for the species as a whole and for individual populations. The proportion of polymorphic loci (P) and the mean number of alleles per locus (A) were calculated using the computer program BIOSYS-1 (Swofford and Selander, 1981). The allelic frequency, effective number of alleles and expected heterozygosity were calculated manually because the species is a tetraploid but expresses both tetrasomic and disomic inheritance patterns. No computer program was available to handle such mixed allozyme data. Other genetic parameters estimated, following the formulae of Nei (1973, 1975) and Hedrick (1985), were total gene diversity (H_T), expected gene heterozygosity (H_e), intrapopulation diversity (H_s), interpopulation gene diversity (D_{st}), the coefficient of gene differentiation (G_{st}), coefficient of absolute gene differentiation (Dm), and coefficient of intrapopulational gene diversity (R_{st}). The complement index (CI) (Gottlieb, 1975) was used to determine the most representative population of the taxon in terms of allelic richness. Nei's (1978) unbiased genetic identity (I) and distance (D)

were calculated using the computer program GDD (Ritland, 1989). The phylogenetic dendrogram with unweighted pair group arithmetic mean (UPGMA) was plotted using the computer program GDb(Ritland, 1989), while a rooted Wagner Tree dendrogram was plotted using the BIOSYS-1 program (Swofford and Selander, 1981).

4.2.3. *The average number of alleles per locus (A)*

'A' is a genetic parameter which estimates the number of alleles per locus in a population (Moran and Hopper, 1987), indicating the allelic richness and evenness in a population (Brown and Weir, 1983). The major disadvantage of this statistic is its dependence on the sample size (Ayala and Kiger, 1984; Hedrick, 1985; Moran and Hopper, 1987). The parameter is calculated as the mean of the observed number of alleles divided by the number of assayed loci for each population. That is:

$$\bar{A} = \frac{n}{m} \qquad (4)$$

where n is the total number of alleles observed, and m is the number of loci assayed.

The average for the species is thereafter obtained by summing up means from all populations and dividing by the number of sampled populations. Its variance was estimated following Nei and Raychoudhury (1974), as:

$$V(A) = \frac{A(1-A)}{m} \qquad (5)$$

Allelic frequency which is the proportion of an individual allele to the total observed was calculated from genotypic frequency, assuming tetrasomic inheritance, as:

$$p_i = p_{i1}+p_{i2}+p_{i3} \qquad (6)$$

$$\text{where } p_{i1} = G_{ii} + a\sum_{i=1}^{n} G_{ij},$$

$$p_{i2} = G_{ii} + b\sum_{i=1}^{n} G_{ij},$$

$$p_{i3} = G_{ii} + c\sum_{i=1}^{n} G_{ij}$$

where *Gii* is the genotypic frequency of homozygotes with allele *pi;*
Gij is the frequencies of all heterozygotes with that allele;
n is the number of heterozygotes and;
a, b or *c* are the proportions of allele *pi* in each category of heterozygotes of a tetraploid, represented numerically as 0.25, 0.50 or 0.75, respectively; the parameter *p* should only appear as single, double or triple alleles in a heterozygote of a tetraploid.

4.2.4. The proportion of polymorphic loci (P)

Cavalli-Sforza and Bodmer (1971), cited in Hedrick (1985) defined a genetic polymorphism as, "the occurrence in the same population of two or more alleles at one locus, each with appreciable frequency". In a popultion survey, the magnitude of what is an appreciable frequency must be delineated. Thus, the proportion of polymorphic loci (P) is a statistic expressing the number of loci with two or more alleles, the frequency of the most common allele is arbitrarly set smaller than 0.99 or smaller than 0.95. The limit is often set on the basis of sample size. The proportion of loci polymorphic is one of the parameters used to determine genetic variation in natural populations using isozyme loci polymorphism. Here, the upper limit for the most common allele at a locus was set at 0.95, because most of the sample sizes used were less than 100 individuals. The parameter was estimated (Hedrick, 1985) as:

$$\bar{P} = \frac{x}{m} \quad (7)$$

where x is the number of loci polymorphic in the sample, and; m is the number of loci assayed, including monomorphic loci. The average polymorphism for the species was then calculated from Equation (7) as:

$$\bar{\bar{P}} = \frac{\sum_{i=1}^{m} \bar{P}}{s} \quad (8)$$

where s is the number of populations of the species examined and other terms are defined as above.

The average proportion of polymorphic loci, however, is dependent on the number of individuals studied, hence the need for a more appropriate measure of genetic variability (Li and Graur, 1991).

4.2.5. *Genetic Diversity*

Gene diversity is synonymous with expected heterozygosity in a randomly mating population (Nei, 1973); otherwise, it refers to the probability of non-identity in random and non-random mating populations (Weir, 1990). Heterozygosity as a genetic parameter is important because it separates the individuals into two categories: homozygous and heterozygous. However, Hedrick (1985) explained two weaknesses of heterozygosity as a parameter: first, it has limited sensitivity in the presence of more than two alleles, since the upper limit is fixed at unity; second, the theoretical properties of its distribution are complicated. Here, gene diversity and expected heterozygosity has been used synonymously. The gene diversity for *A. karroo* was calculated assuming that the pair of isoloci of the two genomes was from a single diploidized

locus. It has also been assumed that the populations are randomly mating, i.e. in Hardy-Weinberg equilibrium. Thus, the frequency of a homozygote was calculated following Waples (1988) and Hedrick *et al.* (1991), as:

$$\bar{H}e = 1 - \sum_{i=1}^{n} p_i^2 q_i^2 \qquad (9)$$

where *pi* and *qi* are the mean frequencies of leading and variant allele at the isoloci summed over *n* alleles, and the other terms are as defined above.

In a homozygote of an autotetraploid, the four alleles have the same frequency and the probability of forming a genotype is the product of the frequencies of four alleles. Therefore, for those isozyme loci with the four comigrating alleles in a homozygote, the estimate of the expected heterozygosity was calculated using formula of Hedrick (1985) for autotetraploids, as:

$$\bar{H}e = 1 - \sum_{i=1}^{n} p_i^4 \qquad (10)$$

where all terms are as defined above.

As it was observed that all the four alleles were segregating in all polymorphic loci, formulae (9) and (10) are assumed identical in this case.

The effective number of alleles (A_e) in a population, which is the reciprocal of the frequency of the homozygotes, was calculated following Ayala and Kiger (1984), as:

$$\bar{A}e = \frac{1}{\sum_{i=1}^{n} p_i^4} \qquad (11)$$

where all terms are as defined above.

Effective number of alleles ($\bar{A}e$) is best perceived as the number of alleles that, were they expressed in equal frequencies, would equate to the heterozygosity value under random mating. Ayala and Kiger (1984) demonstrated that the effective number of alleles is more suitable than estimated mean expected heterozygosity as a parameter for measuring changes in genetic variation. However, Nei (1987) has cautioned about the unsual characteristics of the effective number of alleles. He further noted that the statistic is only equal to the actual number of alleles when the latter have the same frequencies.

Gene diversity for each population was estimated as a weighted sum of the number of loci sampled from the estimates of allele frequency (Table 4.1), and the formula for estimation of heterozygosity (Equation 9), as:

$$\bar{H}e = \frac{\sum_{i=1}^{m} 1 - \sum_{i=1}^{n} p_i^4}{m} \qquad (12)$$

where all terms are defined as above.

The average gene diversity for the species was thereafter obtained as a weighted sum of the gene diversity of all the populations assayed, following Equation (12), as:

$$\bar{H}e = \frac{\sum_{i=1}^{s} \frac{\sum_{i=1}^{m} 1 - \sum_{i-1}^{n} p_i^4}{m}}{s} \qquad (13)$$

where all terms are defined as above.

The total gene diversity (H_T), which is a measure of the mean of heterozygosity expected under random mating (Nei, 1973; 1975; Hamrick *et al.*, 1992), was calculated for each locus as:

$$\bar{H}T = 1 - \sum_{i=1}^{n} p_i^4 \qquad (14)$$

where $p_i = (\sum p_i/s)$ representing the weighted allele frequencies over s populations.

The mean total gene diversity for the species was calculated from Equation (14), as:

$$\bar{H}T = \frac{\sum_{i=1}^{m} 1 - \sum_{i=1}^{n} p_i^4}{m} \qquad (15)$$

where the terms are defined as above.

Gene diversity at a locus within a population (H_e) was computed from single locus heterozygosity (Equation 10) and weighted over the number of loci to estimate the intrapopulational gene diversity (H_s), following Weir (1990), as:

$$\bar{H}s = \frac{\sum_{i=1}^{m} 1 - \sum_{i=1}^{n} p_i^4}{m} \qquad (16)$$

where $\bar{H}s$ is an estimate of the average amount of genetic variation maintained within any one population, or the proportion of loci at

which an individual can be expected to be heterozygous, and m is the number of loci sampled.

The overall gene diversity across populations within the species was computed from Equation (16) as a weighted sum for all the (s) populations assayed, following Hamrick and Godt (1989), as:

$$\bar{H}s = \frac{\sum_{i=1}^{s} \frac{\sum_{i=1}^{m} 1 - \sum_{i=1}^{n} pi^4}{m}}{s} \qquad (17)$$

where all terms are defined as above.

In a panmictic population, where there is no gene differentiation among populations, all alleles are expected to be equally distributed over the entire geographical range, and H_T should be equal to H_s (Yeh, 1981), which is the average gene diversity within a population. Due to natural changes in gene frequency caused by selection, mutation, genetic drift and gene flow, actual populations differ from the ideal.

Interpopulation gene diversity (D_{st}) is the absolute measurement of gene differentiation among populations (Nei, 1973; 1987), and was calculated for every locus as:

$$D_{st} = H_T - H_s \qquad (18)$$

The parameter (D_{st}) is a modification of Wright's F-statistic (Wright, 1965), expanded to cater for loci with multiple alleles since the original formula, [$1-F_{it} = (1-F_{is})(1-F_{st})$], will not hold in a situation where more than two alleles are expressed at a locus (Nei, 1973). F_{it}, F_{is} and F_{st} are deviations of genotypes from Hardy-Weinberg equilibrium, which is more complex when applied to polyploids. The complexities, e.g., overlap of allelic products leading to either under or overestimations of genotypic

frequencies in multi-allelic polyploids with homoeologous genes have been discussed by Asins and Carbonell (1987) and Waples (1988).

Nei (1973, 1975) defined an absolute measure of gene differentiation, (D_m). D_m is the average minimum genetic distance between populations and it is independent of of gene diversities within populations. It excludes comparisons of populations with themselves and is appropriate for comparing gene differentiation in different organisms. It was estimated as:

$$D_m = \frac{sD_{st}}{s\text{-}1} \qquad (19)$$

where all terms are as defined above.

The coefficient of gene differentiation (G_{st}) is the index expressing interpopulation variation relative to total diversity, and was estimated from Equation (18) as:

$$G_{st} = \frac{D_{st}}{H_T} \qquad (20)$$

The above estimate of G_{st} from equation (20) should be used within one population and is only useful when comparing other populations with similar breeding systems (Nei, 1973). The coefficient of gene differentiation (G_{st}) varies from 0 to 1 and is equivalent to Wright's F_{st} (gene differentiation among populations), as demonstrated by Nei (1973).

Coefficient of intrapopulational gene diversity (Rst) is the index of genetic diversity due to absolute gene differentiation among populations relative to absolute gene differentiation among populations (Nei, 1973). The coefficient of intrapopulational gene diversity was computed from D_m (Nei, 1973), as:

$$R_{st} = \frac{D_m}{H_s} \qquad (21)$$

4.2.6. *Genetic identity*

Genetic identity (Nei, 1972) is a parameter which indicates the probability that two alleles either sampled within a population, or one taken from each population, are identical. The probability of identity of two randomly chosen genes in a population X is:

$$j_x = \sum_{i=1}^{n} p_i^2{}_x \qquad (22)$$

while in a population Y it is:

$$j_y = \sum_{i=1}^{n} p_i^2{}_y \qquad (23)$$

The probability of identity of a gene from X and Y is (Nei, 1973; 1987):

$$j_{xy} = \sum_{i=1}^{n} p_{ix} p_{iy} \qquad (24)$$

where all terms are as defined previously.

Thus, the normalized identity of genes between X and Y with respect to one locus is defined as:

$$I_j = \frac{j_{xy}}{\sqrt{j_x j_y}} \qquad (25)$$

and for all loci is defined as:

$$I = \frac{J_{xy}}{\sqrt{J_x J_y}} \qquad (26)$$

which is the arithmetic mean of the sum of jx, jy and jxy, respectively, over all loci, including monomorphic loci. Nei (1978; 1987) demonstrated

that through expectation maximization, assuming multinomial sampling and random mating using a large sample size, unbiased estimates of genetic identities, hence genetic distances may be obtained.

4.2.7. Genetic distance

Genetic distance (Nei, 1972; 1978; 1987) is the applied index of genetic differentiation. It is useful as a measure of the accumulated number of gene substitutions per locus (i.e net codon differences) and is defined as:

$$D = -\log_e I \qquad (27)$$

where I is the genetic identity.

Genetic distance varies from zero (which implies all genes are identical) to infinity. The extent to which D exceeds zero indicates the degree of genetic divergence between the populations being compared.

The complement index (CI; Gottlieb, 1975) is a statistic indicating a population possessing the highest proportion of all the alleles identified within a taxon. It was estimated as a parameter to determine the most representative population of the 12 populations sampled. The assumption of the statistic (Gottlieb, 1975) is that the genetic changes in a population are mainly effected through alleles which are neither frequent nor rare at 95% criterion. The index was calculated as:

$$CI = \frac{k_i}{\sum_{i=1}^{n} k_i} \qquad (28)$$

where *ki* is the number of alleles at polymorphic loci with a frequency ≥1% in the population that is neither unique nor ubiquitous; summation of *ki* is the total number of such alleles in all populations. An allele may be defined as unique if it is present in

fewer than 5% of the populations sampled. A ubiquitous allele is one present in more than 95% of the sampled populations.

4.3. Results and Discussions

4.3.1. *Allelic frequency*

Table 4.1 presents the observed alleles and their frequency for the 10 loci studied in 12 populations. All alleles were polymorphic except for *Adh-2* and *6-Pgd-2* at Manzingwenya, S. Africa. According to the allele classification of Moran and Hopper (1987), alleles occurring in more than one population with a frequency greater than 10% are considered common, alleles occurring in one population only at 10% are localized, and those alleles occurring in less than 10% are considered rare. At most loci of *A. karroo,* two alleles were common except *6-Pgd-2*. The species also has multiple alleles, some of which fitted the description of rare and localized genes. The most outstanding alleles in the rare and localized category were *f* at *Aat-1* and *Sdh-1, d* at *Adh-2,* allele *c* at *Aat-3* and allele *a* at *Dia-1*. All the loci segregated for at least three or more alleles in a population. While it is possible that inadequate sample sizes could have marred identification of these localized alleles in some populations (Nei, 1975), it is also possible that other environmental forces might have had a major contribution in selecting against these alleles (Table 4.1).

When the distribution of most common alleles at all loci were correlated with the geographic factors latitude, longitude, altitude, and mean annual rainfall (Table 2.1), significant positive correlations were observed (Fig. 4.1) between the frequency of allele *Adh-2b* and either longitude ($r = 0.68$; $P < 0.01$) or mean annual rainfall ($r = 0.77$; $P < 0.01$). The frequencies of *Aat-3a* and *Sdh-1d* were negatively correlated with latitude ($r = -0.60$; $P<0.05$, and $r = -0.53$; $P<0.10$, respectively). Brain (1986) found that

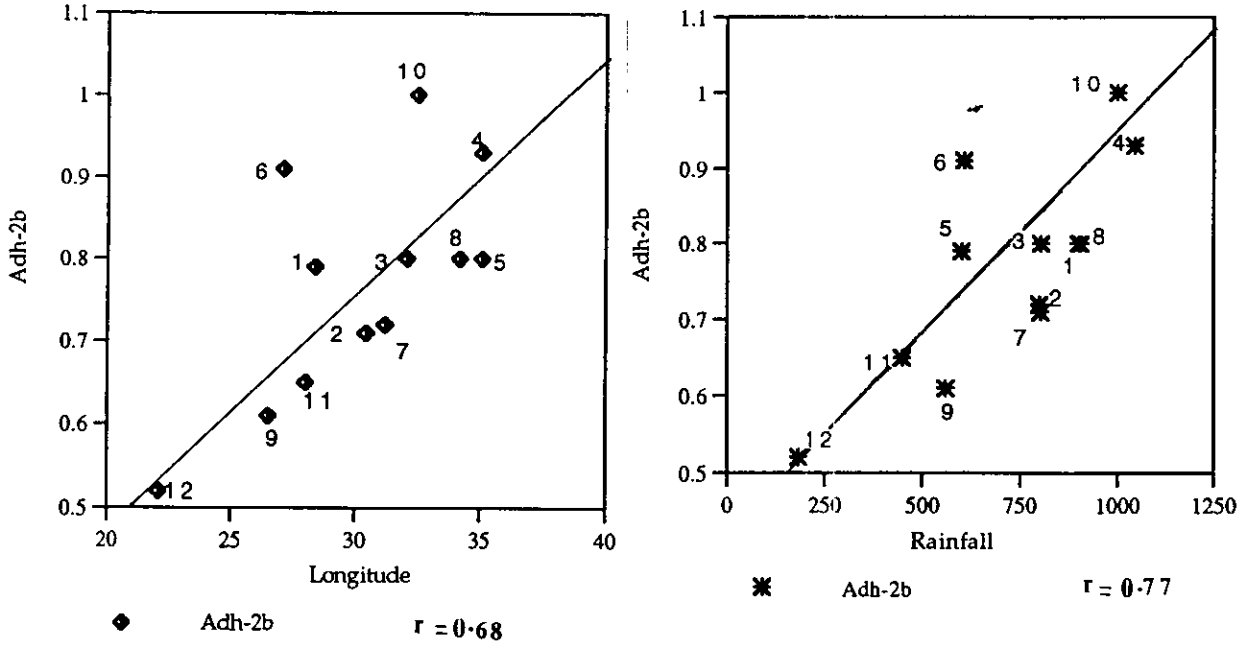
Table 4.1. Allele frequencies at 10 loci and expected heterozygosity (He) in *A. karroo*. Populations are abbreviated from names in Table 2.1.

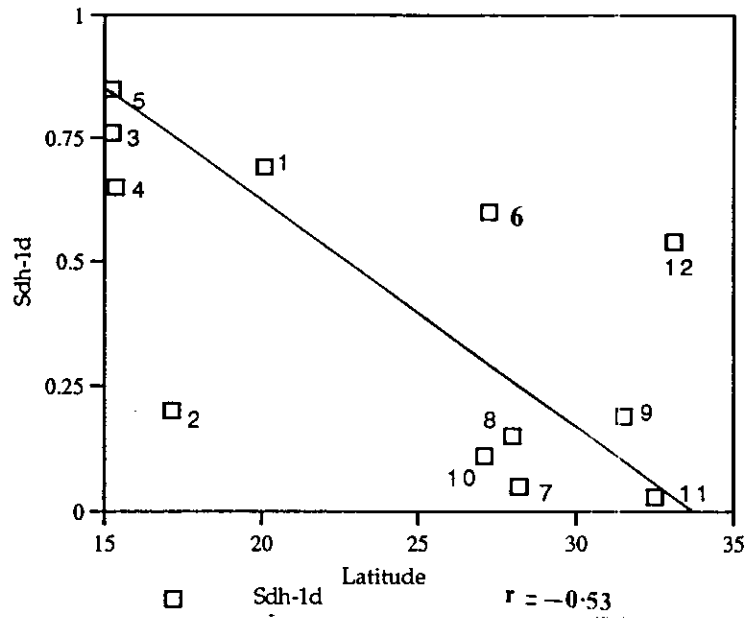
Populat.		P.Alb	Quee.	Cint.	Hluh.	Baba.	Manz	Kroo.	Zom	Dedza	UVE	Muto	Lusa.
No.		12	9	11	8	7	10	6	4	3	1	2	5
Sample	Size	23	65	50	80	75	30	31	80	80	120	80	25
Locus	Allele												
Aat-1	a	0.02	0.01	0.00	0.06	0.06	0.04	0.05	0.01	0.0	0.02	0.01	0.06
	b	0.48	0.59	0.61	0.56	0.52	0.33	0.55	0.50	0.58	0.58	0.55	0.58
	b"	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	c'	0.0	0.05	0.02	0.08	0.14	0.0	0.0	0.18	0.10	0.10	0.21	0.19
	c	0.06	0.23	0.29	0.23	0.21	0.30	0.24	0.22	0.24	0.22	0.16	0.17
	d'	0.02	0.0	0.0	0.0	0.0	0.14	0.0	0.0	0.0	0.0	0.0	0.0
	d	0.40	0.11	0.08	0.06	0.05	0.05	0.14	0.09	0.08	0.08	0.07	0.0
	e	0.01	0.0	0.0	0.01	0.02	0.14	0.05	0.0	0.0	0.00	0.0	0.0
	f	0.01	0.0	0.0	0.0	0.0	0.0	0.07	0.0	0.0	0.0	0.0	0.0
	He	**0.92**	**0.88**	**0.86**	**0.89**	**0.92**	**0.98**	**0.95**	**0.93**	**0.89**	**0.89**	**0.91**	**0.86**
Aat-2	a	0.0	0.04	0.03	0.03	0.04	0.11	0.02	0.01	0.0	0.05	0.03	0.0
	b	0.56	0.45	0.43	0.24	0.27	0.56	0.54	0.53	0.48	0.46	0.42	0.44
	c	0.44	0.51	0.54	0.73	0.69	0.33	0.44	0.46	0.52	0.49	0.55	0.56
	He	**0.89**	**0.89**	**0.88**	**0.72**	**0.77**	**0.89**	**0.88**	**0.88**	**0.87**	**0.90**	**0.88**	**0.86**
Aat-3	a	0.61	0.67	0.69	0.49	0.56	0.71	0.74	0.58	0.55	0.57	0.47	0.48
	b	0.39	0.33	0.31	0.50	0.44	0.29	0.26	0.42	0.45	043	0.53	0.52
	c	0.0	0.0	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	He	**0.84**	**0.78**	**0.77**	**0.88**	**0.86**	**0.74**	**0.69**	**0.86**	**0.87**	**0.86**	**0.87**	**0.87**
Adh-1	a	0.34	0.32	0.29	0.32	0.29	0.38	0.33	0.34	0.34	0.34	0.31	0.35
	b	0.31	0.34	0.39	0.41	0.41	0.20	0.27	0.32	0.40	0.34	0.31	0.39
	c	0.35	0.34	0.30	0.26	0.30	0.31	0.39	0.33	0.24	0.27	0.28	0.26
	d	0.0	0.0	0.01	0.01	0.0	0.11	0.01	0.01	0.02	0.05	0.10	0.0
	He	**0.96**	**0.96**	**0.96**	**0.96**	**0.96**	**0.97**	**0.96**	**0.96**	**0.96**	**0.97**	**0.97**	**0.96**
Adh-2	a	0.48	0.19	0.11	0.14	0.23	0.0	0.09	0.06	0.14	0.18	0.25	0.20
	b	0.52	0.61	0.65	0.80	0.72	1.00	0.91	0.93	0.80	0.79	0.71	0.80
	c	0.0	0.18	0.08	0.05	0.05	0.0	0.0	0.01	0.01	0.03	0.01	0.00
	d	0.0	0.0	0.0	0.01	0.0	0.0	0.0	0.0	0.01	0.0	0.0	0.0
	n	0.0	0.02	0.16	0.0	0.0	0.0	0.0	0.0	0.04	0.0	0.03	0.0
	He	**0.87**	**0.86**	**0.83**	**0.58**	**0.72**	**0.0**	**0.29**	**0.27**	**0.44**	**0.60**	**0.74**	**0.59**
β–Est-1	a	0.08	0.14	0.03	0.19	0.16	0.01	0.01	0.14	0.13	0.16	0.05	0.04
	b	0.73	0.66	0.13	0.37	0.53	0.54	0.46	0.44	0.57	0.48	0.18	0.16
	c	0.02	0.06	0.65	0.32	0.06	0.12	0.36	0.22	0.24	0.05	0.56	0.37
	d	0.17	0.14	0.19	0.12	0.25	0.33	0.17	0.21	0.06	0.30	0.21	0.43
	e	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.01	0.0	0.0
	He	**0.72**	**0.82**	**0.81**	**0.97**	**0.92**	**0.90**	**0.94**	**0.96**	**0.89**	**0.94**	**0.90**	**0.95**
Dia-1	a	0.0	0.0	0.0	0.0	0.0	0.0	0.05	0.0	0.0	0.0	0.0	0.0
	b	0.0	0.0	0.02	0.07	0.12	0.0	0.10	0.11	0.19	0.13	0.11	0.17
	c	0.01	0.02	0.02	0.01	0.0	0.18	0.0	0.02	0.03	0.01	0.01	0.01
	d	0.62	0.29	0.14	0.15	0.09	0.33	0.20	0.20	0.26	0.23	0.30	0.20
	e	0.21	0.10	0.25	0.06	0.05	0.26	0.05	0.01	0.06	0.06	0.08	0.12
	f	0.16	0.59	0.57	0.71	0.74	0.23	0.60	0.66	0.46	0.57	0.51	0.50
	He	**0.85**	**0.87**	**0.89**	**0.74**	**0.70**	**0.98**	**0.87**	**0.80**	**095**	**0.89**	**0.92**	**0.93**
Sdh-1	a	0.09	0.08	0.06	0.02	0.12	0.0	0.0	0.0	0.0	0.0	0.09	0.0
	b	0.14	0.47	0.49	0.28	0.26	0.32	0.0	0.0	0.0	0.0	0.14	0.0
	c	0.20	0.20	0.30	0.54	0.56	0.53	0.32	0.30	0.19	0.21	0.54	0.04
	d	0.54	0.19	0.03	0.15	0.05	0.11	0.60	0.65	0.76	0.69	0.20	0.85
	e	0.02	0.03	0.12	0.01	0.01	0.04	0.08	0.05	0.05	0.10	0.03	0.06
	f	0.01	0.03	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	He	**0.91**	**0.95**	**0.93**	**0.91**	**0.90**	**0.91**	**0.86**	**0.81**	**0.67**	**0.77**	**0.91**	**0.48**
6-Pgd-1	a'	0.24	0.25	0.26	0.30	0.28	0.29	0.20	0.28	0.25	0.25	0.25	0.13
	a	0.38	0.51	0.47	0.40	0.39	0.31	0.25	0.62	0.50	0.46	0.50	0.33
	b'	0.17	0.20	0.23	0.24	0.26	0.28	0.24	0.07	0.18	0.25	0.22	0.33
	b	0.21	0.04	0.04	0.06	0.07	0.12	0.31	0.03	0.07	0.04	0.03	0.21
	He	**0.97**	**0.93**	**0.94**	**0.96**	**0.97**	**0.98**	**0.98**	**0.85**	**0.93**	**0.95**	**0.94**	**0.97**
6-Pgd-2	a'	0.17	0.19	0.21	0.23	0.03	0.0	0.29	0.01	0.04	0.03	0.0	0.12
	a	0.66	0.75	0.77	0.39	0.71	1.00	0.55	0.68	0.76	0.81	0.49	0.76
	a"	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.01	0.0	0.03	0.42	0.08
	n	0.17	0.06	0.02	0.38	0.26	0.0	0.16	0.30	0.20	0.13	0.09	0.04
	He	**0.82**	**0.68**	**0.65**	**0.95**	**0.75**	**0.00**	**0.90**	**0.78**	**0.67**	**0.57**	**0.91**	**0.67**

five (k, l, m, n, o) bands at the peroxidase locus of *A. karroo* were correlated with geography. The increase in frequency of *Adh-2b* allele from west to east, and with rainfall, is consistent with earlier observations by Marshall *et al.* (1974), Roose and Gottlieb (1980) and Gottlieb (1981) that the enzyme *Adh* is induced by moisture conditions. However, in a similar study, Brown *et al.* (1974) found no defined pattern of the *Adh* locus in a natural populations of *Bromus mollis*. The direction of the increase in the frequency of *Adh* enzyme here suggested that most homozygotes occurred in sites with higher precipitation. The *Sdh-1d* allele was common in most populations, but its negative correlation with latitude can be interpreted in terms of more *Sdh-1* alleles (*a-f*) in the south, thus reducing its frequency there. Also, the eastern populations of Manzingwenya, Babanango and Hluhluwe showed a higher frequency of allele *Sdh-1c*, suggesting an environmental influence. The latitudinal pattern of change in the *Sdh-1* alleles was general but not conclusive explanation in that Mutorashanga, a population in the northern latitude expressed more alleles of *Sdh-1* in relationship to other populations in that region. This would appear to rule out the possibility that these alleles were lost during species' migration from the south northwards. Another possible explanation has been recurrent polyploidization of the species as noted in section 3.3.4.13., but verification of such a possibility requires further studies.

The complement index (CI; Gottlieb, 1975) was used to identify the most representative population among the 12 sampled (Table 4.2). Hluhluwe population exhibited the highest complement index (0.95), followed by populations from Cintsa River and Mutorashanga, each with a CI of 0.91. Gottlieb (1975) suggested that major changes in genetic structure of a plant population are effected through the non-ubiquitous and non-unique alleles. The assumptions of CI (Gottlieb, 1975) is that ubiquitous alleles are neutral to the selective pressures while the rare

Figure 4.1. Distribution of Adh-2b and Sdh-1d alleles of *A. karroo* relative to geographical factors.





Population numbers are as in Table 4.1.

alleles are too few to effect changes in populations, thus, it is the non-ubiquitous and non-unique alleles that play crucial role in genetic changes of a population.

Table 4.2. Complement indices of 12 populations of *A. karroo*

Pop. No.	12	9	11	8	7	10	6	4	3	1	2	5
Tot. allel	37	38	39	41	37	33	35	37	35	38	39	33
Ubi. allel	20	20	20	20	20	20	20	20	20	20	20	20
Uni. allel	3	1	0	1	0	1	1	0	0	0	0	0
Non-ubiq.	14	17	19	20	17	12	14	17	15	18	19	13
CI	0.67	0.82	0.91	0.95	.81	0.57	0.67	0.81	0.71	0.86	0.91	0.62

Pop. No. refers to population numbers given in Table 2.1. Tot. allel. = total alleles, Ubi. allel. = ubiquitous alleles, Uni. allel. = unique alleles, and Non. ubiq. = non-ubiquitous and non-unique alleles.

The mean number of alleles per locus averaged over the 12 populations was 3.7 (Table 4.3). Generally, a high mean number of alleles per polymorphic locus has been associated with monocots (A=3.26; Hamrick and Godt, 1989), but data accumulated recently demonstrate that some dicots and gymnosperms also have high mean numbers of alleles per locus. Some examples of tree species reported to have high average numbers of alleles per locus are *Pinus taeda* (3.89 to 4.78, Conkle, 1981), *Gleditsia triacanthos* (3.2, Schnabel and Hamrick, 1990) and *Maclura pomifera* (4.0, Schnabel *et al.* 1991). Here, the Hluhluwe and Mutorashanga populations had the highest average number of alleles per locus, at 4.1 and 3.9, respectively. The lowest mean number of alleles per locus was observed in the Lusaka and Manzingwenya populations (3.3), which comprised 25 and 31 samples, respectively. Across all populations, a significant positive correlation existed between the mean number of alleles per locus and sample size ($r = 0.63$; $P < 0.05$). This observation is consistent with previous reports; for example, Nei (1975) and Brown and

Table 4.3. Distribution of genetic diversity estimates (with standard errors) for 12 populations of *A. karroo*.

Population	Numb. in Tab. 2.1.	N	$\bar{A}$	$\bar{Ae}$	$\bar{P}$	$\bar{Ho}$	$\bar{He}$
Prince Albert	12	23	3.7 ± .35	8.0	100	0.70 ± .10	0.88 ± .02
Queenstown	9	65	3.8 ± .28	7.2	100	0.72 ± .08	0.86 ± .03
Cintsa River	11	50	3.8 ± .27	6.7	100	0.69 ± .10	0.85 ± .03
Hluhluwe	8	80	4.1 ± .32	7.0	100	0.74 ± .10	0.86 ± .04
Babanango	7	75	3.6 ± .37	6.5	100	0.69 ± .09	0.85 ± .03
Manzingwenya	10	30	3.3 ± .47	5.2	80	0.68 ± .11	0.74 ± .09
Kroonstad	6	31	3.5 ± .35	6.0	100	0.70 ± .11	0.83 ± .07
Zomba	4	80	3.7 ± .28	5.3	100	0.71 ± .10	0.81 ± .06
Dedza	3	80	3.5 ± .29	5.3	100	0.68 ± .10	0.81 ± .05
Umguza Valley	1	120	3.8 ± .34	6.0	100	0.73 ± .09	0.83 ± .05
Mutorashanga	2	80	3.9 ± .30	9.5	100	0.75 ± .09	0.90 ± .02
Lusaka	5	25	3.3 ± .32	5.4	100	0.68 ± .09	0.82 ± .05
Mean		62	3.7	6.5	98.3	0.71	0.83

Num. in Tab. 2.1. refers to population numbers given in Table. 2.1. Sample sizes (N), mean number of alleles per locus (A), effective number of alleles (Ae), percentage loci polymorphic (P) at 0.95% level, observed (HO) and expected heterozygosities (He).

Weir (1983) have demonstrated that this parameter is dependent on sample size. However, this relationship is not direct: Umguza Valley population, which had the largest sample size, did not exhibit the highest mean number of alleles. The relationship was influenced by the variation at some loci, e.g. *Dia-1, Sdh-1* and *6Pgd-1* (Table 4.1), which displayed alleles which were present in some regions and not in others. Hamrick *et al.* (1981) noted that the number of alleles in a population sample expresses the allelic richness and the value is usually higher in widespread species. Brown and Weir (1983) indicated that genetic diversity is also dependent on the mean number of alleles per locus. Populations with an

evenly distributed number of alleles have been demonstrated to express high variablity (Ayala and Kiger, 1984).

The high allelic richness at Hluhluwe and Mutorashanga populations could be explained partially by the historical background of the sites. Hluhluwe area, which had the highest number of alleles, has been reserved as a National Park, while Mutorashanga area has been a mining reserve. Both these reservations might have limited genetic erosion due to exploitation, which might have occured more easily at other sites.

The effective number of alleles (Table 4.3) was higher than the average number of alleles; this could be due to the polyploid nature of the species. Waples (1988) noted that, in alloploids, the level of heterozygosity may be biased upwards when the isoloci are not exactly identical, or downwards when double heterozygotes are scored as homozygotes due to the technical problem of recognizing them.

4.3.2. The proportion of polymorphic loci

The proportion of polymorphic loci (P) were 80% in Manzingwenya and 100% in the remaining populations (Table 4.3). The average polymorphism (95% criterion) for the populations was 98% and for the species was 100%, which is exceptionally high compared to the 50% reported by Hamrick and Godt (1989) for plant species generally, 95% for *A. tortilis* (Olng'otie 1992), and 90% for *A. albida* (Joly *et al.*, 1992). Fincham (1972) and Hamrick and Godt (1989) attributed most genetic diversity among species to the proportion of loci polymorphic rather than the number and the frequency of alleles at a locus. In addition, Gottlieb (1981), Ayala and Kiger (1984) and Hedrick (1985) concluded that the low frequency alleles in a population exist primarily in heterozygotic form. It

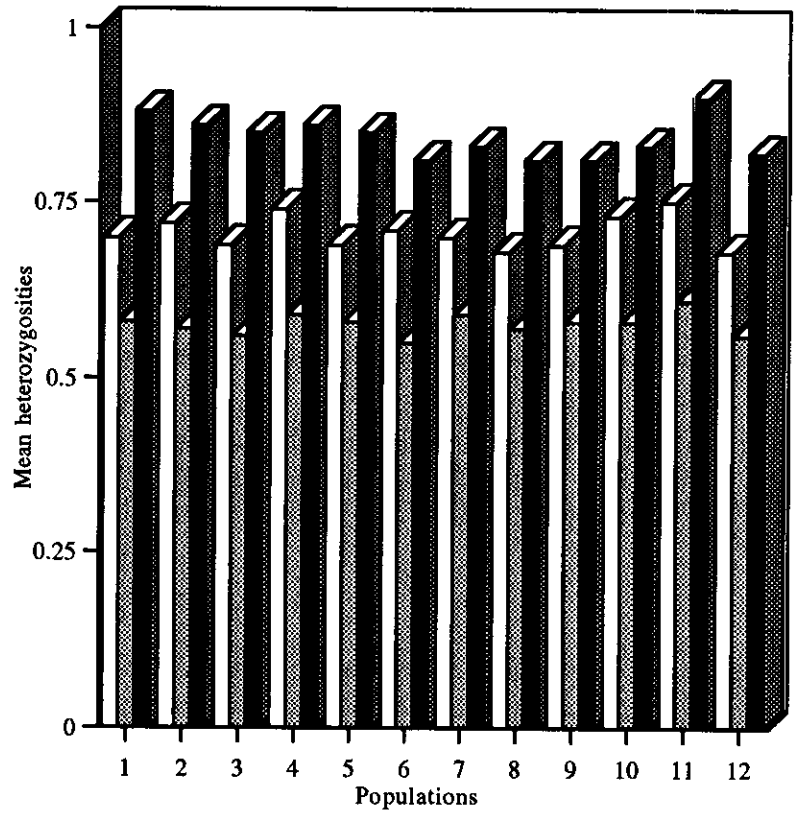
then follows that in a multiallelic species such as *A. karroo,* if every allele has high frequency, their contribution to homozygosity will be very low, and a high level of polymorphism will result.

4.3.3. Genetic diversity

All populations assayed exhibited a very high level of genetic diversity (Table 4.3). Observed heterozygosity (H_o) ranged from 68% in the Manzingwenya population to 75% in the Mutorashanga population. The mean observed heterozygosity was 71%, and the theoretical expected heterozygosity assuming tetrasomic inheritance (H_e = 83%) was higher than that observed. The lowest expected heterozygosity was found at Manzingwenya (74%) and the highest at Mutorashanga (90%). These observations are consistent with the fact that, at Manzingwenya, two loci were monomorphic. A comparison of mean heterozygosity levels for different populations is illustrated in Figure 4.2. The heterozygosity estimated assuming Hardy-Weinberg equilibrium (H_e H.W.) is much lower than that assuming tetrasomic inheritance (H_e tet.). The observed heterozygosity (H_o) is intermediate between that of the two assumptions.

Estimates of other gene diversity statistics are presented in Table 4.4. The values for H_T ranged from 97% (*Adh-1*) to 64% (*Adh-2*). The mean total gene diversity was 88% (± .03). A similar trend was observed for the intrapopulational gene diversity (H_s), which was greatest at *Adh*-1 locus (96%) and least at *Adh-2* locus (57%). It is unlikely that the heterozygosity would have been lowered appreciably if the other six loci considered in Chapter Three were scored, since all except α-esterase were highly polymorphic. The mean intrapopulational gene diversity was 84% (± .04). Among the populations, loci *Sdh-1, Adh-2, β-Est-1, Dia-1* and *Aat-1* (in descending order) were the most variable, as evidenced by coefficients of gene differentiation, G_{st} and D_m (Table 4.4). Two loci, *Adh-1* and *6-Pgd-1,*

Figure 4.2. Heterozygosity levels in 12 populations of *A. karroo*.



□ Ho

▒ He (H.W).

■ He (tet.).

Ho = Observed heterozygosity
He (H.W) = Expected " (Hardy-Weinberg)
He (tet.) = Expected " (tetrasomic inheritance)
Population numbers are as in Table 4.1.

Table 4.4. Genetic differentiation at 10 loci across 12 populations of *A. karroo*.

	Gene diversity estimates					
Locus	$\bar{H}T$	$\bar{H}s$	$\bar{D}st$	$\bar{D}m$	$\bar{G}st$	$\bar{R}st$
Aat-1	0.96	0.91	0.05	0.06	0.05	0.06
Aat-2	0.89	0.86	0.03	0.03	0.03	0.04
Aat-3	0.85	0.83	0.02	0.03	0.03	0.03
Adh-1	0.97	0.96	0.00	0.01	0.01	0.00
Adh-2	0.64	0.57	0.08	0.09	0.12	0.15
β-Est-1	0.96	0.89	0.06	0.07	0.07	0.08
Dia-1	0.92	0.87	0.05	0.06	0.06	0.06
Sdh-1	0.94	0.84	0.10	0.11	0.11	0.14
6-Pgd-1	0.96	0.95	0.01	0.01	0.01	0.01
6-Pgd-2	0.73	0.69	0.04	0.04	0.05	0.06
Mean	0.88	0.84	0.05	0.05	0.05	0.06
s.e	0.03	0.04	0.01	0.01	0.00	0.01

Total gene diversity (HT), intrapopulation diversity (Hs), interpopulation gene diversity (Dst), coefficient of gene differentiation (Gst), absolute coefficient of gene differentiation (Dm) and coefficient of interpopulational diversity relative to intrapopulational gene diversity (Rst).

were approaching fixation level, as indicated by both coefficients of gene diversity in all populations. The mean coefficient of gene differentiation among populations was 5.3%, which is comparable to that reported for populations of most conifers (Conkle, 1981; Hamrick and Godt, 1989; Millar and Marshall, 1991). From the above parameter, it is estimated that 94.7% of the genetic variation occurred within the populations. When R_{st} was compared to G_{st}, a similar trend of genetic diversity was evident, indicating that most genetic diversity was retained within the populations. A chi-square goodness-of-fit test, to compare observed with expected heterozygosities could not be performed because the potential number of genotypes (see Equation 2 above) exceeded the samples procured.

Conifers have been considered the most genetically variable tree species, with a mean expected genetic diversity of 27% (Mitton, 1983; Hamrick and Godt, 1989). Most of that genetic diversity occurred within the populations. The high level of diversity is thought to be maintained by widespread distribution, an efficient gene flow mechanism, and high fecundity (Brown and Moran, 1981; Hamrick *et al.*, 1981; Loveless and Hamrick, 1984; Hamrick and Godt, 1989). Among the conifers, some higher levels of genetic diversity have been reported, e.g. *Pseudotsuga menziesii* (59%, Brown and Moran, 1981), *Picea abies* and *Pinus taeda* (each 34%, Hamrick *et al.*, 1981). However, it is evident that some species among the angiosperms have higher levels of genetic diversity than those reported for conifers, with estimates of 45% for *A. albida* (Joly *et al.*, 1992) and *A. tortilis* (Olng'otie, 1992), 73% for *Maclura pomifera* (Schnabel *et al.*, 1991) and 83% for *A. karroo* (this study).

A. karroo expressed very high genetic diversity compared to the level of 30% total gene diversity previously reported for plant species in general (Hamrick *et al.*, 1992). This higher level of diversity could result from the presence of multiple alleles due to the segmental tetraploid nature of the species (Chapter Three). Reports of electrophoretic studies in other segmental allotetraploids are scarce and, where available, data are either not scored because of complicated patterns (Bayer and Crawford, 1985; Bayer, 1989), or focus mainly on electrophoretic and taxonomic divergence (Barrington, 1990; Cai and Chinnappa, 1991). Most studies involving a comparison of genetic diversity in polyploids and diploids have concluded that there is higher variation associated with higher ploidy level (Bayer, 1989; Ness *et al.* 1989, Shore, 1991). Similarly, lower levels of genetic diversity, ranging from 1.7% to 30%, have been reported for Australian acacias which are mostly diploids (Coates, 1988; Moran *et al.*, 1989a&b; Muona, 1989). Other factors which could have contributed to high genetic

diversity include the species' mating system and mode of seed dispersal (Hamrick *et al.*, 1981; Loveless and Hamrick, 1984; Hamrick *et al.*, 1992).

Although the mating system of *A. karroo* has not been studied, its reproductive phenology (Chapter One, section 1.4) indicates the qualities of an outcrossing species. Studies conducted in other acacias (Moffett, 1956; Kenrick and Knox, 1985; 1989; Moran *et al.* 1989a; 1989b; Muona *et al.*, 1991; Sedgley *et al.*, 1992) suggest that most species in the genus are predominantly outcrossing.

The main pollinating agents of the acacias are animals (primarily insects), but the efficiency of these pollinators is known to vary. Loveless and Hamrick (1984) and Murawski and Hamrick (1991) indicated that the efficiency of bees in long distance pollen transfer varies and depends on flying habits of the species involved, explaining why plants pollinated by some bee species usually have low genetic differentiation. Otherwise, most plants which are zoomophilous tend to display highly genetically structured populations (Brown and Moran, 1981; Hamrick *et al.* 1984), determined by the low efficiency of gene dispersal. The low coefficient of gene differentiation here (G_{st}, 5.3%) suggests, similarly, high efficiency in gene transfer. In the field, *A. karroo* exhibits asynchronous flowering among different trees and sequential floral maturation on the same capitate inflorescense. If the species is self-incompatible and flowering pattern is not synchronized among related trees to encourage consanguinity, then the mating system would favour random transfer of pollen, increasing genetic diversity and the distribution of alleles. Coefficients of differentiation lower than that estimated here have been reported for some tropical trees such as *Metrosideros* species (Aradhya *et al.*, 1991) *Bertholletia excelsa* and *Swatzia simplex* var. *ochnacea* (Bawa and Krugman, 1991), which are animal pollinated also.

A. karroo is a colonizing species (Wells *et al.* 1986; Scott, 1991), indicating that the seed dispersal mechanisms must be very effective in order to compete for new sites. It is generally accepted that, like most acacias with dehiscent pods, *A. karroo's* seeds are dispersed primarily by wind and gravity (Coe and Coe, 1987). These modes of dispersal are less efficient in gene distribution when the seeds are heavy; it is most likely that herbivores which feed on the species and ingest the seeds contribute to long distance seed dispersal and, hence, gene transfer. Hamrick and Godt (1989) found that trees with animal-ingested seeds are genetically more variable than those with seeds that are dispersed by other mechanisms.

4.3.4. Genetic identity

The genetic identity statistics of Nei (1972; 1978), used as a measure of similarity, are presented in Table 4.5. According to the pair-wise comparison matrix, Dedza, Zomba and Umguza Valley were the most closely related populations. Umguza Valley is slightly closer to Dedza than to Zomba (genetic identities 0.99 and 0.98, respectively). The value of the statistic ranged from 0.77 to 0.99; the mean genetic identity was 0.90 with a cophenetic correlation of 0.87.

Genetic distances, which are functions of genetic identities, are presented in Table 4.6. Generally, the Prince Albert population appeared to be most divergent from other populations. The maximum divergence was observed between Prince Albert population and Lusaka population, with a high genetic distance of 0.27 in comparison to a mean genetic distance for all populations of 0.11. The correlation of genetic and geographical distances (measured on a map) ($r = 0.11$) was positive but not significant, indicating that factors other than simply distance between populations are involved in determination of genetic structure.

Table 4.5. Matrix of standard genetic identities (Nei, 1978) for pair-wise comparisons of 12 populations of *A. karroo* over 10 loci.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1 Prince Albert (12)	1.00	0.89	0.79	0.80	0.82	0.84	0.83	0.81	0.84	0.84	0.84	0.77
2 Queenstown (9)		1.00	0.86	0.88	0.94	0.91	0.91	0.92	0.93	0.94	0.83	0.87
3 Cintsa River (11)			1.00	0.88	0.90	0.88	0.93	0.91	0.91	0.90	0.90	0.92
4 Hluhluwe (8)				1.00	0.94	0.80	0.89	0.91	0.90	0.90	0.90	0.88
5 Babanango (7)					1.00	0.89	0.93	0.97	0.97	0.98	0.86	0.94
6 Manzingwenya (10)						1.00	0.94	0.92	0.93	0.94	0.80	0.90
7 Kroonstad (6)							1.00	0.95	0.95	0.95	0.84	0.94
8 Zomba (4)								1.00	0.98	0.98	0.86	0.94
9 Dedza (3)									1.00	0.99	0.85	0.96
10 Umguza Valley (1)										1.00	0.85	0.97
11 Mutorashanga (2)											1.00	0.87
12 Lusaka (5)												1.00

Numbers in brackets refer to populational numbers in Table 2.1 and Fig. 2.1.

Table 4.6. Matrix of standard genetic distances (above diagonal) and corresponding standard errors (below diagonal) for pairwise comparisons of 12 populations of *A. karroo* over 10 loci.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1 Prince Albert (12)	---	0.12	0.24	0.22	0.19	0.17	0.19	0.21	0.17	0.17	0.17	0.27
2 Queenstown (9)	0.05	---	0.15	0.13	0.07	0.09	0.09	0.08	0.07	0.06	0.19	0.14
3 Cintsa River (11)	0.15	0.12	---	0.13	0.11	0.13	0.08	0.09	0.09	0.10	0.11	0.09
4 Hluhluwe (8)	0.12	0.09	0.06	---	0.07	0.22	0.11	0.10	0.11	0.11	0.11	0.13
5 Babanango (7)	0.13	0.08	0.06	0.04	---	0.12	0.07	0.03	0.03	0.02	0.16	0.06
6 Manzingwenya (10)	0.10	0.06	0.06	0.07	0.05	---	0.07	0.09	0.08	0.06	0.23	0.11
7 Kroonstad (6)	0.11	0.07	0.03	0.05	0.02	0.04	---	0.05	0.05	0.06	0.17	0.07
8 Zomba (4)	0.13	0.07	0.03	0.06	0.01	0.04	0.03	---	0.02	0.02	0.15	0.06
9 Dedza (3)	0.13	0.08	0.05	0.06	0.01	0.02	0.02	0.01	---	0.01	0.16	0.05
10 Umguza Valley (1)	0.13	0.08	0.06	0.06	0.01	0.03	0.02	0.00	0.01	---	0.16	0.03
11 Mutorashanga (2)	0.12	0.11	0.05	0.06	0.08	0.07	0.08	0.08	0.10	0.10	---	0.14
12 Lusaka (5)	0.17	0.14	0.05	0.07	0.03	.033	0.02	0.03	0.04	0.03	0.11	---

Numbers in brackets refer to populational numbers in Table 2.1 and Fig. 2.1.

In a review of electrophoretic analyses and plant systematics, Gottlieb (1977a) concluded that most conspecific populations have high mean genetic identities, above 0.90, while congeneric plant species have reduced genetic identities, varying from 0.50 to 0.60. If pair-wise comparisons of genetic identities and distances are made according to Gottlieb's criterion, it suggests that some populations of *A. karroo* have diverged to subspecies or variety level (0.61-0.89). From the evidence in Table 4.5 and 4.6, the three populations of Umguza Valley, Dedza and Zomba are within the conspecific level. The genetic identity pattern exhibited by the three populations may be related to geographical distance (Fig. 2.1), as these populations are closer to each other than to the other populations. However, this correlation between genetic distances and geography was not consistent across the populations: the Mutorashanga population, that occurred centrally among the other three populations identified above, had a low genetic identity (0.86) with the nearest of the three.

A second group of populations (Babanango, Queenstown, Manzingwenya, Lusaka, Kroonstad, Cintsa River and Hluhluwe) also falls within the levels of conspecificity. Some of the members of this second group are closely connected to the first group, though with reduced mean pair-wise genetic identity. This observation demonstrates that most populations are interrelated, suggesting a possibility of clinal variation. A distant relationship (0.80-0.89) was evident between Prince Albert and the other populations, except Cintsa River and Lusaka. A conspecific relationship was registered between Cintsa River and Lusaka populations, which are geographically distant. The latter observation may evidence moderations of genetic identity by similar ecological factors.

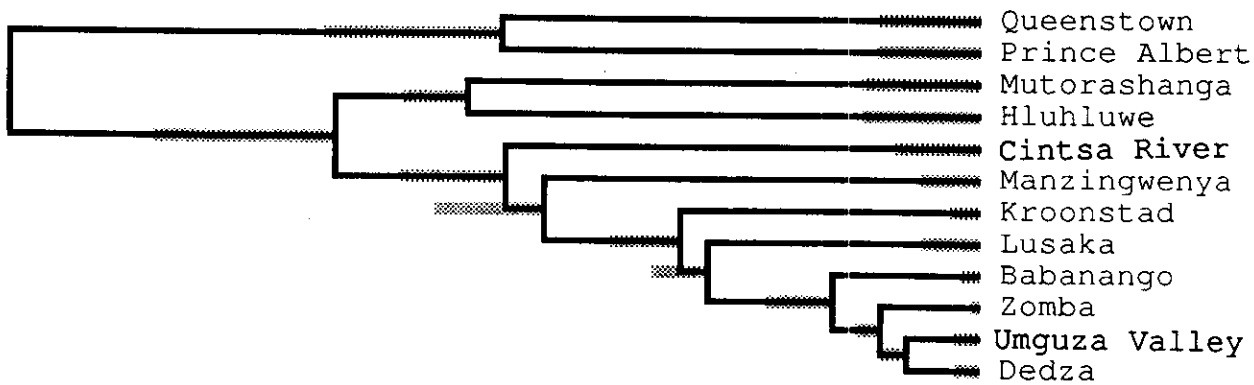
More recent studies (Crawford, 1990; and references therein) have supported the margins of genetic identities suggested by Gottlieb (1977a), but divergence at enzyme loci is often uncorrelated with speciation

(Crawford, 1985). Interesting results have been provided on the divergence of various species, e.g. Soltis (1985) reported observations from *Heuchera americana, H. parviflora, H. pubesens* and *H. villosa,* which are morphologically variable but exhibited mean genetic identities of 0.98 to 0.99 between the first two and the second two species, respectively, and 0.83 to 0.85 between the two sets. Similar results were documented for various species of *Coreopsis* (Crawford and Smith, 1984) and *Antennaria* (Bayer, 1989), whilst Ness *et al.* (1989) found identities of up to 1.00 between polyploid and diploid *Antennaria* species. Conversely, Gottlieb (1973a; 1973b) and Roose and Gottlieb (1976), in their analyses of *Stephanomeria, Clarkia,* and *Tragopogon* species, respectively, reported cases where the subspecies were morphologically or chemotaxonomically identical, but the mean genetic identities were very low (minimum 0.28). Further experimental evidence about the relationship between electrophoretic data and plant speciation have been discussed in reviews by Crawford and Wilson (1979), Gottlieb (1981; 1982) and Crawford (1983; 1989; 1990).

Different plant species have undergone speciation at different stages but the general consensus is that plants which have had rapid and recent speciation, such as progenitor and derivative species, develop little divergence at allozyme loci. Geographic modes of speciation involve gradual transformation of genetic constitution; thus, the divergence depends on duration of separation and the presence of natural barriers to cross breeding. A number of speciation processes have been recognized (Crawford, 1989; 1990); for example, changes in floral structure, chromosome rearrangements, mutations, gene duplication, hybridization and polyploidization. The rate at which these transformations are incorporated in population development influences the trend of speciation.

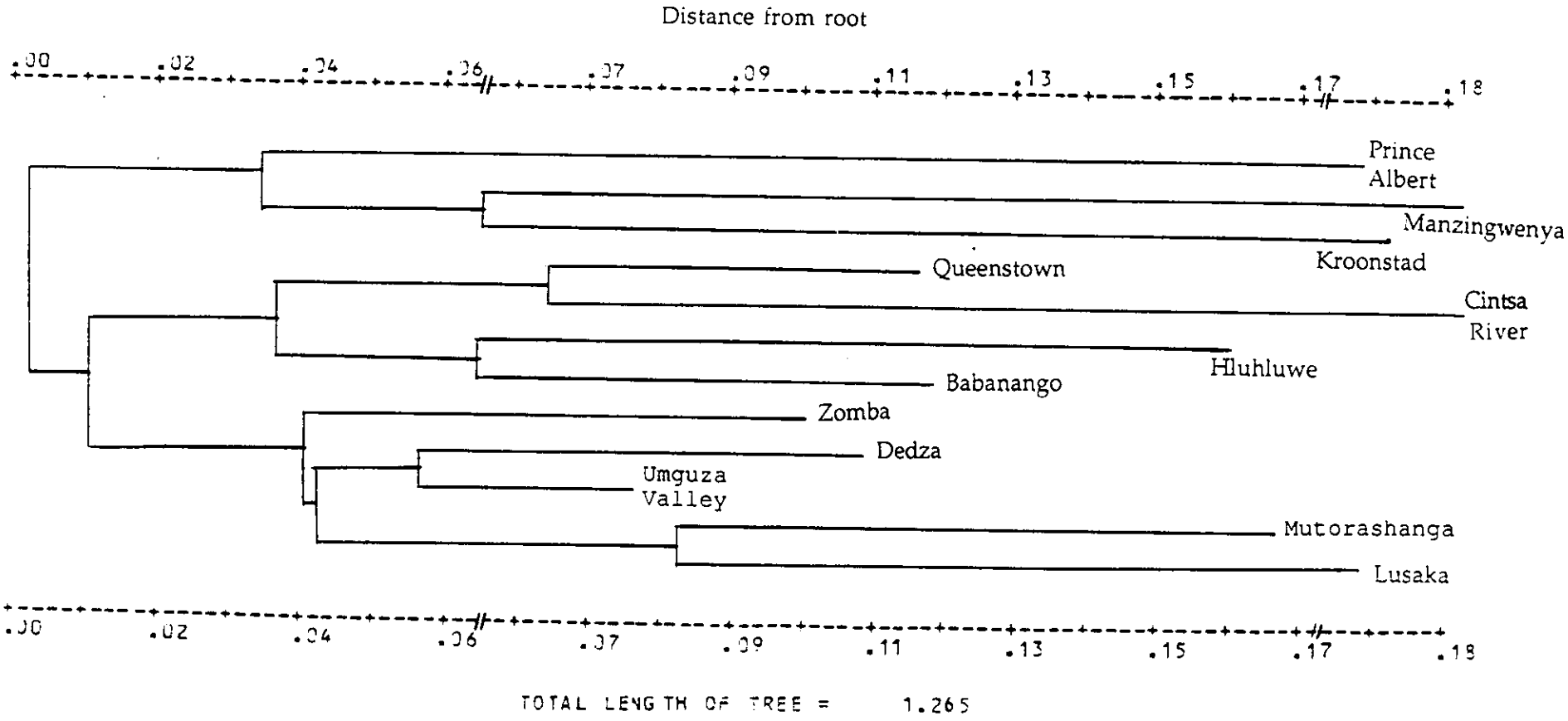
Dendrograms of the genetic relationship for *A. karroo* are illustrated by Figures 4.3 and 4.4. Figure 4.3 is clustered by the unweighted pair group of arithmetic means (UPGMA) of Sneath and Sokal (1973), while Figure 4.4 is the Wagner Tree (Farris, 1972) clustered by rooting the dendrogram at the mid point of the longest path. Both dendrograms were constructed using distance matrices (Table 4.6). The underlying assumption of UPGMA when applied to molecular data is that the expected rate of gene substitution is constant, leading to equal evolution on each branch of the tree (Nei, 1987). However, Nei (1987) cautioned that construction of a true evolutionary tree is difficult and may result in topological and branch length errors, because: (1) the distance measure could be subject to stochastic errors, and; (2) the number of possible trees rises rapidly with increases in the operational taxonomic units (OTUs) (see Appendix III).

Figure 4.3. UPGMA dendrogram of 12 populations of *A. karroo*.



The longest branch has a genetic distance of 0.27.

Figure 4.4. Rooted Wagner Tree dendrogram of 12 populations of *A. karroo*.



Thus, the most likely tree is chosen on the likelihood of genetic relationship and other biological information. UPGMA is known to give superior trees compared to other methods (see Nei, 1987), especially in the presence of stochastic errors and large distantly related pairs. The divergence of the cluster groups (Fig. 4.3) is significant when the shaded error bars on the UPGMA cluster occupy less than half the branch length (Nei and Roychoudhury, 1974; Ritland, 1988). According to Fig. 4.3, only two cluster groups exhibited significant divergence; these clusters are (1) Queenstown and Prince Albert and (2) the remaining populations. This result is further evidence that most variation is within the populations rather than among them.

The Wagner Tree differs from UPGMA in that it is designed to estimate a realized gene tree by considering various possible evolutionary paths. The Wagner Tree construction operates by trying to find the shortest possible tree that will connect the given OTUs. The OTUs are added sequentially to the tree by testing each of the branching possibilities, whilst minimizing the tree length. Although a Wagner tree rooted at the mid-point of the longest branch that connects two OTUs assumes a constant rate of evolutionary change, as does UPGMA, their construction algorithms differ, thus, there is the possibility of obtaining an alternative tree to that expected from UPGMA. Here, the Wagner tree gave a cladogram that closely corresponds to the natural distribution of the populations (OTUs) studied, in that geographically proximate populations are clustered on the same branches. The branches of the Wagner tree provided are scaled (see Li and Graur, 1991) and their lengths are proportional to the number of changes in the distance matrix (note that due to limited space the program changes the scale to fit the tree). The questionable cluster is the group involving Prince Albert, Kroonstad and Manzingwenya. Kroonstad is situated on the western side of Drakensburg

Ranges, Prince Albert is in the south of Karoo region and Manzingwenya is an eastern coastal populations. The Drakensburg Ranges are physical barriers to gene flow of *A. karroo* because it does not grow on frosty sites. Nei (1987) reported that recovering a true genetic tree is is not easy with molecular data: (1) the data is liable to stochastic errors; (2) many loci (>50) need to be considered to increase the accuracies, although minor deviations in the topology of the constructed tree from the true one do not invalidate the tree. The link between Prince Albert and Kroonstad on the Wagner tree of *A. karroo* may be justified since there is no obvious barrier to gene flow, but the link to Manzingwenya may be due to above-mentioned errors, or may suggest that populations are interconnected by intermediaries within the altitudinal range around the mountains. The Wagner Tree illustrated here for *A. karroo* showed some resemblence to an earlier cluster of *A. karroo* populations identified by Brain (1985; 1989) in a study involving peroxidase locus with samples collected within the Republic of S. Africa. The exceptional observations here are those showing the Kroonstad - Manzingwenya link; the northern populations were not included in Brain's (1989) study.

4.4. Conclusions

The general impression from this study is that *A. karroo* is genetically very diverse, with diversity maintained by the widespread distribution of the species and its polyploid nature. As with other tree species, most variation is found within populations. The estimate of low variation among the populations is supported by the low coefficient of gene differentiation. The possibility of maintaining such a low variation among populations depends on the efficiency of gene flow, a factor which is

usually supported by effective cross pollination and seed dispersal mechanisms.

Alternatively, the low differentiation evident in *A. karroo* could have resulted from an expansion of originally smaller populations. The species could, for example, originally have occupied smaller patches of land, restricted by its inability to grow under canopies of taller tree species (Acocks, 1988). However, due to continuous human interventions within the species range, e.g. preparation of land for agriculture, settlements and continuous grazing, the ecological balance could have been disturbed by the removal of primary forest trees. These conditions would have created a favourable environment for a colonizing species such as *A. karroo*. The settlements would have encouraged grass-fires and dispersal of animal-ingested seeds, two natural processes that enhance seed germination in acacias. Further, branches of *A. karroo* were frequently cut for homestead fencing or seeds planted for live fences (Sim, 1906), a system that assisted seed distribution.

Periodic outbreaks of droughts also could have played a role in seed dispersal, in that herbivores which feed on *A. karroo* could be covering long distances for fodder and water, dispersing ingested seeds along their trail. The almost bare grounds following overgrazing and droughts are liable to be swept by heavy rains, carrying away debris including seeds, and finally distributing them along the water courses. Such rapid widespread colonization have recently been documented for *Lantana camara* (Cilliers and Mesar, 1991; Danton *et al.*, 1991), *Prosopis juliflora*, *Leucaena leucocephala* (National Academy of Sciences, 1980) and *Thevetia nerifolia* (per. obser.) in some tropical countries.

Together, these factors could have contributed to rapid expansion of *A. karroo*, minimizing the differentiation between populations. The observed moderate divergence could be a result of gradual geographical

speciation. The high level of heterozygosity is maintained by multiple alleles in a likely allotetraploid genomes, which also safeguard the species against the occurrence of genetic drift and fixation. The genetic diversity within the populations could be enhanced by a possible introgression that might have taken place in the species range and the likely multiple polyploidization discussed in Chapter Three. The variation in banding patterns in certain regions could possibly be a guide to detecting the most likely parents of *A. karroo*, presuming they still exist.

The similarity between populations of *A. karroo* in terms of allozyme loci contrasts with phenotypic differences in form and growth reported earlier (Chapter One). However, such poor correlations are not restricted to this species. Most studies correlating allozyme divergence with different quantitative traits have yielded mixed results (Bush and Smouse, 1992): for example, studies of *Tragopogon* species (Roose and Gottlieb, 1976), *Pinus rigida* (Guries and Ledig, 1982) and *Pseudotsuga menziesii* (El-Kassaby, 1982) found no correlation between allozyme diversity and morphological variation. Elsewhere, positive associations between allozyme divergence and morphological traits have been reported, for growth of various trees (Mitton *et al.* 1981), yield of *Avena barbata* (Hamrick and Allard, 1972), and quantitative characters related to resistance to herbivory and environmental stress in *Pinus edulis* (Mopper *et al.*, 1991).

While sampling according to allozyme diversity should ensure adequate conservation of most genetic variation, selection for morphological characteristics will provide the necessary phenotypes required for different purposes at various sites. At every sampling site, the distance between the sampled trees is an important factor to be considered in sampling strategy, but that can only be defined with a clear view of

family structure and mating systems, which are the subjects of the next chapter.

CHAPTER FIVE

MATING SYSTEM WITHIN A POPULATION OF *A. KARROO* HAYNE

5.1. Introduction

Mating systems play an important role in determining the genetic composition of future generations. When random mating is assumed, genotypic frequencies can be estimated according to Hardy-Weinberg equilibrium, but this is a rare situation in nature. All mating systems that are assortative tend to increase the frequency of homozygotes in a population, hence deviating from the theoretical equilibrium: selfing and consanguineous mating contribute most to this phenomenon (Hartl and Clark, 1989). Consanguineous mating has been reported in a number of plant species (see for example, Ritland, 1984; 1989; Ritland and El-Kassaby, 1985; Muona *et al.*, 1991; Barrett and Husband, 1990). Apart from determining the relationship of gametes joined at fertilization, mating systems influence the genetic structure of populations, the levels of gene pool homogenization and the degree to which populations may be subdivided genetically in response to selection and genetic drift (Hamrick and Schnabel, 1985; Adams and Birkes, 1991).

The number of plant populations for which mating parameters have been determined have risen recently, mainly because of increased awareness of the diversity of plant mating systems, and the number of genetic markers available under isozyme systems (Loveless, 1992). However, this general trend does not apply equally to *Acacia* species. Of more than 1000 *Acacia* species known (Doran *et al.*, 1983; Muona, 1989), only a few have been studied for mating systems; these include *A. mearnsii* Willd., *A. mollissima* Willd., *A. auriculiformis* Cunn. ex Benth, *A. crassicarpa* Cunn. ex Benth, *A. melanoxylon*

R.Br., *A. mangium* Willd., and *A. tortilis* (Moffett, 1956; Moran *et al.*, 1989a; Muona *et al.*, 1991; Wickneswari and Norwati, 1992; Olng'otie, 1992).

Although most of the above studies indicated that high outcrossing rates are widespread in acacias, the range for all species (18-99%) and the specific selfing rate reported for *A. tortilis* (Olng'otie, 1992) suggested the presence of inbreeding. Moreover, Moran *et al.* (1992) readily produced viable seed by selfing polyploids of *A. cowleana* and *A. holosericea* thus suggesting self-compatibility or an expression of apomictic reproduction, that was not previously expected among acacias.

Bias can be introduced into the results of such studies by the materials and methods used. Charlesworth (1988) and Adams and Birkes (1991) explained that the estimated selfing rate is more often a measure of viable selfed progeny than the number of flowers that are self-fertilized. Recent investigations of self-compatibility in *A. retinodes* (Kenrick and Knox, 1985; Kenrick *et al.*, 1986), *A. auriculiformis* and *A. mangium* (Sedgley *et al.*, 1992) revealed that there was no significant difference in pollen tube viability between controlled self- and cross-fertilized flowers, except between individual trees. Generally, *A. retinodes* has been found to be highly self-incompatible (Kenrick and Knox, 1985, 1989), and low seed set has been reported for selfed individuals of *A. auriculiformis* and *A. mangium* (Ibrahim, 1991; cited by Sedgley *et al.*, 1992). The diversity of mating systems reported for other acacias necessitated initiation of a similar study for *A. karroo*, to provide the information base necessary for its genetic conservation and management.

5.1.2. Types of mating systems

Plants exhibit five classes of mating patterns, namely: predominantly self-fertilizing, predominantly outcrossing, mixed selfing and outcrossing,

apomixis and selfing of gametophytes (Richards, 1986; Brown, 1989; Brown *et. al.*, 1989).

5.1.2.1. Predominantly selfing

This mating system is characteristic of plants with outcrossing rate less than 10%, and it has been estimated that they form 20% of all higher plant species (Brown, 1989). Within the populations of selfing plants, variability is usually very low but not depauperate, owing to limited outcrossing and segregation in the progeny of the few heterozygous maternal plants available (Allard, 1989). Polygenic mutations that do occur in selfed progenies may sometimes reintroduce further variation (Barrett and Shore, 1987; Lande and Schemske, 1987). Among forest trees, high selfing rates have been reported for *Picea omorica* (Geburek, 1986; but see Kuittinen and Savolainen, 1991) and *Pinus resinosa* (Fowler, 1965; Fowler and Morris, 1977).

5.1.2.2. Predominantly outcrossing

This mating system characterises plant species with a self-fertilization rate below 5% (Brown, 1989). In some cases, outcrossing can occur within a small subpopulation, or in the form of biparental inbreeding, with similar consequences for panmixia as selfing (Bawa, 1992). However, most plant species that are intolerant to self-fertilization have developed either pre- or post-zygotic rejection of selfing (e.g. Moffett, 1956; Eldridge and Griffin, 1983; Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987). In acacias, some of the few species studied suggest that selfing is discouraged by adopting inbreeding depression, e.g. *A. mearnsii* (e.g. Moffett, 1956) or self-incompatibility at the ovule, e.g. *A. retinodes* (e.g Kenrick and Knox, 1989; Sedgley *et al.* 1992), evoking the possibility of a gametophytic self-incompatibility. However, these traits have been found to vary among trees within a population (Moffett, 1956; Kenrick and Knox, 1989).

5.1.2.3. Mixed selfing and outcrossing

This is considered the most widespread mating system in plants (Brown, 1989; Bawa, 1992; Arroyo and Uslar, 1993), but due to its temporal and spatial variability, its frequency is difficult to quantify. The resulting progenies are usually a mixture of selfed- and cross-pollinated sibs. Understanding this mode of mating is important because, apart from being widespread, it explains the biological, environmental and evolutionary trends that precede establishment of self- and cross-fertilization as a predominant mating pattern. An example of a widely studied genus with many species that express a mixed mating system is *Eucalyptus* (Brown *et al.*, 1975; Moran and Bell, 1983, Peters *et al.*, 1990; Moran, 1992).

5.1.2.4. Facultative or obligate apomixis

Apomixis is a form of asexual reproduction that has risen as a substitute to the sexual, and does not involve nuclear or cellular fusion (Riley, 1949). Apomixis is described as obligate if it is the only method of reproduction. In facultative apomictic plants, normal sexual reproduction is also encountered. Stebbins (1950) classified the two main types of apomixis as vegetative and agamospermy. In vegetative apomixis, vegetative parts are involved in the reproduction, while in agamospermy only parts related to floral structure are involved. Some examples of apomictic plants are found among *Taraxacum, Panicum, Agave,* and *Citrus* species (Stebbins, 1950; Asker and Jerling, 1992; Lancaster, 1993). Among tree species, apomixis has been reported in *Alnus rugosa, Eugenia jambos* (Stebbins, 1950) and among east Asian dipterocarps (Ha *et al.*, 1988). Most apomictic reproduction occurs among polyploids, particularly, with odd number of chromosomes (Stace, 1989; Asker and Jerling, 1992). However, vegetative and adventitious apomixis is known to occur in diploid species like *Agave, Lilium* and *Citrus* (Stebbins, 1950). Mixed

outcrossing and apomixis has been suggested as the best mating model (Brown, 1989) because heterosis is not compromised in apomixis as it is in selfing.

5.1.2.5. *Intragametophytic mating or haploid selfing*

This mating mode occurs when gametes of a single bisexual gametophyte successfully unite to form a zygote (Brown, 1989; Ranker, 1992). Intragametophytic mating is usually associated with large inbreeding coefficients (Soltis and Soltis, 1986; Ranker, 1992). The mating mode is mainly found among homosporous ferns and allied lower plants (Soltis and Soltis, 1986; Ranker, 1992), and it is in contrast to intergametophytic fertilization which is the basis of selfing in higher plants.

5.1.3. *Relevance of mating system information to population management*

The mating system of a species can be studied at two levels (Brown, 1989): the individual level, where the major components are the rate of selfing and fertility; and the populational level, where the major concern is the future genotypic composition of the population. In forestry, knowledge of mating patterns at both levels is important when sampling parental trees to establish breeding and gene conservation populations. Knowledge of mating systems is also necessary in designing multiplication populations, i.e. seed orchards, to achieve the intended packaging of required genetic traits in progeny (Adams and Joly, 1980; Ritland and El-Kassaby, 1985; Adams, 1992). However, the mating system itself is under genetic control and exhibits temporal and spatial variation within and among populations (Brown and Weir, 1983; Brown *et al.*, 1989; Adams, 1992; Mitton, 1992). Thus, a number of studies have reported variation in outcrossing rates within and among populations of various tree species (e.g. Hamrick and Schnabel, 1985:

Schemske and Lande, 1985; Ritland and El-Kassaby, 1985; Moran *et al.*, 1989a; Muona, 1989). The generally high genetic variability within populations of tree species, and the depression of inbreds, indicate that the outcrossing mating pattern is common (see Mitton, 1983; Bawa *et al.*, 1985a; Bousquet *et al.*, 1987; Hamrick and Loveless, 1988; Loveless, 1992; Bawa, 1992).

5.1.4. Methods used in studying mating systems

Different approaches have been used to gain an understanding of mating systems in plants and estimate parameters which describe mating systems. The most conventional method used is controlled pollination to assess self-compatibility and to produce full-sibs (Bawa *et al.*, 1985a; 1985b; Ellis and Sedgley, 1992; Perez-Nasser *et al.*, 1993). Other approaches involve using morphological markers of recessive traits to estimate fixation index or heritability (Fyfe and Bailey, 1951; Moffett, 1956). Open or controlled pollinated progenies are produced and maintained in experimental plantings, where they are monitored for specific traits. However, problems such as dominant expression of a trait, or possibilities of the traits being selection targets, have always been associated with morphological markers (Brown, 1989).

The other interest in mating system studies has been that of estimating male fertility and pollen dispersal. Whereas female fertility could be approximated by direct count of ovules or seeds, male fertility has been more complicated to assess in most plant species (Perez-Nasser *et al.*, 1993). Initial studies to understand gene flow through pollen using morphological and physical markers, or simulation with fluorescent dust, were marred with occasional pitfalls (Adams, 1992, and references therein). Otherwise, where direct numeration has been possible, the ovule-pollen ratio has been used to

estimate fertility levels in some plant species (Cruden, 1977, cited by Perez-Nasser *et al.*, 1993).

The use of isozyme markers in mating system studies is more recent and amenable more to accurate estimates, using models based on allozyme inheritance (Brown and Allard, 1970; Ritland and Jain, 1981; Ritland, 1983; Brown *et al.*, 1989; Adams, 1992). Ritland (1990) has outlined the development of this model-based approach and improvements made to obtain accurate estimates of mating system parameters. One direct use of isozymes in mating system analyses has been to identify plants with rare alleles within a population with the intention of monitoring the gene flow and inheritance patterns (Yazdani *et al,* 1989). The method has been successful in understanding male fertility and gene flow. However, the approach has its disadvantages: first, rare genotypes should be very restricted in number, so as to allow easy modelling; second, the position of the rare genotype might not allow generalizations about the population (Adams, 1992); third, inheritance of fertility and choice of mates by genotypes, which is still poorly understood, can bias the results (Namkoong and Bishir, 1987; Adams, 1992); and fourth, the population must be small enough to identify genetically all potential parents.

The analysis of mating systems of whole populations using models based on isozyme inheritance also has its complexities; the outstanding problem is that of discerning parents unambiguously (Brown and Allard, 1970). In gymnosperms, contributions from both parents have been identified owing to the existence of a maternal haploid megagametophyte and a diploid embryo that can be assayed adjacently (Hamrick and Schnabel, 1985; Fu *et al.*, 1992). Alleles that are unique compared to the maternal genotype can then be assigned to the male parent. In angiosperms, maternal genotype has been inferred from large progeny arrays (Brown and Allard, 1970). Another, but less commonly applied, procedure for angiosperms is to use the endosperm

(Brown *et al.*, 1985). The endosperm in angiosperms is triploid, with two maternal contributions to one paternal (Richards, 1986), a situation which complicates the inferences compared to gymnosperms. It is also interesting to note that endosperms in acacias like in most other legumes degenerate early in the developmental stage of the embryo (Smith, 1981). These methods based on progeny array and endosperms are biased in some cases to the most common genotype among the progenies (Ritland, 1990).

In addition to the above mentioned drawbacks, the model approach sometimes suffers from bias caused by the number and allelic composition of the loci analysed (Ritland and Jain, 1981), or correlation of paternity (Ritland, 1984; Hedrick and Ritland, 1990), or both. In cases where the allelic diversity has been very low, and most loci were monomorphic, the isozymes have been less useful in mating system analyses (Ritland and Ganders, 1987). Some plants do develop full-sib progenies due to deposition of single-pollen load by a pollinator, or from sequential visits by several pollinators from a single pollen source (Epperson and Clegg, 1987; Ritland, 1989). Such matings can easily inflate estimates of selfing rate unless they are partitioned into their constituents (Ritland, 1984; Hedrick and Ritland, 1990). Brown (1989) classified the four aspects of correlated paternity as: (1) biparental inbreeding; (2) self-fertilization; (3) correlation of selfing; and (4) correlation of outcrossing. In most acacias, the most likely cause of correlated paternity is deposition of pollen as a polyad (Kenrick and Knox, 1982; 1985; Muona *et al.*, 1991), but other forms of related mating listed above cannot be ruled out. Some of these short-falls of the model approach could be reduced as better-fitting models are developed (Adams, 1992).

When the maternal genotypes are unambiguously identifiable, a model can be formulated for the mating system estimation. The pollen gene thereafter can be differentiated according to the possible sources and the observed genotypes in a population (Ritland, 1990; Adams and Birkes, 1991;

Adams, 1992). Ritland and Jain (1981) demonstrated that, by increasing the number of loci considered for mating system analysis to four and above, the variances of parameters estimated were much reduced. Further, multiallelic loci have expressed less variable estimates than have diallelic loci.

The objectives of this chapter are: (1) to determine the general mating system within a population of *A. karroo*; (2) to estimate outcrossing rates for individual parent trees; (3) to investigate whether there exists heterogeneity of outcrossing rates among the loci assayed, and; (4) to formulate future sampling strategies based on an understanding of the mating system.

5.2. Materials and Methods

5.2.1. Plant materials

One population of *A. karroo*, from Umguza Valley, was used for mating pattern studies (Table 2.1). Forty trees were sampled from the original 120 trees previously used for genetic diversity studies (Chapter Four). All trees were identified by unique numbers and mapped on the ground (Fig. 5.1). Selection of the 40 tree sample was based primarily on seed yield rather than on the distribution of mother trees in the field. Only trees with enough seeds to raise 60 or more progeny were selected for analysis.

Progeny analysis by electrophoresis is useful in inferring maternal genotypes and outcrossed pollen alleles (Brown and Allard, 1970). However, the number of progenies required for this inference varies with ploidy, heterozygosity level and gene diversity in a population. Like families with excessive heterozygosity, polyploids cannot satisfactorily express maternal genotypes unless large numbers of offsping are analysed, because of the presence of multiple alleles. Ritland (1991) suggested a minimum array of six sibs per tree for diploids, while Brown (1989) recommended a minimum of 15 progenies. Since *A. karroo* is a polyploid with high heterozygosity and

multiple alleles, a 60 progeny array from each tree was considered sufficient. The progenies were analysed electrophoretically for the six polymorphic enzyme systems, encoded by 10 gene loci, previously identified in Chapter Four. However, variability of sibs within families at gene loci encoding 6-phosphogluconate dehydrogenase was low and it was not used further in the analyses.

5.2.2. *Statistical analysis*

Mating system parameters were estimated using the MLTET (see glossary) computer program for tetraploids (Ritland[3], Unpub.). The program is based on the mixed-mating model of Ritland and Jain (1981). Developed from the single locus model of Fyfe and Bailey (1951), the model reduces bias by introducing multi-locus analysis. The usage of multiple loci is more reliable than that of single loci and has the advantage of discerning some outcrosses unambiguously at one or more loci (Shaw *et al.*, 1981). The estimates are based on the fact that progeny of each maternal genotype (family) form a genetic array from ovules that outcross with probability t to a pollen pool with frequency p, and self-fertilize with probability $1-t$.

The MLTET program introduces three major operational methods: Newton-Raphson, maximum-likelihood (ML) (Brown and Allard, 1970; Brown *et al*, 1975) and expectation-maximization (EM) (Ritland and Jain, 1981; Cheliak *et al.*, 1983; Weir, 1990). Newton-Raphson operation, which is fast, is applied together with ML to find valid estimates (at times, $t>1.0$) of populational single (t_s), multilocus (t_m) and multilocus family (t_f) outcrossing rates, and the single locus inbreeding coefficient (F) of maternal parents. The expectation-maximization (EM) algorithm, which is slower but more reliable, is used to estimate pollen (p) and ovule (o) gene frequencies (Ritland, 1991 Unpub.). The EM algorithm is also advantageous in that it estimates selfing

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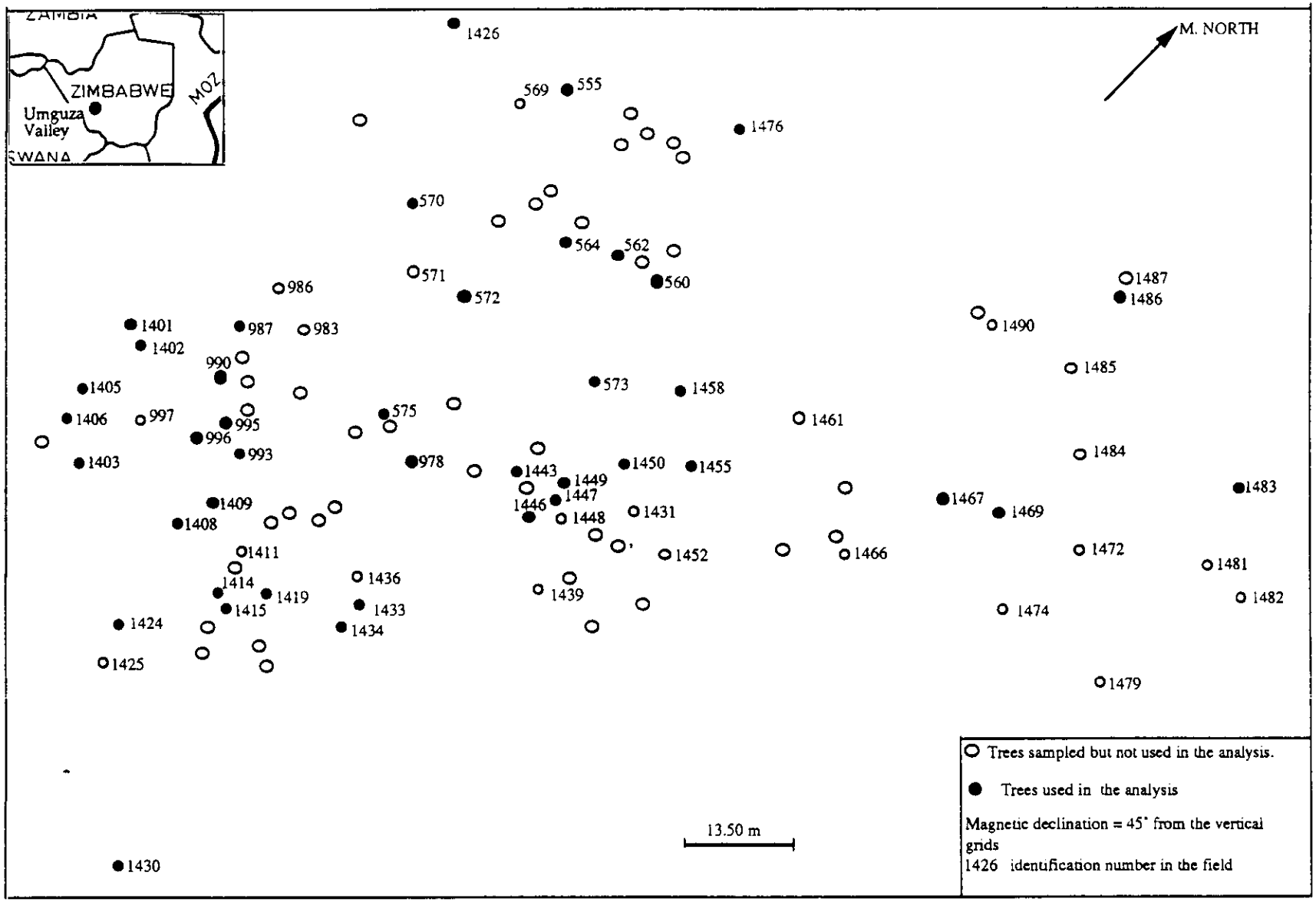

Figure 5.1. Distribution of trees used for analysis of mating system in *A. karroo*, at Umguza Valley, Zimbabwe.

and outcrossing rates depending on available genotypes, independent of populational genotypic equilibrium (Weir, 1990; Xie *et al.*, 1991). Both ML and EM procedures used in the program require or assume initial values of t and p, which are used in iterations until the values stabilize.

The pollen gamete genotypes observed in a sample of progeny from a maternal genotype can be attributed to three sources (Adams, 1992): self-fertilization, cross-fertilization to males within the neighbourhood, and cross-fertilization to males outside the neighbourhood. Adams and Birkes (1991) proposed a model that takes into account pollen contributed from outside the maternal neighbourhood; however, due to difficulty in separating pollen within from those outside in a natural population, the mixed mating model of Ritland and Jain (1981) was considered appropriate for the estimates here. In order to estimate mating system parameters, the mixed mating model assumes certain conditions.

5.2.2.1. Assumptions of mixed mating model

The accuracy in estimation of mating system parameters is maximized by assuming the conditions described by Ritland and Jain (1981) and Brown *et al.* (1989) as follows:

(1) plants within a population are assumed to mate at random;

(2) the pollen pool is assumed to be homogeneous over all the maternal plants;

(3) segregation within a locus is assumed not to be linked to other loci;

(4) it is assumed that no selection, mutation or other genetic changes have occurred to the progeny between fertilization and time of the assay;

(5) progeny of each maternal plant are assumed to form genotypic classes identically distributed as multinomial random variables;

(6) segregation in the heterozygous maternal plants is assumed to be strictly Mendelian in 1:1 ratio for both pollen and ovule production.

The model uses the pooled aggregate of ovule and outcrossing gene frequencies to estimate the mating system parameters for the population. The standard errors were estimated using 250 bootstraps (see Glossary) sampled among the families.

The basis of the computation is the maximum-likelihood estimate of outcrossing rate *t*, obtained as a numerical solution to the following equation (Brown *et al.*, 1989; Weir, 1990):

$$\sum_{i=1}^{n} \frac{Oi}{t} = \sum_{i=1}^{n} \frac{(Ni-Oi)Gi}{1-Git} \qquad (5.1)$$

where O_i = the observed number of detected outcrosses;

N_i = the total sample size from maternal genotype;

n = the number of maternal genotypes;

t = outcrossing rate;

G_i = the detection probability.

Since Gi requires an assumption about the frequencies of pollen multilocus genotypes, an approximate *t* is usually specified for computation. When multiple loci are used, the detection probability of outcrosses increases (Shaw and Allard, 1981) and the equation 5.1 simplifies to:

$$t = \sum_{i=1}^{n} \frac{Oi}{(G\Sigma Ni)} \qquad (5.2)$$

where G is computed as $(1-p_u)$, which is the product over loci of the single locus probability that the pollen allele will also be present in the maternal plant. The parameter p_u is the frequency of the maternal allele in the pollen pool, and the remaining terms are defined as above.

To understand the general mating pattern of *A. karroo*, outcrossing rates for all 12 populations (Chapter 4) were estimated directly from Wright's fixation indices (F = 1-Ho/He), using the data from Table 4.3. Outcrossing rates were computed from fixation indices following Nei and Skayudo (1958), as:

$$t = \frac{(1-F)}{(1+F)} \qquad (5.3)$$

where F is Wright's fixation index.

The results were compared to the mating system parameters obtained from the multi- and single-locus model approach for the single population from Umguza Valley.

To estimate the gene flow (N_m), the coefficient of gene differentiation (Gst, Table 4.4) was used for computation (Slatkin and Burton, 1989), as:

$$N_m = \frac{(1-G_{st})}{4G_{st}} \qquad (5.4)$$

where G_{st} is the Nei's estimation of Wright's F_{st} (genetic differentiation among populations, Table 4.4).

A chi-squared goodness-of-fit test (Sokal and Rohlf, 1981) was used to test the consistency of genotypic classes with the expectations under random mating. The test was also conducted to confirm the deviations of sibs of maternal trees homozygous at *Adh-2* locus from expectation under complete inbreeding. A t-test (Sokal and Rohlf, 1981) was used to investigate the heterogeneity of pollen-ovule allele frequencies.

The most likely genetic structure of the sample population was determined using a computer program for spatial autocorrelation analysis (see Glossary) (Heywood, Unpub.). Only alleles which were moderately frequent (not fixed or rare) were used for this analysis. Alleles were weighted as: 0 - not present; 1 present in heterozygote form, and; 2 - homozygous state. Individual trees mapped on Fig. 5.1 were used to construct a Gabriel network

(Sokal and Oden, 1978; Heywood, 1991; Epperson, 1992). The statistical significance of the departure of Moran's coefficient of autocorrelation from its expected value $(n-1)^{-1}$ was tested by the standard normal deviates (Sokal and Oden, 1978).

5.3. Results and Discussion

Single and multilocus estimates of outcrossing rates, and estimates of the frequency of the most common ovule and pollen alleles at different loci, are presented in Tables 5.1, 5.2 & 5.3.

Table 5.1. Single and multilocus estimates of outcrossing rate *t*, with standard errors and sample sizes, for *A. karroo* from Umguza Valley.

LOCUS	t + s.e	sample size
Aat-1	0.93 ± 0.01*	2263
Aat-2	1.22 ± 0.32*	2263
Aat-3	0.61 ± 0.12*	2263
Adh-1	1.99 ± 0.01*	2263
Adh-2	0.58 ± 0.07*	2263
Sdh-1	0.54 ± 0.05*	2263
β–Est-1	0.53 ± 0.05*	2263
Dia-1	0.67 ± 0.05*	1898
ts	0.72 ± 0.02*	2263
tm	0.90 ± 0.02*	2263
ts«	0.68 ± 0.02*	2263
tm«	0.88 ± 0.02*	2263
Fixation indixes		
f	0.11 ± 0.13	2263
vf	0.35 ± 0.16	2263
g	0.29 ± 0.23	2263
h	-0.03 ± 0.22	2263

« outcrossing rates when the two loci *Adh-1*, with high fixed heterozygosity, were excluded. f = 2 gene fixation index; vf = variance of f; g & h = 3 and 4 gene fixation indices, respectively. *significant deviations from t = 1 ($p<0.05$). Total sample size was reduced by missing data or germiation failure.

5.3.1 Outcrossing rates

The mean estimate of outcrossing rate from the single-locus model was 0.72, while the estimate from multilocus model was 0.90. The estimate at *Adh-1* loci was high and was interpreted as a failure of truncation of the program. Although such an outcome may be equated to unity in estimates of outcrossing rates, Brown *et al.* (1985) cautioned that such action may overestimate or underestimate the rates. Since the loci sampled were adequately large, the accuracy was not compromised by excluding a single

Table 5.2. Estimates of outcrossing rates based on Wright's fixation indices (equation 5.3) for *A. karroo* populations.

Population	Fixation indices (F)	Outcrossing rates (t)
Prince Albert (12)	0.19	0.68
Queenstown (9)	0.16	0.72
Cintsa River (11)	0.19	0.68
Hluhluwe (8)	0.14	0.75
Babanango (7)	0.19	0.68
Manzengwenya (10)	0.12	0.78
Kroonstad (6)	0.16	0.72
Zomba (4)	0.15	0.72
Dedza (3)	0.13	0.74
Umguza Valley (1)	0.13	0.76
Mutorashanga (2)	0.17	0.71
Lusaka (5)	0.18	0.69
Mean	0.16	0.72

Numbers in brackets refer to population nuumbers on Table 2.1.

locus (Ritland[3], Unpub.). When locus *Adh-1* was excluded from the estimates, the outcrossing rates dropped to 0.68 for single-locus and 0.88 for multilocus models (Table 5.1). A comparable outcrossing rate of $t = 0.72$ for all populations was obtained using Wright's fixation index. There were no major

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differences between outcrossing rates calculated using fixation indices, which ranged from 0.68 to 0.78 across the 12 populations (Table 5.2).

The eight polymorphic loci used in the estimation displayed variable outcrossing rates, ranging from t = 0.53 to 1.99 for *β–Est-1* and *Adh-1* loci, respectively (Table 5.1). "Biologically unreasonable" (t > 1.0) outcrossing rates (Brown *et al.*, 1985) were expressed by two loci, *Aat-2* (1.22) and *Adh-1* (1.99). Brown *et al.* (1985) explained that this departure does not invalidate the mixed mating model; the major causes of the discrepancy were nominated as sampling error and disassortative mating. In this study, one factor which most likely contributed to estimates of outcrossing rates above t =1.0 was high heterozygosity. The three loci showing predominantly outcrossing rates (t > 0.90) had expressed tendencies towards fixed heterozygosity (Chapter Three). Generally, these loci expressed 2-4 segregating alleles, with most genotypes as heterozygotes. This created the difficulty of discerning paternity accurately and hence the failure of the iterations of log-likelihood to stabilize at the *Adh-1* locus. All single-locus estimates of outcrossing rates were significantly different from t =1.0 (i.e. [t+2 s.e] < 1.0, p<0.05), favouring the mixed mating model.

Table 5.3. Estimates of the frequencies (with standard errors), of the common alleles in the outcrossed pollen pool and ovules.

LOCUS	POLLEN	OVULE
Aat-1 b	0.55 ± 0.01	0.48 (0.02)
Aat-2 b	0.55 ± 0.04	0.41 (0.02)
Aat-3 b	0.56 ± 0.02	0.56 (0.02)
Adh-1 a	0.42 ± 0.03	0.30 (0.02)
Adh-2 b	0.87 ± 0.02	0.89 (0.03)
Sdh-1 d	0.70 ± 0.02	0.68 (0.04)
β-Est-1 b	0.45 ± 0.03	0.36 (0.02)
Dia-1 e	0.61 ± 0.02**	0.38 (0.03)

Significant difference between ovule and pollen frequencies (**p<0.01).

The inbreeding coefficient calculated here reached 11% (Table 5.1), and biparental inbreeding rate was estimated at 20%; biparental inbreeding is estimated as the difference between multilocus and single-locus outcrossing rates. However, these inbreeding coefficients appeared less accurate as they were characterized by large standard errors. The mean Wright's fixation index calculated for all populations was 16%, which was within the range of those reported for mixed-mating plant species (Slatkin and Burton, 1989).

A comparison of maternal ovule and pollen pool gene frequencies indicated that they were not significantly different, except for the *Dia-1* locus ($p<0.01$, Table 5.3). This suggested that the pollen pool allele frequencies were homogenous over maternal genotypes. However, a chi-square goodness-of-fit test for genotypic proportion of progenies at *Aat-3* and *Adh-2* loci indicated a significant departure ($X^2 = 571.4$ with 4 degrees of freedom at *Aat-3*) from the expected equilibrium (Tables 5.4 & 5.5). Several genotypic classes had less than five individuals at *Adh-2* locus, a condition that did not allow the goodness-of-fit test to be carried out. The *Aat-3* and *Adh-2* loci were used for genotypic equilibrium tests because they had fewer alleles, which also reflected a tetrasomic inheritance pattern, making it possible to determine expected genotypic equilibrium (Appendix II). The departure from expected equilibrium at the *Aat-3* locus was aggravated by the presence of genotypes

Table 5.4. Genotypic proportions observed at the diallelic *Aat-3* locus in *A. karroo*.

Algebraic genotype	a^4	$4a^3b$	$6a^2b^2$	$4ab^3$	b^4	Total
Genetic genotypes	aaaa	aaab	aabb	abbb	bbbb	5
Number observed (O)	322	371	1077	354	15	2139
Number expected (E)	133.69	534.75	802.13	534.75	133.69	2139.01
Maternal genotypes ob.		aaab	aabb	abbb		3
Number observed		9	30	1		40

Table 5.5. Genotypic proportions observed at the triallelic *Adh*-2 locus in A. karroo.

AGe.	a^4	$4a^3b$	$4a^3c$	$6a^2b^2$	$6a^2c^2$	$12a^2bc$	$10ab^2c$	$12abc^2$	$6b^2c^2$	b^4	$4ab^3$	$4ac^3$	$4b^3c$	$4bc^3$	c^4	Total
GGe.	aaaa	aaab	aaac	aabb	aacc	aabc	abbc	abcc	bbcc	bbbb	abbb	accc	bbbc	bccc	cccc	15
Obse.	21	150	0	239	1	2	2	0	29	1775	23	0	4	11	1	2258
Expe.	29	114	114	171	171	343	286	343	171	29	114	114	114	114	29	2256
MGe.	-	-	-	aabb	-	-	-	-	bbcc	bbbb	abbb	-	-	-	-	4
Obse.	-	-	-	4	-	-	-	-	1	28	7	-	-	-	-	40

Abbreviations are from terms in Table 5.4 above.

with null alleles (Appendix IV), which could not be classified. The heterogeneity observed at *Dia-1* may have been caused by the problem of discerning parental alleles correctly. Some genotypes at this locus expressed three alleles which varied only in staining intensities. Brown and Allard (1970) cautioned that such multiple alleles can cause bias and complexity in isozyme analysis.

Table 5.6. Isozyme estimates of multilocus outcrossing rates (tm) of *Acacia* species.

Species	No. of Pop.	No. of Loci	Mean tm %	Range tm %	Reference
A. auriculiformis	2	14	92	92-93	Moran *et al.*, 1989
A. auriculiformis	2	6	79	67-95	Wickneswari & Norwati, 1992
A. crassicarpa	2	10	96	93-99	Moran *et al.*, 1989
A. melanoxylon	2	12	90	86-100	Muona *et al.*, 1991
A. tortilis	10	13	35	18-53	Olng'otie, 1992
A. karroo	1	7	88	88	This study (Multilocus estimate)
A. karroo	12	10	72	68-78	This study (Fixation index estimate)

Mating system parameters have been estimated for five *Acacia* species using isozyme systems (Table 5.6). Of these, three species are of Australian origin and only two, including *A. karroo*, are from Africa. The Australian acacias studied have displayed a high outcrossing rate (Moran *et al.*, 1989;

Muona *et al.* 1991; Moran, 1992; Wickneswari and Norwati, 1992). For the African *A. tortilis*, the mean estimate of outcrossing rate was low, implying a high level of inbreeding (t = 0.35, Olng'otie, 1992). *A. karroo* (this study) has expressed an outcrossing rate (0.88) which is intermediate between those of *A. tortilis* and the Australian acacias.

Unlike Australian acacias studied (cited above), which are diploid, *A. tortilis* and *A. karroo* are polyploids. Polyploids are known to tolerate higher levels of inbreeding than diploids because the duplicated alleles shelter sibs from inbreeding depression (Schemske and Lande, 1985; Shore and Barrett, 1987). The large difference between outcrossing rates in *A. tortilis* and *A. karroo* could possibly be accounted for partially by the difference in their polyploid nature: while the former behaved as a typical autopolyploid for the loci analysed (Olng'otie, 1992), the latter expressed itself partially as an autopolyploid and as an allopolyploid (Chapter Three).

Another important observation from this study was that the results concurred with the mixed mating pattern which has been demonstrated to be prevalent among woody species (Loveless and Hamrick, 1984; O'Malley and Bawa, 1987, Hamrick and Godt, 1989; Bawa, 1992; Arroyo and Uslar, 1993). The inbreeding coefficient calculated here reached 11%, which is comparable to that reported for diploid *Acacia* species (Muona *et al*, 1991). Biparental inbreeding rate was estimated at 20%, and was relatively high. These coefficients of inbreeding were also associated with high standard errors, which could possibly be attributed to an ambiguous assignment of multiple alleles to parents. Correlated mating is a character expected in most acacias, as has been demonstrated for *A. melanoxylon* (Muona *et al.*, 1991).

However, in the presence of lethal alleles, even the present selfing level estimated at 31%, as a total of biparental inbreeding (20%) and actual selfing (11%), can have adverse effects on sibling viability. Evolution of mating systems in plants is considered to be influenced by the fitness differentials

between selfed and outcrossed progeny (Mitton, 1992). Thus, tree species have evolved various adaptive measures to avoid sib mortality due to selfing. Some Australian acacias have adopted self-incompatibility (Kenrick and Knox, 1989); the gymnosperms and eucalypts are known to show inbreeding depression (Adams and Joly, 1980; Eldridge and Griffin, 1983, Geburek, 1986; Loveless, 1992, Moran, 1992).

On the contrary, pioneering plant species may either incorporate or lose genes to promote selfing following migration (Schemske and Lande, 1985; Barrett and Shore, 1987). Some plant species that have gone through bottlenecks have avoided mortality by gradual elimination of lethal genes (Fowler, 1965; Namkoong and Bishir, 1987; Millar and Westfall, 1992). Barrett and Husband (1989) presented detailed evidence for changes in floral morphology of *Eichhornia* and *Bidens* species to encourage selfing following colonization. Other plants, for example, *Impatiens capensis* (Waller and Knight, 1989) and *Glycine argyrea* (Brown *et al.*, 1986) have developed both chasmogamous and cleistogamous flowers to produce outcrossed and inbred sibs. In polyploid species, the inbreeding barrier can be evaded by a gradual break-down of self-incompatibility in combination with tolerance to a high genetic load (Lande and Schemske, 1985; Barrett and Shore, 1987). If the selfing rate in *A. karroo* is as high as that demonstrated by this study, the adaptation is possibly promoted by its polyploid nature, correlated mating, and as a pioneering strategy.

Heterogeneity of estimates over loci analysed is well documented (e.g. Ritland and El-Kassaby, 1985; Moran *et al.*, 1989; Wickneswari and Norwati, 1992). Brown *et al.* (1985) attributed this phenomenon to inequality of sampling variances at different loci. In *A. karroo,* the variation at different loci was conspicuous, as different loci exhibited different inheritance patterns (Chapter Three). Elsewhere, low single-locus outcrossing rate estimates have

been reported for *Pithecellobium pedicellare* for both excessively heterozygous and homozygous loci (O'Malley and Bawa, 1987).

Environmental factors encouraging selfing have been reported by several investigators (e.g. Levin, 1981; Bawa, 1990; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987; Adams, 1992). Among the common factors described by these workers, low tree density, is a phenomenon likely to affect breeding patterns in *A. karroo*. Within its geographical range, *A. karroo* is most often found as scattered trees, with only occasional formation of thickets in some microsites (Acocks, 1988). Together with the fact that this species is entomophilous, there appears to be a cause for it to adopt selfing as a means of perpetuation whenever pollinators are rare. Asynchronized flowering (pers. obser.) between trees might also limit cross pollination.

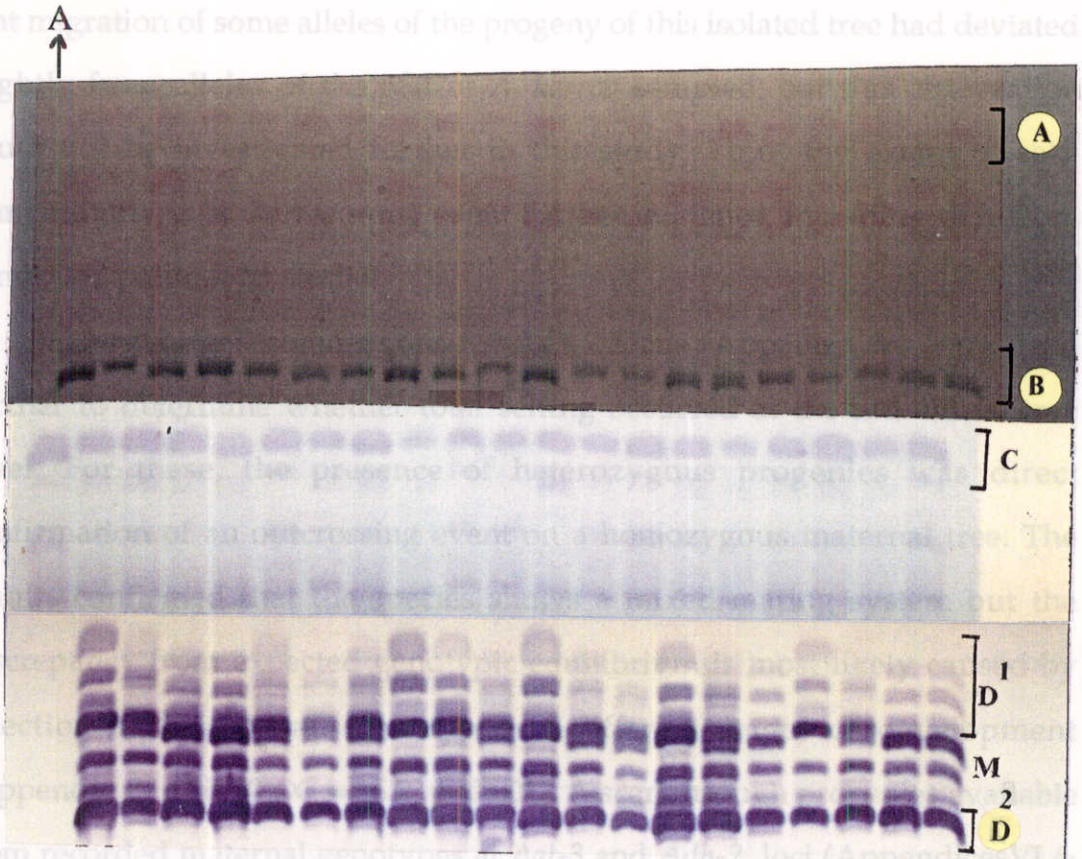


Figure 5.2. Progeny array from an isolated mother tree showing homozygosity at different loci. **A** *β-Est-1*; **B** *Sdh-1*; **C**: *Dia-1*; **D** Adh-1 & 2. All samples expessed single alleles at *β– Est-1*, *Sdh-1*, *Dia-1* and *Adh-2*, therefore segregation at *Adh-1* locus is likely to be a result of multiple alleles. The arrow indicates anodal migration.

However, a similar study of entomophilous *Pithecellobium pedicellare* (Mimosoideae), with an estimated density of two trees per hectare, reported a mean outcrossing rate of 0.95 (O'Malley and Bawa, 1987). Further, results consistent with a high outcrossing rate were recently reported by Hamrick and Murawski (1990) for *Platypodium elagans* which grows at low densities. The observations above demonstrate that there can be ample gene flow where there is insect pollination, even when trees are widely scattered.

In this study, allozyme analysis of sibs from an isolated tree revealed complete homozygosity at some loci, suggesting that *A. karroo* was able to tolerate inbreeding. A low-level variation was seen within the family arrays at *Adh-1* locus (Fig. 5.2), but this could be interpreted as the outcome of independent segregation of alleles at a multiallelic locus. It was noted also that migration of some alleles of the progeny of this isolated tree had deviated slightly from alleles of the rest of *A. karroo* sampled, but this observation could not be investigated further in this study. Thus, the extent of self-compatibility in *A. karroo* must await further evidence, including that from controlled pollination studies.

Maternal trees homozygous for *Adh-2* locus (Appendix V) were used further to determine whether total selfing occurred at the individual tree level. For these, the presence of heterozygous progenies was direct confirmation of an outcrossing event on a homozygous maternal tree. The results confirmed that the species shows a mixed mating system but the discrepancy from expected genotypic equilibrium is most likely caused by selection against certain genotypes at different stages of development (Appendix IV and V) ($X^2 = 16.2$, $p<0.001$). A scan through progenies available from recorded maternal genotypes at *Aat-3* and *Adh-2* loci (AppendicesVI & V) indicated that certain genotypes were far less frequent than expected. The missing genotypes could not be attributed to inbreeding only because some belonged to heterozygous classes. Inbreeding *per se* is known not to cause loss

of genetic diversity within a population unless associated with selection against inbreds (Hedrick, 1985; Falconer, 1989), but it can cause a seriόus decrease in fitness (Eldridge and Griffin, 1983; Geburek, 1986). Moreover, several investigators have indicated that, considering resource allocation, selfing without pollen discounting could be one of the most efficient mating systems (see Brown *et al.*, 1975, and references therein; Schemske and Lande, 1985; Brown, 1989; Ritland, 1991), except when inbreeding depression is operational.

The level of gene flow (N_m) estimated from the G_{st} value was 4.47, which is within the range found for those outcrossing insect-pollinated species such as eucalypts (Moran, 1992), but lower than that of most wind-pollinated plant species (values ranging from 5.3 to 37.8; Hamrick, 1987). Any N_m above unity indicates that there is appreciable gene flow between populations (Slatkin and Burton, 1989). Thus, the inbreeding level of *A. karroo* is high compared to those rates reported for diploid acacias and predominantly outbreeding gymnosperms (Muona, 1989; Moran, 1992), but the gene flow estimate is high enough to sustain appreciable genetic diversity (Slatkin and Burton, 1989), as was demonstrated by the coefficient of gene differentiation (Chapter Four).

Although the number of maternal trees sampled for this study (40) was relatively few, as a random sample their genotypic composition should reflect classes within the adult and offspring populations. In this study, only 4 genotypes at *Adh-2* and 3 at *Aat-3* loci were found in the adult trees, suggesting that certain genotypes were favoured. The observed progeny genotypes were numerous compared to those found for the maternal samples (Tables 5.3 & 5.4). A study by Schnabel and Hamrick (1990), of *Gleditsia triacanthos*, found no significant genotypic differences between age classes, other than increased heterozygosity, suggesting intensive selection against homozygotes.

The number of maternal genotypes observed in this study could offer an alternative explanation to the apparently high selfing rate demonstrated here. It is possible that the limited number of genotypes among parental trees could exaggerate the selfing rate. The homozygotes could be allozygous, i.e. genotypes with alleles that are identical-by-state but not by descent. Hamrick and Murawski (1990), working with *Platypodium elagans*, found that 10% of the progenies were sired by neighbours, and 50% or more of the sibs were sired by about 10 parents, although long distance flow of pollen (>750 m) was also recorded (see Hamrick, 1992). Similarly, *A. karroo* could be outcrossing, but with a reasonable proportion of sibs sired by parents which are nearby and with the same genotypes, but not necessarily related. Homogametic mating (mating by choice between plants of the same genotype at a locus) is also a possibility, as has been determined in maize (Bijlsma *et al.*, 1986). This form of mating occurs when flowering onset is controlled by a single locus.

It is possible that genotypic selection takes place at two stages in this species. The first selection takes place at the time of fertilization, where a pollen of a certain genotypic composition is not encouraged to develop (Appendices IV and V). A second selection may occur during or after the seedling recruitment stage and might be dependent on environmental factors. The latter suggestion could be verified by analysing tissue taken directly from the plants of different age groups, including maternal plants; however, in this study, such tissues were not analysed. Selection can be detected by demographic verification of the departure of genotypic frequencies from the expected equilibrium (Ennos, 1989). Using this method, it has been demonstrated that relative fitness is never constant for any particular genotype throughout its life stages nor in different environments (Ennos, 1989, and references therein). Here, it has been demonstrated that not all expected progenies were found, and the frequencies of parental genotypes

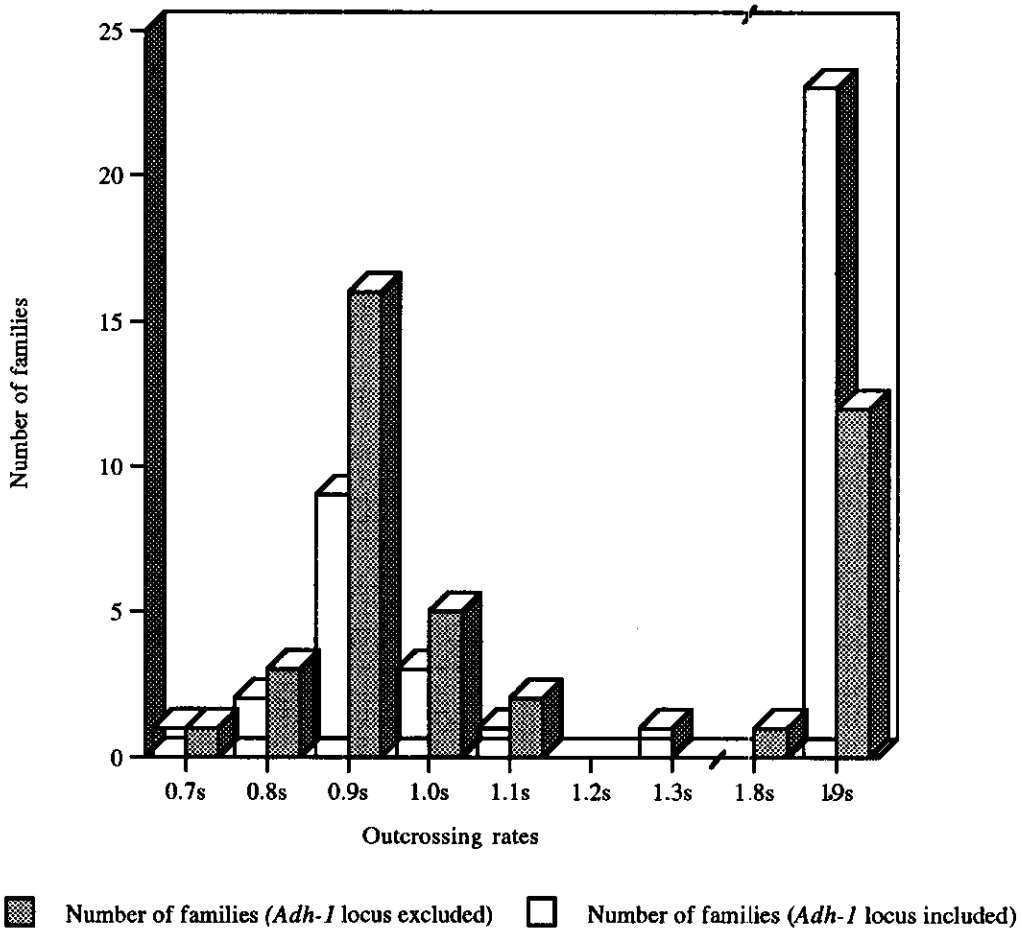
were much reduced. The most important question remains: why were some genotypes favoured?

5.3.2. Family heterogeneity in outcrossing rates

Single-locus and multilocus estimates were computed to test for individual tree variation in outcrossing rates. The multilocus estimates ranged from 0.71 to 1.99 (Fig. 5.3 and Appendix VI), while single-locus estimates ranged from 0 to 1.99 (Appendix VI). These results were less reliable than the populational estimates because iterations could not converge for 12 (23 families when *Adh-1* locus is included) families in the multilocus model (Fig. 5.3). The unreliability of the results was reflected also on the large standard errors of estimates of outcrossing rates for individual trees. Single locus outcrossing rates could not be estimated for a number of individual trees because paternities were too ambiguous. Such individual trees expressed outcrossing rate estimates at 0 or 1.99 level (Appendix VI). These results further stress the need for larger sample sizes for analysis at many loci to estimate accurately mating system parameters in polyploids. Although further analyses of variance could not be computed due to non-convergence of the log-likelihoods in those families, the observations suggested the existence of variation in outcrossing rates among the families (Fig. 5.3). Schwarzmann and Gerhold (1991) found that the variance estimates for single-tree outcrossing rates were negatively correlated with the number of progeny analysed.

Several studies of mating systems have reported heterogeneity in outcrossing rates among families in a population. The heterogeneity can either be temporary, or spatial, or both (Moran and Brown, 1980; Brown *et al., 1985*; Hamrick and Schnabel, 1985; Schwarzmann and Gerhold, 1991; Bush and Smouse, 1992). Family heterogeneity in outcrossing rates may be due to genetic or environmental factors, or both. Individual genetic differences influence traits like fertility, sexual asymmetry and pollen-ovule compatibility

Figure 5.3. Distribution of outcrossing rates in *A. karroo*.



(Ross, 1985; Kenrick and Knox, 1989; Sims, 1993). The environmental factors are likely to include the availability of pollinators and their foraging behaviour, pollen carry-over, temperature and even physiological status of the tree (Levin, 1981).

The results of the genetic spatial autocorrelation analysis is illustrated in Fig. 5.4a-c. All allozymes were variable in pattern, indicating that there was no linkage disequilibrium. The values were small with opposing signs, indicating lack of genetic spatial pattern. Positive autocorrelations indicate homogeneity; negative autocorrelations mark dissimilarities. Most widespread plant populations are expected to show effects of isolation by distance (Wright, 1969; Heywood, 1991). The presence of such a trend is usually marked by high order negative autocorrelations (Sokal and Oden, 1978). In some populations, significant positive and negative Moran's coefficients can both be found, e.g. Schnabel and Hamrick (1990). Only populations in panmictic equilibrium, or in small but heterogenous environmental patches with strong localized selective forces, are expected to display small positive and negative autocorrelation. In *A. karroo,* the spatial autocorrelation was not significant but, considering the species weediness, effective gene flow is a likely explanation, whether through seed, pollen or both. Epperson (1992) noted that, in each generation, gene flow tends to reduce correlations, which are again reinforced by temporal selection favouring different genotypes in different environments.

5.3.4. Rare genotypes in A. karroo.

Figure 5.5. shows some of the rare genotypes mentioned above. Whilst some were expected, according to the segregation ratios, their diminished number is a question worthy of further investigation. It is possible that these genotypes have low fitness and are hence liable to be targeted for elimination.

Figure 5.4a. Distance-corrected correlograms for allozyme variation of *A. karroo*.

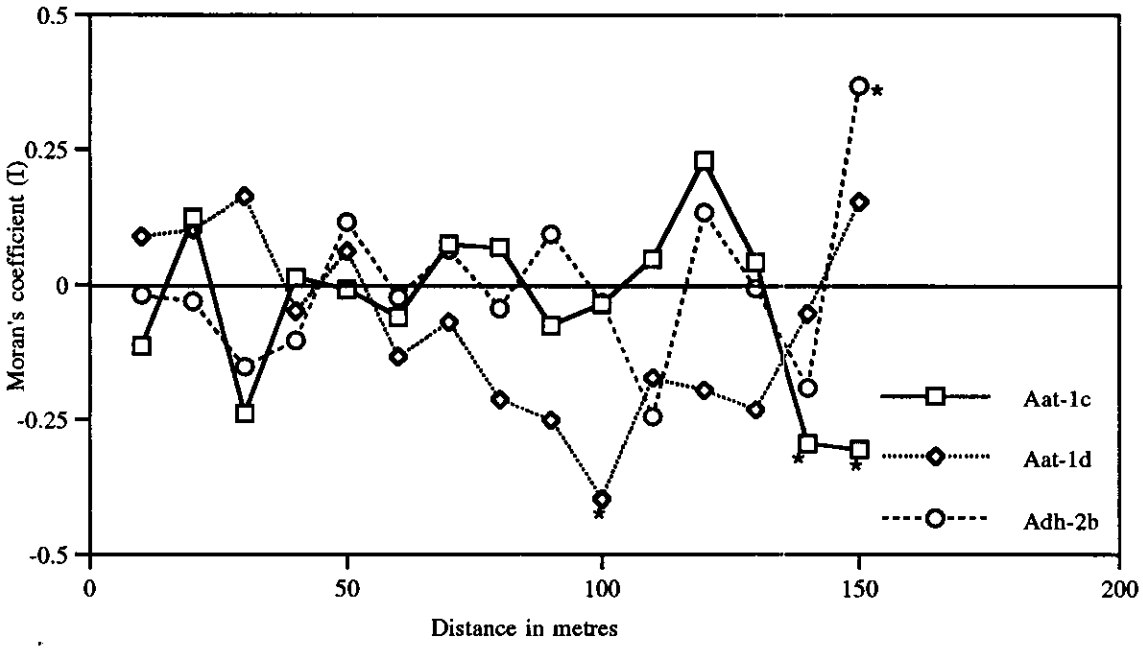
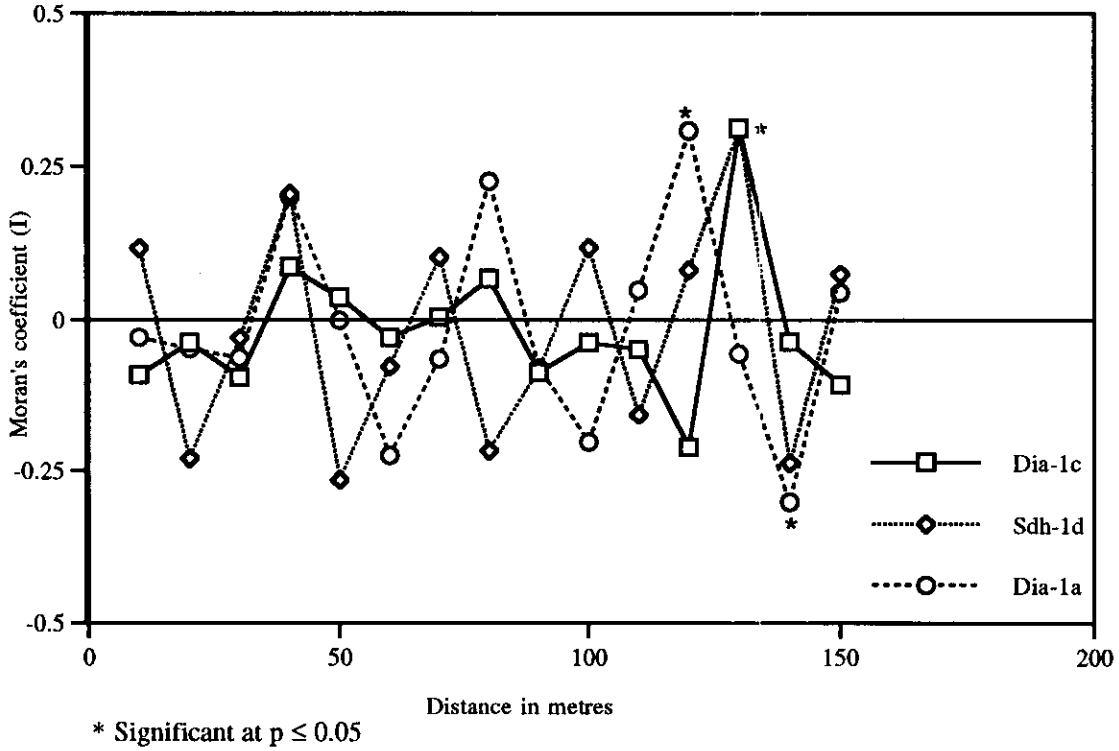
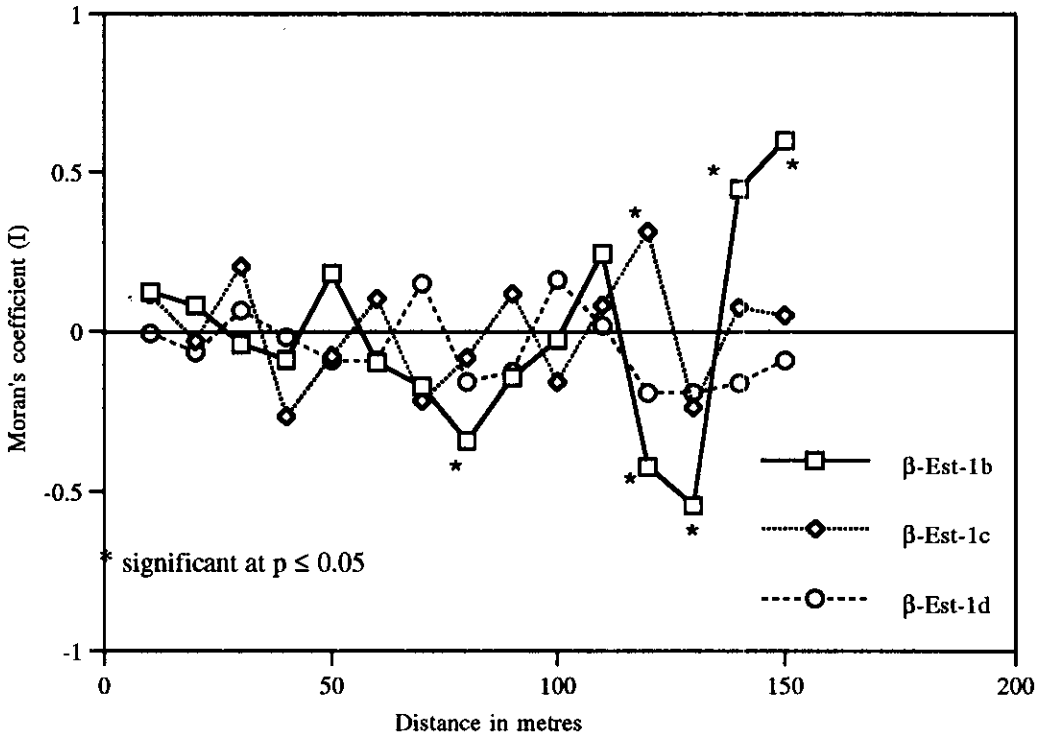


Figure 5.4b. Distance-corrected correlograms for allozyme variation of *A. karroo*.



* Significant at $p \leq 0.05$

Figure 5.4c. Distance-corrected correlograms for esterase allozymes of *A. karroo*.



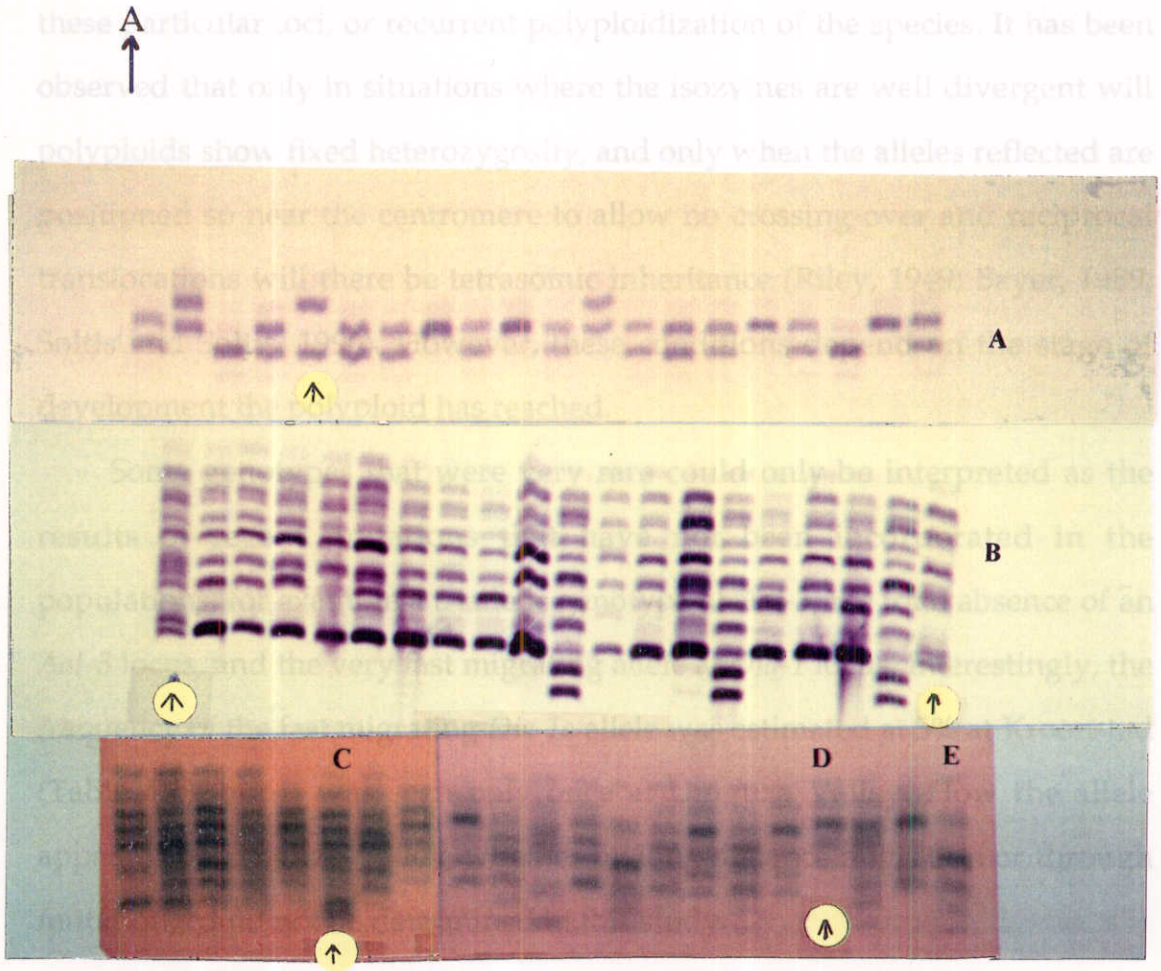


Figure 5.5. Some of the rare genotypes of *A. karroo* (marked with arrows). **A** an offspring with alternating alleles (ccee) at *Sdh-1* locus (1476); **B** two progenies with homoeologous bands at *Adh-2* locus (1447); **C** a genotype with three alleles at *Aat-3* locus (1455); **D** a progeny with no *Aat-3* locus (1430); **E** progenies with alternating alleles (accc) at *Aat-3* locus (1430). Note tree numbers are given in brackets because some of these characters segregated within families. The arrow indicates anodal migration.

For example, it was clear that inheritance of alternating alleles was notably rare, except at *Dia-1* and *β-Est-1* loci. Unexpectedly, for the *Sdh-1* locus where six alleles were recorded, this inheritance pattern was frequent in populations that originated south of 30°S latitude.

The frequency of null alleles was another factor that was difficult to explain. They were more prevalent at *Aat-3* locus and showed an inheritance trend in some families. Some families also exhibited tetrasomic inheritance

with four alleles expressed. It is difficult to pinpoint the biological causes of these observations, but it could be related to the divergence of genomes at these particular loci, or recurrent polyploidization of the species. It has been observed that only in situations where the isozymes are well divergent will polyploids show fixed heterozygosity, and only when the alleles reflected are positioned so near the centromere to allow no crossing-over and reciprocal translocations will there be tetrasomic inheritance (Riley, 1949; Bayer, 1989; Soltis and Soltis, 1990). However, these conditions depend on the stage of development the polyploid has reached.

Some genotypes that were very rare could only be interpreted as the results of recent mutations that have not been incorporated in the populations, for example a triallelic genotype at *Aat-3*, the total absence of an *Aat-3* locus, and the very fast migrating allele at *Dia-1* locus. Interestingly, the frequency of the fast migrating *Dia-1a* allele was estimated at 5% at Kroonstad (Table 4.1), but it was seen only once at Umguza Valley. How the allele appeared at Umguza Valley, whether by long distance migration or through mutation, could not be determined in this study.

5.4. Conclusions

A. karroo is predominantly outcrossing, but shows a high level of effective selfing. The estimated biparental inbreeding was higher than the actual selfing, suggesting the presence of correlated mating. However, clarification of the degree of true selfing must await controlled pollination studies. Evidence for the outcrossing nature of the species was further supported by the level of gene flow. Thus, *A. karroo* fits a description of a mixed-mating species (Schemske and Lande, 1985). Provided that there is neither inbreeding depression nor self-incompatibility, the mixed-mating system could be a guarantee for high fecundity. Given the average outcrossing rates observed,

tolerance of consanguineous mating could ensure successful fertilization of most ovules under optimum environmental conditions. It is likely that such a strategy might have supported the widespread distribution of *A. karroo*, and is reflected in its potential as a weed in some areas.

Individual trees displayed various levels of outcrossing. Maternal genotypes heterozygous or homozygous at a locus persistently produced more progenies with similar genotypic patterns. This tendency was responsible for either very high or very low single-locus outcrossing estimates for some families. It was this manifestation of defined genotypic classes which suggested that the outcrossing mating pattern was a matter of chance rather than the rule for this species, or that maternal genotypes predetermine the genotypic classes of their progeny. However, the existence of allozygous homozygotes together with homogametic mating within the populations could bias the outcrossing rate estimates downwards, leading to false conclusions.

As discussed above, progenies inheriting alternating alleles were rare and observed maternal genotypes were fewer than expected, giving the impression of non-independent segregation of alleles. Under such conditions, it is possible to suggest that reduction in genotypic variability due to rigorous selection has created a situation where most genotypes are identical not by descent but by state. The possibility of such a state was emphasized by the observation that two sibs with similar alleles at a locus could nevertheless differ, either by expressing or not expressing the interallelic heterodimers. This observation is consistent with the proposal that the species is a segmental tetraploid (Chapter Three) and that alleles at a locus could be different in homology, such that homodimers fail to interact. Thus, the question of self-compatibility in *A. karroo* could not be resolved here, and will have to await controlled pollination studies.

The single locus estimates of outcrossing rate were variable; this could be expected because all the loci displayed a variable number of alleles and inheritance patterns (Chapter Three). Together they imply various levels of sampling variances and experimental error. This could be because alleles are transmitted as a set, but the behaviour of each at fertilization is independent of others at different loci, unless there is linkage disequilibrium.

The evidence gained from the mating system estimates, spatial genetic structure, genotypic selection and the genetic diversity in this study provide basic information about the level of genetic organization within a population of *A. karroo*. This limited knowledge can be used as a basis for genetic management interventions, some of which are discussed in Chapter Six.

CHAPTER SIX

CONCLUSIONS

These isozyme studies of *A. karroo* presented intricate and intriguing challenges, occasionally revealing some genetic aspects that are theoretically well documented, but seldom illustrated in natural populations. Some of these challenges might not have been adequately addressed due to our limited understanding of, and the complexities involved in, the interpretation of isozyme patterns in polyploids, especially on polyacrylamide gels. This chapter aims to emphasize and summarize the importance of some of these rare observations in terms of: (1) the suitability of the sampling strategies applied in this study; (2) the ploidy level as characterized by isozymes; (3) rare allozyme patterns in the species; (4) the level of genetic diversity; (5) importance of the mating system, and; (6) the implications of the results for the future of the species.

6.1. The sampling and the observed genetic characteristics of *A. karroo*

The use of single seeds from mother trees sampled for isozyme analysis seems to have adequately reflected the genetic diversity within the sampled populations. This was demonstrated by the minimal difference observed between allele frequencies obtained from genetic diversity analysis and with those observed in the study of mating systems for the Umguza Valley population. In the former analysis, 120 seeds representing 120 trees were used, while in the latter, 2263 seeds from 40 trees were analysed (Appendix VII). The number of populations that can be sampled depends on the availability of resources. In this study there were populations that could not be analysed statistically because of small sample sizes, and others which were

not available for various reasons, for example, locations of political disturbances; such populations will still require genetic evaluation. The geographic proximity of populations sampled is also important. When populations are widely separated, details of genetic relationship may not be clearly established. This was demonstrated in the Wagner Tree dendrogram, where the clustering of some populations was not explicit.

Sampling for estimates of mating system parameters requires a different approach from that stated above. For highly heterozygous, multiallelic species, it is necessary to use a large progeny array and many loci to increase the chances of scoring homozygotes, facilitating identification of parental genotypes. For *A. karroo*, mating system parameters for over half of the sampled families could not be estimated because of the difficulties involved in differentiating alleles contributed by pollen from those contributed by the ovules. It is important also that the sampling for mating system studies should be conducted such that family structures can be detected. When samples are selected randomly with great distances between trees, it is possible that such information will be missed. In addition to random sampling, there should be intensive collection of analytical material from certain sub-populations to allow detection of family structures and gene flow. For example, in this study, one maternal tree with a unique allele *c* at *Adh*-2 locus was found; sibs from other maternal trees with a similar allele were scored also, but it was impossible to refer to this tree unequivocally as the paternal parent with the unique allele because other potential parents in the population were not analysed. Furthermore, these populations often consist of some trees that produce male flowers only, and could not be included in the analysis unless tissues other than seeds were used. Such circumstances require that vegetative tissue be included among the specimens collected for analyses.

Cytological evidence indicated that *A. karroo* is a tetraploid. More evidence on the polyploid nature was gained from isozyme inheritance patterns, which also indicated that the species is a segmental polyploid. One important question that was not addressed in this study concerns the ploidy level of sterile trees of *A. karroo*, reported by Ross (1971, 1979). Sterility is known to be prevalent among autopolyploids and triploids (Riley, 1949), and it would be interesting to know which factors are responsible for the infertility in these sterile trees.

In this study, chromosome number and isozyme banding patterns reflecting tetrasomic inheritance, multiple alleles, fixed heterozygosity, allelic duplication, ghost bands, gene silencing, null alleles, mutations were deduced. These phenomena usually complicate interpretation of isozyme patterns, but are rarely all found within a single species, as was the case in *A. karroo*. Thus, observing them together, as was the case here, assisted in reaching some conclusions about the genetic nature of the species as a polyploid.

Unequal segregation of chromosomes is characteristic of autopolyploids (Riley, 1949; Lewis, 1980), and often detected through cytological studies of dividing cells. Here, in the progeny array analysis, a number of sibs were found to have a variable number of bands, sometimes exceeding the expected four alleles, suggesting that the segregation did not always follow the same pattern. The most likely source of such extra alleles is unequal segregation of chromosomes in the gametes.

The other important observation concerns the fate of silenced alleles in a population. In preceding chapters, evidence of mass silencing of certain alleles within some populations was presented. These alleles, including two homoeologues of *Adh-2b* (Fig 5.4), were not detected in these populations, leading to the conclusion that these alleles were either comigrating, or have been silenced. However, when large progeny arrays were assayed from every

maternal tree to estimate mating system parameters, it was realized that a few families still maintained these alleles which were presumed silenced. For the majority of the genotypes, the positions of these alleles on the gels were always marked by faint bands, usually referred to as "ghost bands" (Gottlieb, 1981). However, these same alleles expressed normal activities in a few families. From that observation, it could be suggested that the characteristic bands often termed ghost bands represent recently silenced alleles in ancient polyploids. Ancient polyploidy has been suspected in a number of plant species, for example, acacias (Khan, 1951, and references therein), ferns (Werth, 1989) and some angiosperms (Soltis and Soltis, 1993). Here, the activity of these bands varied from one family to another, indicating that the silencing is a gradual loss of activity by these alleles in a population. In some cases where their activity was intermediate, it was difficult to decide whether they were absent or active, complicating the genetic interpretation. The tentative conclusion here that ghost bands represent recently silenced alleles must await further empirical verification.

Extremely rare patterns with no repeatability, and those beyond expectation from the observed alleles, were either dismissed as artifacts or, where well expressed, accepted as mutations, as illustrated in Figure 5.5. Some of these expressions could be post-translational transformations of enzymes rather than the genes themselves, but this is difficult to verify unless reflected in sib arrays to which Mendelian segregation laws can be applied to confirm their genetic status (Brown and Moran, 1981).

If the species is a segmental tetraploid, it is possible to propose some explanations for its phenotypic diversity. Recent studies (see Rieseberg and Ellstrand, 1993, for reviews) show that correlations of character between hybrids and original parents are not stable. The traits can be inclined to either of the parents, additive, or totally unique. It is also known that segmental polyploidy represents an unstable stage in speciation. Species of this nature

tend to hybridize and backcross with the progenitors, radiating into different phenotypes. At the same time, other factors known to characterize polyploid species such as gene silencing, gene duplication, and an unequal chromosomal segregation at meiosis, can influence phenotypic expression in an already complicated situation. This study also revealed a possibility of the formation of the species through recurrent polyploidization (Chapter 3). It is possible that all these factors operate, resulting in prolific phenotypes known in the ecological range of *A. karroo*.

The level of total genetic diversity (H_T = 88%) estimated for *A. karroo* was higher than that reported for other plant species. To date, the mean total genetic diversity level reported for all other plant species is estimated at 30% (Hamrick *et al.*, 1992), with only a few species showing levels higher than 50%. However, most of these earlier studies were of diploid species. The other cause for lower diversity reported in polyploids could be the statistical methods used to estimate the level of heterozygosity: in some studies, the probability of homozygotes has been estimated according to the Hardy-Weinberg equilibrium because the species concerned express disomic inheritance (e.g. Jarvie and Barkworth, 1990; Stankovics and Naggy, 1992), which is likely to lead to lower estimates of heterozygosity level for polyploids. In some cases, allotetraploids do exhibit complete fixed heterozygosity, or complexities which do not allow genetic interpretation of genotypes (Ness *et al.*, 1989; Moran *et al.*, 1991); thus *A. karroo* is could be one of the few species in allo- cum-autotetraploid class for which such genetic data have been reported.

From the empirical evidence here, it is possible to attribute the high genetic diversity to the presence of multiple alleles. The genetic diversity is also maintained by an average gene flow (4.47), supported by a mixed-mating system and, possibly, by an efficient seed dispersal mechanism.

6.2. Implications for genetic conservation and improvement programmes of *A. karroo*

The first question that comes to mind is "why conserve a widespread weedy species"? There are a number of relevant answers: (1) where there is pressure on land for other uses, it is impossible for the species to remain widespread indefinitely; (2) being a multipurpose species, exploitation for different purposes can quickly increase demand, outstripping natural production; (3) selective utilization can easily cause dysgenic effects, resulting in loss of the best genotypes; (4) a widespread species is a complex of several genetic groups, with some adapted to specific environments; ecotypes on marginal ranges can easily be lost unless a proper conservation strategy is prioritized.

In this study, a survey of isozyme variation has shown the level and the distribution of the genetic diversity within and between populations. This information is fundamental when sampling the genetic diversity of the species for *ex-situ* conservation. The other most readily applicable result always searched for by workers using isozymes and other molecular markers is that which correlates the genetic variation with phenotypic, physiological, or ecological variables. Such direct correlations are rare in isozyme studies, and did not form the main objective of the investigations here; however, some significant results have been reported, relating allelic distribution to geographical factors such as longitude, latitude and rainfall. Such preliminary observations will require more comprehensive sampling to verify, but is a step in the right direction. Systematic sampling could be organized following the gradient of the environmental factors, e.g. rainfall, temperature, longitude and latitude to confirm or dismiss these correlations. Also, by transfering the

samples and growing them in the reverse conditions to their origin the environmental effect on the putative alleles might be detected.

Although the variation among populations estimated was not great (G_{st} = 5%), there were populations such as Hluhluwe and Mutorashanga which expressed high allelic richness. These two populations are in a game and a mining reserve, respectively. The results seem to suggest that this initial protection of these populations has helped in retaining the allelic richness. Since the original conservation priorities were not plant oriented, additional measures can be taken to strengthen the *in-situ* conservation of the species in the same localities. Also, more *in-situ* conservation populations can be sited in areas where localized alleles are distributed.

The observed variability can be exploited further to develop breeding populations. The evidence presented here relating to variation in fitness of genotypes may be tested by selecting a sample of maternal trees, first on their morphological traits, followed by isozyme analyses to determine their genotypes. Their progeny coud be tested in experimental trials for survival and performance. Since it was observed here that the maternal trees tend to produce or to retain progeny of specific genotypes, such an experiment may help to determine genotypic variation in fitness, which may be useful when roguing breeding populations. Moreover, the knowledge of the mating system and choice of mates would be a valuable asset in designing seed orchards and implementing breeding programmes. The current information available on mapped trees at Umgusa Valley, Zimbabwe could be useful to start such a trial

6.3. Recommendations

The Wagner Tree dendrogram divided *A. karroo* into three cluster groups, representing the level of isozyme divergence. Thus, for successful

conservation and genetic improvement programmes, samples must be collected from all the groups. The high genetic diversity within populations and low genetic differentiation among populations indicate that a few sampled trees should be representative of their populations. For example, 70% of all genotypes expressed in the 12 populations combined (Table 3.2) were seen in the progenies of 40 maternal trees sampled at Umguza Valley. The absent genotypes were contributions from rare or localized alleles, the sampling of which requires a systematic strategy. Results of spatial autocorrelation analysis suggested lack of spatial pattern within the single population studied. If this result is applicable to other populations, then selection within short intervals (20 m) should produce variable samples for genetic conservation and improvement programmes of the species. The mixed-mating system should ensure incorporation of all the alleles into future generations, assuming that all the parental trees in the established population are fertile and contribute effectively to the gene pool.

Since no studies have correlated allozyme genotypes to morphological variants of *A. karroo*, the results obtained here cannot be used as the sole criterion of sampling for genetic conservation and breeding programmes. Other selection criteria must be based on end products expected from the species, e.g. fuelwood, fodder, gum, tannin, timber, ornamental use, adaptability to specific sites.

Here, it was demonstrated that the genotypes observed in progeny were not always consistent with those expected. Some genotypes were selected against while others were favoured. The racemes of *Acacia* species, among them *A. karroo*, are known to consist of several florets of which only a few develop into pods with viable seeds (Gordon-Gray and Ward, 1975; New, 1984; Kenrick and Knox, 1989; Sedgley *et al.*, 1992): such losses, whether mediated by the environment, lethal genes, or ovule-pollen incompatibility could have adverse consequences for conservation populations and

plantation forestry. If such floral abortions are due to genetic effects, it would be appropriate to incorporate isozyme genotypic selection of parental trees, because specific genotypes are likely to produce unwanted progenies, lowering production capacity.

There is an urgent need to study the reproductive biology of the species. Such a study will give more information on the self-compatibility level and shed light on factors associated with it. Moreover, the question of mate preference within plant populations has always been empirically demonstrated in seed orchards and controlled pollination studies (see Kenrick and Knox, 1985; Wheeler and Jech, 1992, and references therein), but it is still vaguely understood. A thorough study of reproductive biology of *A. karroo* would shed more light on this phenomenon, should it be the cause of early genotypic selection (Chapter 5).

In natural populations, studies could be initiated to understand the dynamics of genotypic selection, since it was indicated here that there were more genotypes among the seedling than the adult populations. The other important information that can be deduced in such studies is the role of androdioecy in a species which is partially inbreeding.

This study revealed also the possibilities of environmental selection of genotypes. In the presence of such selection, it will still be adequate to sample trees from populations with localized alleles and replicate them as experimental-cum-conservation stands in different ecological zones. Through such experiments, perhaps, information correlating environmental factors to genotypic and fitness can be derived, and sub-species could be differentiated from ecotypes by their persistent morphological characteristics. Future research should include specific sampling to elucidate possible correlations of genotypes with ecological conditions and phenotypic diversity.

Additional studies should be conducted within sites of introgressions to understand the frequency of hybridizations between *A. karroo* and other

neighbouring *Acacia* species. An isozyme survey on other species such as *A. tenuispina, A. inconflagrabilis* and so on, which have been suggested to hybridize with *A. karroo* should be conducted: perhaps such studies might identify the progenitors of *A. karroo,* which will help in understanding the changes the *A. karroo* genome has undergone as result of hybridization. More sensitive techniques with more genetic markers, such as RFLPs and RAPDs, could be employed but, whatever the technique used, *A. karroo* is likely to present many surprises because of its unstable polyploid nature.

GLOSSARY

Allele. A particular form of a gene at a particular locus (ed. Hillis and Moritz, 1990).

Allozyme. An enzyme that is the product of a particular allelic form of a gene. (Hedrick, 1985).

Androdioecy. Where male and hermaphrodite genets coexist. (Richards, 1986).

Anode. The positive electrode in an electrolytic cell (such as electrophoresis chamber) toward which anions migrate, (anion is a negatively charged molecule). (ed.Hillis and Moritz, 1990).

Assortive mating. Mating that is nonrandom with respect to the phenotypes in a population, i.e., the probality of individuals of two given phenotypes mating is not equal to the product of their frequencies in the population. (Hedrick, 1985).

Biome. A major global ecological unit, or type of flora and fauna formation (e.g. savanna grassland, boreal forest. (Anonymous, 1991).

BIOSYS-1. A computer program for the analysis of electrophoretically detectable allelic variation. (Swofford and Selander, 1981).

Bootstrapping. A statistical method based on repeated random sampling with replacement from an original sample to provide a collection of new estimates of some parameter, from which confidence limits can be calculated. (ed. Hillis and Moritz, 1990).

Bottleneck. A temporary reduction in population size from which future generations are derived. (Hedrick, 1985).

Cathode. The negative electrode in an electrolytic cell (such as electrophoresis chamber) toward which cations migrate, (cation is a positively charged molecule). (ed. Hillis and Moritz, 1990).

Cleistogamy. Where flowers do not open, and are thus inevitably autogamous. (Richards, 1986).

Codon. A sequence of three adjacent nucleotides coding for an amino acid. (Freifelder, 1976).

Coefficient of gene differentiation (G_{st}). The index of genetic diversity due to variation among population relative to total diversity (Nei, 1973).

Coefficient of intrapopulational gene differentiation (R_{st}). The index of genetic diversity due to absolute gene differentiation among populations relative to intrapopultion diversity. (Nei, 1973).

Cofactor. A small molecule essential for activity of an enzyme; usually distinguished from a prosthetic group by being loosely bound, (a prosthetic group is a tightly bound, specific, nonpolypeptide unit required for the biological activity of a protein). (Freifelder, 1976).

Complement index (CI). A statistic expressing the most representative population of a taxon in allelic diversity. (Gottlieb, 1975).

Consanguinity. The sharing of at least one recent common ancestor. (Ayala and Kiger, 1984).

Disomic. Where a chromosome or gene is present twice in a genet. (Richards, 1986).

Ecosystem. A unit which includes all the living organisms and the non-living material within a defined area, the size of which is relatively arbitrary. (Anonymous, 1991).

Electromorph. An electophoretically indistinguishable class of isozymes. Electromorphs represent alleles if all differences between variants result in changes in electrophoretic migration rate. (ed. Hillis and Moritz, 1990).

Electrophoresis. A technique that separates dissolved or colloidal particles subjected to an electrical field according to their mobilities. Electrophoretic mobility depends on the size, three-dimensional geometry, and electrical charge of the particle. (Li and Graun, 1991).

Endosperm. In flowering plants, a tissue specialized for nourishing the developing embryo. (Ayala and Kiger, 1984).

Entomophily. pollination by insects. (Richards, 1986).

Environment. The surroundings of an organism, including both the non-living world and other organisms inhabiting the area. (Anonymous, 1991).

Enzyme. A protein which is a catalyst of biochemical reactions. There are many different kinds, each kind directly promoting only one or a very limited range of reactions. (Anonymous, 1991).

Expected heterozygosity (H_e). It is the probability of non-identical alleles at a locus combining to form a zygote. It is estimated by subtracting the frequency of all homozygotes at a locus from unity (see heterozygosity). (see Hamrick and Godt, 1989)

GDD & GD. A computer program for estimating genetic diversity, distance and constructing a dendrogram. (Ritland, 1989).

Gene. A functional unit of heredity that undergoes Mendelian segregation. (Hedrick, 1985).

Gene diversity (H). A measure of genetic variability in a population. The mean expected heterozygosity (He) per locus in a population. (Li and Graur, 1991).

Gene flow. Interchanging of genetic factors between and within populations as a result of emigration and immigration of individuals (*pollen transfer and propagule dispersal in plants*). (Anonymous, 1991).

Gene pool. The total variety and amount of alleles within a population. (Hedrick, 1985)

Genetic drift. The chance changes in allelic frequency that result from the sampling of gametes from generation to generation. (Li and Graur, 1991).

Genetic load. Reduction in the fitness in a population from the maximum possible because of genetic segregation. (Hedrick, 1985).

Genotype. The genetic constitution of an individual, often stated just for one or two genes. (Hedrick, 1985).

Hardy-Weinberg principle. The relationship in which after one generation of random mating, single-locus genotypic frequencies can be represented as a binomial (with two alleles) or a multinomial (with multiple alleles) function of the allelic frequencies. (Hedrick, 1985).

Heterozygosity (H). The proportion of heterozygotes in a population. (Hedrick, 1985).

Homology. Similarity by common ancestry or genetic relatedness. (Li and Graur, 1991).

Homoeologous. (Of chromosomes) from different parents, having partial, but not complete homology (ability to pair and recombine in a hybrid). (Richards, 1986).

Homozygote. With only one allele at a locus. (Richards, 1986).

Hybrid. The offspring of parents which are not genetically identical. (Anonymous, 1991).

Inbreeding. Nonrandom mating in which the mating individuals are more closely related than individuals drawn by chance from the population. (Hedrick, 1985).

Intrapopulational gene diversity (H_S). The mean diversity within populations, estimated as the mean of expected heterozygosity over all loci assayed. (see Hamrick and Godt, 1989).

Interpopulational gene diversity (D_{ST}). The difference between total gene diversity and intrapopulational gene diversity. (Nei, 1987).

Isozyme. Any of the distinct forms of enzyme that have identical or nearly identical chemical properties but are encoded by different loci. (Li and Graur, 1991).

Linkage disequilibrium. Nonrandom association of alleles at different loci in a population. (Ayala and Kiger, 1984).

Locus. The site of a gene on a chromosome; or, sometimes, the gene and its alleles. (Hedrick, 1985).

Marker. An allele whose inheritance is under observation in a cross a population. (Ayala and Kiger, 1984).

MLTET. Generalized computer program for estimating inbreeding parameters in tetraploids (MLTET-Multilocus-Tetraploid). (Ritland, Unpub.).

Mutation. A structural change in a gene which may give rise to a new heritable characteristic if it occurs in one of the germ cells. (Anonymous, 1991)

Natural selection. The process by which organisms which are not well fitted to their environment are eliminated and those which are well fitted survive to breed and pass on their genes to subsequent generations. (Anonymous, 1991).

Nondisjuction. The failure of homologous chromosomes to separate during meiosis. (Li and Graur, 1991).

Operational taxonomic units (OTU). Any of the extant taxonomic units under study. (Li and Graur, 1991).

Phenotype. The morphological, biochemical, behavioural, or other properties of an organism, putatively indicating ancestral relationships. (Hedrick, 1985).

Polyploid A genet (a genetical individual) with more than two homologous or homoeologous sets of chromosomes. (Richards, 1986).

Population. A group of individuals existing together in time and space and capable of interbreeding. (Hedrick, 1985).

Protein. A complex bio-molecule, made up of one or more chains of amino acids. Where made of several chains, each of these is known as a polypeptide chain. (Anonymous, 1991).

Quaternary structure. Types and modes of interaction between two or more polypeptide chains within a protein molecule with two or more subunits. (Li and Graur, 1991).

Random-mating population. A group of individuals in which the probability of mating with individuals of particular types is equal to their frequency in the population. (Hedrick, 1985).

Reciprocal translocation. A translocation that involves an exchange of chromosome segments between two nonhomologous chromosomes. (Ayala and Kiger, 1984).

Rooted tree. A phylogenetic tree that specifies ancestral and descendant species, thus indicating the direction of the evolutionary path. (Li and Graur, 1991).

Spatial autocorrelation analysis. A statistical approach to test whether the observed value of a nominal, ordinal, or interval variable at one locality is independent of values of the variable at neighbouring localities. (Sokal and Oden, 1978a).

Speciation. The process of species formation. It is thought to occur either when the new species are in the same area (sympatric), in different areas (allopatric), or in adjacent areas (parapatric). (Hedrick, 1985).

Species. A group of populations that is reproductively isolated from other groups. (Hedrick, 1985).

Statistic. An estimate of the value of a particular parameter or measure based on a sample. For example, the estimate of the allelic frequency based on a sample from the population of interest is a statistic. (Hedrick, 1985).

Stochastic process. A process, the outcome of which cannot be predicted exactly from knowledge of initial conditions. However, given the initial conditions, each of the possible outcomes of the process can be assigned a certain probability. (Li and Graur, 1991).

Substrate. The substance acted on by an enzyme. (Freifelder, 1976).

Taxon (pl. **taxa**). A taxonomic group of any rank (e.g., species, genus, kingdom) to which individual organisms are assigned. (Li and Graur, 1991).

Tetrasomic. Where a chromosome or gene is present four times in a genet; tetrasomic inheritence involves three types of heterozygote (simplex, duplex and triplex = Aaaa, AAaa & AAAa, respectively). (Richards, 1986).

Topology. The branching pattern of a phylogenetic tree. (Li and Graur, 1991).

Total gene diversity (H_T). The heterozygosity expected when populations are pooled and random mating takes place. (Ritland, 1988).

Unbiased genetic identity (I) [unbiased genetic distance(D)]. The frequency of identical genes estimated by expectation maximization under the

assumption of multinomial sampling and random mating on a large sample size ($D = -\log_e I$) (Nei, 1987).

Variation. Difference between individuals which may be caused either genetically or environmentally. The differential survival of genetic variants results in the process of natural selection. (Anonymous, 1991).

Zymogram. The pattern on an allozyme electrophoresis gel visualized by histochemical staining. (ed. Hillis and Moritz, 1990).

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Appendix I

Staining procedures for the four isozymes not used.

Alanine aminopeptidase (AAP, E.C. 3.4.11.1)

0.035 M Tris maleate	pH 7.2	100 ml
Alanine-β-naphthyline (free base) dissolved in 1.0 ml DMSO*		20 mg

Incubated at 37°C for 30 mins, poured off and stained with:

Distilled water	100 ml
Fast Black K salt	20 mg

Fluorescent esterase (F-EST, E.C. 3.1.1.1)

0.1 M Na acetate	pH 5	100 ml
4-methylumbelliferyl in acetone		4 mg

Incubated at room temperature and bands read using UV light (254 nm).

Isocitric dehydrogenase (IDH, E.C. 1.1.1.42)

0.1 M Tris-HCl	pH 7.5	100 ml
DL-Isocitric acid		60 mg
MgCl2		50 mg
NADP		20 ml
MTT		15 ml
PMS		4 mg

Leucine aminopeptidase (LAP, E.C. 3.4.11.1)

0.035 M Tris maleate	pH 7.2	100 ml
Leucine-β-naphthylamide		20 mg

Incubated at 37°C for 30 mins, poured off and stained with:

Distilled water	100 ml
Fast Black K salt	20 mg

*DMSO - Dimethyl sulfoxide

Appendix II

Expected genotypic frequencies for an isolocus in an autotetraploid.

Case one: Two alleles

$$a^4+2a^3b+a^2b^2+2a^3b+4a^2b^2+2ab^3+a^2b^2+2ab^3+b^4 =$$

$$a^4+4a^3b+6a^2b^2+4ab^3+b^4$$

Case two: Three alleles

$$a^4+2a^3c+a^2b^2+2a^2bc+a^2c^2+$$
$$2a^3b+4a^2b^2+2ab^3+4a^2bc+4ab^2c+2abc^2+$$
$$2a^3c+4a^2bc+4a^2c^2+2ab^2c+4abc^2+2ac^3+$$
$$a^2b^2+2ab^3+2ab^2c+b^4+2b^3c+b^2c^2+$$
$$2a^2bc+4ab^2c+4abc^2+2b^3c+4b^2c^2+2bc^3+$$
$$a^2c^2+2abc^2+2ac^3+b^2c^2+2bc^3+c^4 =$$

$$a^4+4a^3b+4a^3c+6a^2b^2+6a^2c^2+12a^2bc+12ab^2c+12abc^2+6b^2c^2+b^4+4ab^3+4ac^3+4b^3c+4bc^3+c^4$$

Appendix III

Possible numbers of rooted and unrooted trees for 1-10 OTUs

Number of OTUs	Number of rooted trees	Number of unrooted trees
2	1	1
3	3	1
4	15	3
5	105	15
6	954	105
7	10 395	954
8	135 135	10 395
9	2 027 025	135 135
10	34 459 425	2 027 025

Reproduced from Li and Graun (1991).

Apendix IV

Observed genotypes at *Aat-3* locus of *A. karroo.*

		GENOTYPES OF OFFSPRINGS										
FAM.	M.GEN	1111	1112	1122	1222	2222	1133	1113	3222	2223	3333	Total
560	1122	-	7	27	6	-	-	-	-	-	-	40
996	1122	-	1	55	4	-	-	-	-	-	-	60
987	1122	1	3	30	6	-	-	-	-	-	-	40
978	1122	-	2	11	5	2	-	-	-	-	-	20
1446	1122	-	-	30	9	-	-	21	-	-	-	60
564	1122	-	4	28	11	-	-	1	-	-	-	44
1483	1122	-	11	35	13	1	-	-	-	-	-	60
572	1122	-	8	42	8	1	-	-	1	-	-	60
1402	1122	13	10	22	11	-	-	4	-	-	-	60
1415	1122	1	13	30	4	-	-	11	1	-	-	60
1469	1122	-	14	36	7	-	-	-	-	-	-	60
570	1122	14	13	13	13	-	1	3	3	-	-	60
1408	1122	14	8	20	18	-	-	-	-	-	-	60
555	1122	16	10	17	13	1	-	1	2	-	-	60
993	1122	-	2	36	16	1	-	-	-	-	-	55
995	1122	9	4	39	7	1	-	-	-	-	-	60
990	1122	-	-	50	8	2	-	-	-	-	-	60
1406	1122	-	13	40	7	-	-	-	-	-	-	60
1401	1122	- 12	4	37	5	1	-	1	-	-	-	60
1424	1122	-	4	47	9	-	-	-	-	-	-	60
1426	1122	13	4	24	18	-	-	-	-	1	-	60
1443	1122	8	3	5	4	-	-	-	-	-	-	20
1450	1122	-	14	25	13	-	-	6	2	-	-	60
1455	1122	-	30	23	6	1	-	-	-	-	-	60
1458	1122	-	18	24	9	3	-	6	-	-	-	60
1467	1122	17	3	26	13		-	-	1	-	-	60
1476	1122	-	20	18	7	1	-	14	-	-	-	60
1486	1122	-	10	37	13	-	-	-	-	-	-	60
573	1112	22	6	24	2	-	-	6	-	-	-	60
575	1112	38	4	12	3	-	-	3	-	-	-	60
1403	1112	15	7	35	2	-	-	1	-	-	-	60
1409	1112	12	20	17	9	-	-	2	-	-	-	60
1449	1112	15	4	32	4	-	-	4	1	-	-	60
1414	1112	20	24	14	2	-	-	-	-	-	-	60
1419	1112	20	14	22	1	-	-	3	-	-	-	60
1430	1112	24	12	19	1	-	-	-	-	-	1	60
1433	1112	- 11	12	17	12	-	-	8	-	-	-	60
1434	1112	15	17	20	7	-	-	1	-	-	-	60
1447	1112	12	4	17	24	3	-	-	-	-	-	60
1405	1222	-	1	14	45	-	-	-	-	-	-	60
Σ 40	3	322	371	1077	354	15	1	106	11	1	1	2259

Fam = family number, M. gen. = maternal genotype. 3 represents a null allele in the genotypes
Note the low number of 2222 (15) homozygotes compared to 1111(322).

Appendix V

Observed genotypes at *Adh*-2 locus in *A. karroo*.

		GENOTYPES OBSERVED IN OFFSPRINGS												
FAM.	M.Gen	1111	1112	1122	1133	1123	1223	1222	2222	2223	2233	2333	3333	Total
560	1222	-	9	-	1	1	-	2	25	-	1	-	-	40
996	1222	3	5	25	-	-	-	1	26	-	-	-	-	60
987	1222	-	1	5	-	-	-	5	26	-	3	-	-	40
978	1222	-	-	8	-	-	-	4	7	-	1	-	-	20
1446	1222	3	9	18	-	-	-	3	27	-	-	-	-	60
1414	1222	-	8	9	-	-	-	-	43	-	-	-	-	60
1483	1222	-	8	4	-	-	-	-	48	-	-	-	-	60
564	1122	5	4	14	-	-	-	3	18	-	-	-	-	44
1402	1122	3	12	22	-	-	-	-	23	-	-	-	-	60
1415	1122	3	16	21	-	-	-	-	20	-	-	-	-	60
1469	1122	4	13	18	-	-	-	-	23	-	-	1	-	60
573	2222	-	3	2	-	-	-	1	54	-	-	-	-	60
572	2222	-	-	3	-	-	-	-	57	-	-	-	-	60
570	2222	-	6	-	-	-	-	-	53	-	1	-	-	60
555	2222	-	1	5	-	-	-	-	54	-	-	-	-	60
993	2222	-	7	1	-	-	-	-	46	-	-	1	-	55
995	2222	-	-	8	-	-	-	2	50	-	-	-	-	60
990	2222	-	2	7	-	-	-	-	51	-	-	-	-	60
575	2222	-	1	6	-	-	-	-	53	-	-	-	-	60
1403	2222	-	8	-	-	-	-	-	52	-	-	-	-	60
1405	2222	-	3	-	-	-	-	-	57	-	-	-	-	60
1408	2222	-	4	2	-	-	-	-	54	-	-	-	-	60
1409	2222	-	4	4	-	-	-	-	52	-	-	-	-	60
1419	2222	-	4	5	-	-	-	-	51	-	-	-	-	60
1424	2222	-	-	5	-	-	-	-	55	-	-	-	-	60
1426	2222	-	5	1	-	-	-	-	54	-	-	-	-	60
1430	2222	-	-	6	-	-	-	-	53	1	-	-	-	60
1406	2222	-	2	4	-	-	-	-	54	-	-	-	-	60
1401	2222	-	2	2	-	-	1	2	53	-	-	-	-	60
1433	2222	-	5	3	-	-	-	-	52	-	-	-	-	60
1434	2222	-	2	10	-	-	-	-	48	-	-	-	-	60
1443	2222	-	-	1	-	-	-	-	19	-	-	-	-	60
1447	2222	-	1	-	-	-	-	-	54	-	1	4	-	60
1450	2222	-	2	4	-	-	-	-	54	-	-	-	-	60
1455	2222	-	-	3	-	-	-	-	57	-	-	-	-	60
1458	2222	-	-	2	-	-	-	-	58	-	-	-	-	60
1467	2222	-	-	9	-	-	-	-	51	-	-	-	-	60
1476	2222	-	1	-	-	-	-	-	59	-	-	-	-	60
1486	2222	-	2	-	-	-	-	-	58	-	-	-	-	60
1449	2233	-	-	2	-	1	1	-	26	3	21	5	1	
Σ 40	4	21	150	239	1	2	2	23	1775	4	29	11	1	2258

Fam = family number, M. gen. = maternal genotype. 3 represents a *c* allele in the genotypes. One offspring with a null allele was recorded and is excluded from the Table.

Appendix VI

Estimates of single- and multi-locus outcrossing rates of individual trees of *A. karroo* (standard errors in parenthesis).

	A	B	C	D	E	F	G	H	I	J
1					Columns C to J - single-locus outcrossing rates of individual trees.					
2										
3	Family No.	tm (no Adh-1)	AAT-1	AAT-2	AAT-3	ADH-1	ADH-2	SDH-1	BST-1	DIA-1
4										
5	555	1.99 (0.01)	1.84 (0.00)	1.99 (0.00)	1.28 (0.31)	1.99 (0.01)	0.54 (0.00)	0.70 (0.12)	0.77 (0.11)	1.05 (0.26)
6	560	1.19 (0.11)	1.20 (0.13)	1.82 (0.13)	0	1.99 (0.19)	1.99 (0.00)	0.66 (0.22)	0.74 (0.18)	0.67 (0.00)
7	564	1.99 (0.01)	1.99 (0.01)	1.99 (0.00)	0.14 (0.00)	1.99 (0.01)	0.64 (0.01)	0.54 (0.16)	0.89 (0.16)	0.67 (0.00)
8	570	0.95 (0.48)	1.62 (0.01)	1.00 (0.17)	1.58 (0.32)	1.99 (0.00)	0.12 (0.12)	0.22 (0.09)	0.90 (0.14)	0.95 (0.16)
9	572	0.95 (0.43)	1.57 (0.03)	0.76 (0.12)	0.13 (0.14)	1.99 (0.00)	0.32 (0.18)	0.94 (0.16)	0.51 (0.13)	0.60 (0.13)
10	573	1.00 (0.46)	0.87 (0.51)	0.92 (0.34)	0.73 (0.20)	1.99 (0.01)	0.23 (0.14)	0.64 (0.11)	0.59 (0.13)	0.85 (0.14)
11	575	1.02 (0.44)	0.92 (0.23)	1.49 (0.07)	0.34 (0.12)	1.99 (0.00)	0.64 (0.23)	0.22 (0.09)	0.77 (0.12)	0.64 (0.12)
12	978	0.92 (0.54)	0.57 (0.14)	1.99 (0.00)	0	1.99 (0.02)	0.28 (0.23)	1.00 (0.33)	0	0.67 (0.00)
13	987	1.00 (0.51)	0.76 (0.12)	1.30 (0.31)	0	1.99 (0.01)	1.03 (0.24)	1.03 (0.18)	0.16 (0.08)	0.58 (0.14)
14	990	0.99 (0.49)	1.99 (0.02)	1.38 (0.21)	0	1.99 (0.01)	0.78 (0.26)	0.85 (0.18)	0.12 (0.06)	0.67 (0.00)
15	993	0.89 (0.44)	1.90 (0.00)	0.99 (0.38)	0	1.99 (0.01)	0.14 (0.13)	0.60 (0.13)	0.58 (0.08)	0.48 (0.26)
16	995	0.71 (0.08)	0.80 (0.18)	1.38 (0.31)	0.0 (0.17)	1.99 (0.02)	0.87 (0.30)	0.44 (0.14)	0.28 (0.09)	0.67 (0.00)
17	996	0.86 (0.17)	0.69 (0.12)	1.22 (0.26)	0	1.99 (0.01)	0.43 (0.12)	0.58 (0.12)	0.48 (0.13)	0.67 (0.00)
18	1401	1.87 (0.00)	1.99 (0.01)	1.16 (0.34)	0	1.99 (0.01)	0.22 (0.13)	0.97 (0.12)	0.31 (0.10)	0.57 (0.12)
19	1402	1.99 (0.01)	1.99 (0.00)	0.91 (0.40)	1.37 (0.30)	1.99 (0.00)	0.49 (0.11)	0.44 (0.14)	0.16 (0.09)	1.02 (0.14)
20	1403	1.07 (0.44)	1.29 (0.36)	1.67 (0.16)	0.75 (0.28)	1.99 (0.01)	0	0.79 (0.09)	0.24 (0.09)	0.60 (0.12)
21	1405	1.99 (0.01)	1.99 (0.00)	0.95 (0.34)	0.11 (0.11)	1.99 (0.01)	0.11 (0.10)	1.99 (0.00)	0.87 (0.13)	0.87 (0.15)
22	1406	0.91 (0.38)	0.32 (0.05)	1.99 (0.00)	0	1.99 (0.01)	0.67 (0.26)	0.71 (0.16)	0.52 (0.09)	0.39 (0.10)
23	1408	1.99 (0.01)	1.99 (0.00)	1.99 (0.00)	0.29 (0.31)	1.99 (0.01)	0.23 (0.16)	0.46 (0.12)	0.46 (0.11)	0.48 (0.12)
24	1409	0.99 (0.01)	1.00 (0.29)	1.34 (0.34)	1.00 (0.22)	1.99 (0.01)	0.57 (0.24)	0.81 (0.15)	0.50 (0.11)	0.53 (0.09)
25	1414	0.99 (0.49)	1.76 (0.06)	1.43 (0.19)	0.18 (0.13)	1.99 (0.01)	0.22 (0.13)	0.20 (0.11)	0.27 (0.07)	1.07 (0.12)
26	1415	1.01 (0.30)	1.76 (0.06)	1.77 (0.00)	1.77 (0.29)	1.99 (0.01)	0.40 (0.12)	0.50 (0.12)	0.58 (0.12)	1.12 (0.15)
27	1419	1.93 (0.01)	0.76 (0.30)	1.22 (0.22)	0.43 (0.17)	1.99 (0.01)	0.68 (0.25)	0.32 (0.12)	0.75 (0.13)	0.45 (0.11)
28	1424	1.99 (0.01)	1.99 (0.00)	1.99 (0.00)	0	1.99 (0.01)	0.53 (0.24)	0.19 (0.10)	0.87 (0.10)	1.14 (0.14)
29	1426	0.92 (0.37)	1.46 (0.02)	1.99 (0.00)	0.38 (0.29)	1.99 (0.01)	0.12 (0.11)	0.64 (0.19)	0.80 (0.13)	0.82 (0.12)
30	1430	1.99 (0.00)	0.76 (0.18)	1.58 (0.16)	0.37 (0.17)	1.99 (0.01)	0.64 (0.25)	0	0.93 (0.10)	0.14 (0.13)
31	1433	1.99 (0.01)	0.51 (0.09)	1.99 (0.00)	1.42 (0.20)	1.99 (0.01)	0.35 (0.19)	0.56 (0.14)	0.71 (0.12)	0.61 (0.15)
32	1434	0.95 (0.41)	0.74 (0.11)	1.96 (0.02)	0.80 (0.24)	1.99 (0.01)	1.09 (0.27)	0.92 (0.14)	1.13 (0.15)	0.26 (0.10)
33	1443	0.88 (0.53)	0.69 (0.40)	1.99 (0.13)	1.46 (0.48)	1.99 (0.01)	0.32 (0.35)	0.27 (0.20)	0.21 (0.15)	0.14 (0.13)
34	1446	1.99 (0.01)	0.70 (0.21)	1.99 (0.00)	1.67 (0.26)	1.70 (0.29)	0.70 (0.11)	0.58 (0.11)	0.57 (0.12)	0.36 (0.11)
35	1447	0.98 (0.49)	1.18 (0.11)	1.91 (0.00)	0.82 (0.26)	1.99 (0.00)	0.12 (0.09)	0.54 (0.12)	0.21 (0.09)	0.74 (0.10)
36	1449	0.95 (0.52)	0.97 (0.41)	1.99 (0.00)	1.07 (0.25)	1.99 (0.00)	0.58 (0.09)	1.10 (0.24)	0.44 (0.10)	0.48 (0.15)
37	1450	1.99 (0.01)	0.87 (0.43)	1.99 (0.00)	1.03 (0.28)	1.99 (0.01)	0.44 (0.21)	0.18 (0.14)	0.80 (0.10)	0.42 (0.13)
38	1455	0.91 (0.29)	1.06 (0.46)	1.48 (0.74)	0.36 (0.52)	1.99 (0.01)	0.31 (0.17)	0.11 (0.08)	0.42 (0.11)	0.74 (0.14)
39	1458	0.96 (0.41)	0.84 (0.42)	1.82 (0.35)	0.99 (0.28)	1.99 (0.00)	0.21 (0.15)	0.87 (0.12)	0.61 (0.15)	1.06 (0.16)
40	1467	1.99 (0.01)	1.99 (0.01)	1.83 (0.06)	0.69 (0.34)	1.99 (0.00)	0.95 (0.28)	0.18 (0.11)	0.90 (0.13)	0.76 (0.15)
41	1469	0.99 (0.46)	0.85 (0.31)	1.99 (0.00)	0.51 (0.25)	1.99 (0.00)	0.56 (0.12)	0.35 (0.12)	0.44 (0.12)	0.80 (0.09)
42	1476	0.96 (0.46)	1.38 (0.42)	1.99 (0.00)	1.90 (0.20)	1.99 (0.00)	0	0.65 (0.11)	0.49 (0.12)	1.08 (0.14)
43	1483	1.11 (0.26)	1.12 (1.99)	1.99 (0.00)	0	1.24 (0.37)	1.26 (0.24)	0.54 (0.13)	0.24 (0.08)	0.10 (0.13)
44	1486	0.93 (0.44)	0.75 (0.13)	1.99 (0.00)	0	1.99 (0.00)	1.99 (0.00)	0.48 (0.10)	0.38 (0.10)	0.66 (0.14)
45	tm	0.88 (0.17)								
46	ts	0.67 (0.20)	0.93 (0.04)	1.22 (0.15)	0.63 (0.50)	1.99 (0.01)	0.58 (0.07)	0.54 (0.05)	0.53 (0.05)	0.67 (0.05)

Estimated outcrossing rates range from 0-1.99.

Appendix VII

Estimated allele frequencies at 8 enzyme loci at Umguza Valley.

Allele	Sample size - 120 trees	Sample size - 40 trees
Aat-1a	0.02	0.10
Aat-1b	0.59	0.55
Aat-1b"	0.00	0.00
Aat-1c'	0.10	0.10
Aat-1c	0.22	0.12
Aat-1d'	0.00	0.10
Aat-1d	0.00	0.00
Aat-1e	0.04	0.03
Aat-1f	0.00	0.00
Aat-2a	0.05	0.07
Aat-2b	0.46	0.55
Aat-2c	0.49	0.36
Aat-2n	0.00	0.02
Aat-3a	0.57	0.56
Aat-3b	0.43	0.41
Aat-3n	0.00	0.03
Adh-1a	0.34	0.41
Adh-1b	0.33	0.22
Adh-1c	0.28	0.24
Adh-1d	0.05	0.09
Adh-2a	0.18	0.13
Adh-2b	0.79	0.87
Adh-2c	0.03	0.00
Adh-2d	0.00	0.00
Adh-2n	0.00	0.00
Sdh-1a	0.00	0.00
Sdh-1b	0.00	0.01
Sdh-1c	0.21	0.09
Sdh-1d	0.69	0.70
Sdh-1e	0.10	0.19
Sdh-1f	0.00	0.01
β-Est-1a	0.16	0.13
β-Est-1b	0.48	0.44
β-Est-1c	0.06	0.12
β-Est-1d	0.30	0.24
β-Est-1e	0.00	0.05
Dia-1a	0.00	0.00
Dia-1b	0.13	0.15
Dia-1c	0.01	0.04
Dia-1d	0.23	0.14
Dia-1e	0.06	0.05
Dia-1f	0.57	0.62