The Conservation Genetics of the Clanwilliam Cedar *(Widdringtonia cedarbergensis)*

by
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Abstract

Widdringtonia cedarbergensis is an endangered conifer species in the western Cape, South Africa. This species is under serious threat of extinction and is being actively managed by Cape Nature Conservation in a small section of the Cedarberg mountains in an attempt to boost population numbers with a seedling replanting scheme and preservation of adults from fires. This study set out to determine levels of genetic diversity and fitness within and among populations of the Clanwilliam cedar for the following reasons: (1) to assess the level of genetic diversity; (2) to screen the seed source for the replanting programme; (3) to locate vigorous seedling sources for replanting and (4) to determine the effect of population size on genetic diversity and fitness.

Starch gel electrophoresis was employed to assess levels of genetic variation within and among seven populations of *W.cedarbergensis*. *W.nodiflora* and *W.schwarzii*, two congeneric species, were incorporated into this section of the thesis as benchmarks against which to compare levels of genetic variation in *W.cedarbergensis*. The three species are different with regard to biology and distribution and predictions as to their population genetic structures were set up accordingly. A major difference in their biology is the resprouting behaviour of *W.nodiflora* in response to fire. Fitness components in populations of *W.cedarbergensis* were divided into reproductive and "ecological" traits, and seedling growth traits. Reproductive and ecological traits were measured in the field and seedling growth traits were obtained from a seedling growth experiment carried out in the glasshouse. Overall, 15 potential fitness traits were measured. The same seven populations were tested for differences in these fitness traits. This enabled an assessment of the seed source used for replanting, as well as alternative seed sources. All fitness variables were tested for a significant relationship with genetic variation measured as heterozygosity. The populations were rated according to population size, density and isolation and correlated with heterozygosity to determine whether there was any relationship.

Seventeen enzyme loci were resolved for each species. Estimates of genetic diversity showed that *W.cedarbergensis* and *W.schwarzii* had low allelic variation. This was attributed to the effect of bottlenecks. High levels of inbreeding and population substructuring were found in *W.cedarbergensis* which suggested that trees were selfing possibly due to limited pollen movement between trees as a result of tree isolation and fine-scale fragmentation incurred by fires. Although allelic diversity in *W.nodiflora* was extremely high, high levels of inbreeding were found within populations which was attributed to selfing among resprouted ramets of the same genet. Tests for differences in fitness traits between populations revealed no population as the most consistently fit for reproductive and ecological traits for the fittest population, although two populations, DG and CPS, were consistently found to have the most vigorous seedlings. The replanting seed source, MB, showed adequately vigorous seedlings.

Four out of fifteen measures of fitness were found to co-vary with heterozygosity. These were embryo abortion fraction, germination rate, shoot biomass and total biomass. Seeds and seedlings were more vulnerable to the effects of inbreeding than traits related to fecundity. These four traits, in turn, co-varied with other traits. The relationship between germination rate and seedling:parent ratio, in particular, indicated that genetic phenomena are impacting the demography of populations. Reductions in fitness occurred in several traits below 30%

heterozygosity and became critical below 25% heterozygosity. 30% heterozygosity occurred below a population size scale of 4 (4000 individuals) and 25% heterozygosity occurred below a population size scale of 1 (250 individuals). Population size, therefore, seemed important in maintaining the genetic diversity of populations. The effect of population density on fitness was not effectively examined in this thesis and deserves further attention.

The results of this thesis had several implications for conservation and active management. My recommendations were the following: (1) two populations, DG and CPS, are the best seed sources for the replanting programme and should supplement the current and most accessible seed source (MB); (2) Replanting should be aimed at boosting seedling:parent ratios in small populations such as WB and KK, as well as closing gaps between trees and clumps of trees as far as possible to facilitate pollen movement and therefore outcrossing; (3) the bottleneck is at a critical stage where adult tree survival is of profound importance in ensuring seedlings are outbred and every effort should be made to reduce mortality due to fire.

CHAPTER ONE. INTRODUCTION

Context of the study

Widdringtonia cedarbergensis (the Clanwilliam Cedar) is a conifer species endemic to the Cedarberg mountains in the south western Cape, South Africa. It is currently listed as endangered in the Red Data Book (Hall & Veldhuis 1985) and is a rare example of an endangered plant species that has the attention of the public on a similar scale to the Cheetah or the Black Rhino. The reasons for its conservation status are multiple but one factor stands above the rest - fire. Populations of *W.cedarbergensis* suffer from extensive mortality owing to its sensitivity to fire. Fire is an important factor in the Cape fynbos[1] vegetation which is dominant in most of the Cape mountains. Not being adapted to fire (by resprouting or serotiny), *W.cedarbergensis* is now almost confined to rocky outcrop refuges where the trees are protected from fire. Further, these populations have been subject to intensive logging in past years. The wood is very hard and durable and was therefore useful for building, shipping, fuel and telegraph poles. Archival documents give accounts of extensive populations of the Clanwilliam Cedar in the last century. Logging was stopped around the turn of the century although populations have not recovered their numbers since. *Widdringtonia cedarbergensis* has thus been fragmented into a number of substantially smaller populations. What remains of this species today is guarded by Cape Nature Conservation (CNC) who manage the Cedarberg catchment area. Bottleneck pressures remain, however, since wild fires are a regular feature of annual summer drought. A similar problem exists for a congeneric species, *W.schwarzii*, although it is far better protected from fire by virtue of the broken topography of the Kouga and Baviaanskloof mountains to which it is endemic.

[1]temperate heathland belonging to the Cape Floristic Kingdom

Management of the Clanwilliam cedar is difficult because management strategies of wilderness areas in the south-western Cape include planned fires for optimal regeneration of other fynbos species. To combat this problem, a replanting scheme implemented by CNC has been initiated in a restricted area of the Cedarberg called Welbedacht where seeds have been collected predominantly from a plantation on the Middelberg. Further, adult trees in this restricted area are protected from intense fires by controlled patch burning with cool fires to eliminate fuel loads in their vicinity.

This thesis has three major aims: (1) to investigate whether bottleneck effects have had a genetic impact on *W.cedarbergensis*; (2) to apply the information gleaned from this study to current management activities as far as possible and (3) promote conservation genetics in future studies in South Africa.

Key Questions

I used protein electrophoresis to answer the following questions surrounding the conservation of the Clanwilliam cedar:

1. **Is there evidence for genetic erosion in the Clanwilliam cedar?**

I approached this question by comparing levels of genetic variation in the Clanwilliam cedar with two congeneric species, *W.nodiflora* and *W.schwarzii*. The biology and distribution of these three species differ and predictions about their population genetic structures were made accordingly. Deviations from these predictions, therefore, would serve to highlight potential problems in their conservation genetics. The impact of size reduction of the metapopulation is investigated as well as the effect of fragmentation of the metapopulation into smaller subpopulations. Since *W.nodiflora* is a root crown resprouter, I further investigate the effect of resprouting on population genetic structure, a trait which, to my knowledge, has not yet been studied in relation to population genetics.

2. **Does genetic variation have an impact on fitness in the Clanwilliam cedar?**

I considered reproductive, ecological and seedling characteristics in populations of *W.cedarbergensis* as potential fitness traits and firstly established whether they varied between populations. I followed this up by determining the relationship between protein

heterozygosity and the various fitness components. This aspect of the study also served to screen the Middelberg plantation (the seed source for the replanting programme) for reproductive, ecological and seedling fitness. At the same time, it served to locate potentially healthier seed sources among the natural populations.

3. **Does population size have an impact on genetic variation and fitness?**

The effect of population size on levels of genetic variation and fitness components were considered, testing the prediction that smaller populations are genetically eroded. I also attempted to determine whether there was a critical population size below which genetic variation and fitness were dramatically reduced.

Thesis Structure

The remainder of this introductory chapter is a literature review on the background of the genus *Widdringtonia* and its evolution as well as the differences between the four species within the genus. I also give the rationale for a genetic study in the context of current conservation biology trends and the needs of *W.cedarbergensis*.

In Chapter Two, I outline the sampling and electrophoretic procedures carried out in this study. A whole chapter was dedicated to laboratory procedures and field sampling to lighten other chapters and because it was a major hurdle to overcome to make this thesis possible.

In Chapter Three, levels of genetic variation in *W.cedarbergensis*, *W.nodiflora* and *W.schwarzii* are reported, testing the hypothesis that *W.cedarbergensis* is genetically depauperate as a result of an intensifying bottleneck. The role of resprouting in population genetic structure is considered.

In Chapter Four, differences in ecological and reproductive fitness components are tested with particular reference to the planting out programme.

In Chapter Five, differences in seedling fitness components are tested, with particular reference to the planting out programme.

In Chapter Six two hypotheses are tested: (1) that genetic variation has an impact on fitness components and (2) that population size has an impact on genetic variation and therefore fitness. I ask whether certain fitness components are more important than others and whether there is a critical population size. Close attention is paid to the relevance of the findings to management activities.

The final chapter, Chapter Seven, brings together the conclusions drawn from this thesis, considers the extent to which questions have been answered and suggests directions for further research.

The Genus Widdringtonia

The genus *Widdringtonia* which is confined to southern Africa, consists of some of the most valued conifer species in the region. With the current classification, there are three species within this genus: *W.cedarbergensis* Marsh (the Clanwilliam cedar), *W.nodiflora* (L.) Endl. (the Mountain Cypress) and *W.schwarzii* (Marloth) Mast (the Willowmore cedar). There is a putative fourth species, *W.whytei* which is the national tree of Malawi, indicating its economic importance to the country as source of softwood (Pauw 1992).

Systematics and Taxonomy

Widdringtonia was first described by Brogniart in 1883 as *Pachylepis*, a name which had already been used by Lessing for a genus of the Compositae. In 1841 it was called *Parolinia* by Endlicher, a name which had also been used before, by Webb, to name a genus of the Cruciferae. Endlicher therefore renamed his genus *Widdringtonia* after Captain Widdrington of the Royal Navy who had taken a keen interest in gymnosperms (Chapman 1961). Although they are considered the African analogues of true Cypresses of southern Europe, Asia and Western North America because of their strong resemblance to this group in foliage and habit, the closest relatives to *Widdringtonia* are *Callitris* from Australia and the monotypic *Tetraclinis* of North Africa and Malta (Chapman 1961). *Widdringtonia* is the sole representative of the Cupressaceae in southern Africa.

The Widdringtonias are evergreen trees or, depending on which species, shrubs. They are primarily distinguished from both *Tetraclinis* and *Callitris* by the oppposite arrangement of their decussate leaves compared with the whorled arrangement in *Callitris* and *Tetraclinis*. Delimitations between these genera have confused experts, however, and on one occasion, a member of *Callitris* introduced at a mission station at Stockenstrom was classified as a separate species within *Widdringtonia* (Masters 1905). Delimitations within the genus have been even more confusing owing to lack of character variation, particularly in *W.nodiflora*.

The first detailed account of the genus was compiled by Masters (1905) who also described a new species *W.mahoni* from the eastern highlands of Zimbabwe, making six *Widdringtonia* species (ie. *W.juniperoides* Endl., *W.schwarzii* Marloth., *W.cuppressoides* Endl., *W.whytei* Rendle, *W.mahoni* Mast. and *W.equisetiformis* Mast.). Since this classification, the genus has undergone numerous taxonomic changes. For example, *W.mahoni* was later included as *W.whytei* by Rendle (1911). In 1933 Stapf maintained six species namely, *W.whytei*, *W.cuppressoides*, *W.stipitata*, *W.dracomontana*, *W.schwarzii* and *W.juniperoides*. Stapf stated that all of these species were distinctive except for *W.stipitata*.

Local geographic forms were in the past recognised as species, however the degree of variation appears such that there are no constant differences separating them as species. On the basis of this Marsh (1966), in the most recent revision of *Widdringtonia*, reduced the number of *Widdringtonia* species to three. *W.whytei* Rendle was included with *W.dracomontana* in *W.cuppressoides* (L.) Endl. *W.cuppressoides* was originally described from the Cape mountains. Marsh (1966) could find no distinguishing character to separate the range variants of *W.cuppressoides*. She did, however, recognise geographical races. *W.cuppressoides* was renamed *W.nodiflora* in 1972 when it was discovered that the type specimen of *Brunia nodiflora* L. (Bruniaceae) was a twig of *W.cuppressoides*, but this did not involve a change in the species concept put forward by Marsh (1976).

The collapse of *Widdringtonia* into three species has generated much interest in the systematics of the group. This is mainly in conjunction with the wide ranging species, *W.nodiflora*. Many botanists have remarked in particular on the presence of two very different forms on Mt. Mulanje (Rendle 1893), currently classified under *W.nodiflora*. These

two forms are the former *W.whytei* and the *W.nodiflora* more typical of the Cape mountains. The former is the tall majestic form known as the Mulanje Cedar which has impressed many visitors to Mulanje (Rendle 1893, Chapman 1961). The latter is the "dwarf" shrubby form which coppices vigorously after fire. In a recent study, Pauw (1992) found sufficient morphological differences in bark characteristics, in seedling and cone morphology as well as in adult growth form between the two forms for them to be recognised as two separate species. This distinction was important since seed had been indiscriminately collected from the most accessible form (ie. the dwarf form) for silvicultural purposes under the misconception that seed from either form would produce the ideal tall form. Four species are therefore recognised in thesis, the three put forward by Marsh (1966) as well as *W.whytei* as distinguished from *W.nodiflora* by Pauw (1992). For the purposes of this project, *W.whytei* is recognised as distinct from *W.nodiflora*.

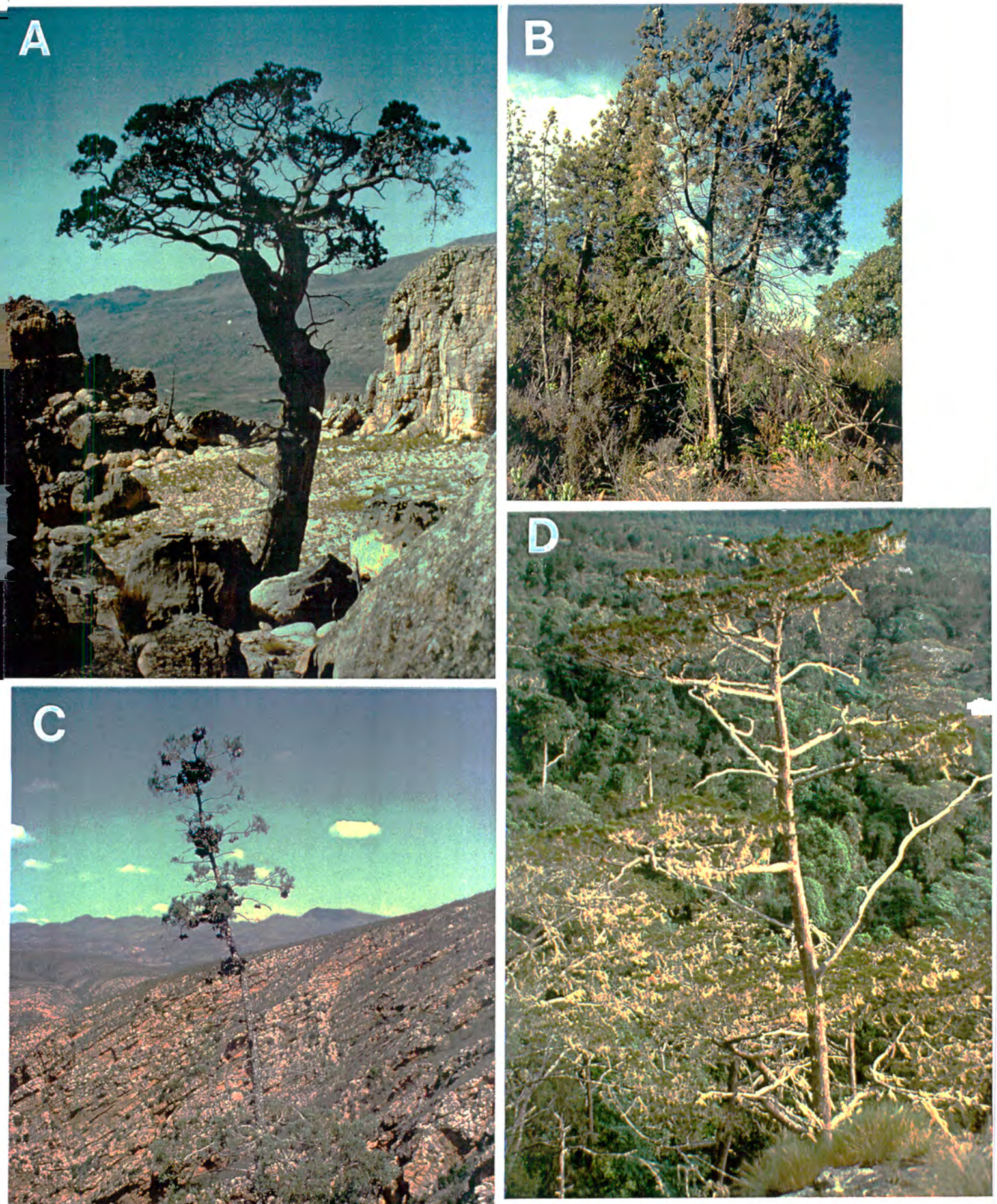


Figure 1.1. The natural habitats of (a) *W.cedarbergensis*, (b)*W.nodiflora*, (c) *W.schwarzii* and (d) *W.whytei*.

Widdringtonia cedarbergensis (Figure 1.1a)

W.cedarbergensis is commonly known as the Clanwilliam cedar because it is restricted to the Cedarberg of the Clanwilliam district in the western Cape (Figure 1.2a). It is described as a rare tree usually 5 to 7m in height, but up to 20m in protected areas (Palgrave 1977). It is typically associated with low shrub fynbos (Manders 1985). The features which distinguish this species from the rest of the genus are the tubercled margins along the margins of the female cone and the large ovoid, triquetrous and obscurely winged seeds (Marsh 1966). *W.cedarbergensis* is, in fact, the only species which has large heavy seeds with a vestigial wing. (Table 1.1). This species is considered threatened should the causal factors persist (Hall & Veldhuis 1985).

Widdringtonia nodiflora (Figure 1.1b)

W.nodiflora is commonly known as the Mountain Cypress and is distributed from the Cape Peninsula in the Cape, South Africa to Malawi in central Africa (Figure 1.2b). *W.nodiflora* is the only shrubby species within the genus, coppicing after fires. It is described as being 4 to 6 m in height, occurring at high altitudes on mountain sides, among rocks and in gullies. The communities in which it grows are chiefly fynbos communities. The distinguishing features of *W.nodiflora* are the smooth to wrinkled margins of valves of the female cone which are not tubercled as in *W.cedarbergensis* (Marsh 1966). The seeds have a conspicuous reddish wing (Table 1.1). This species is not listed as endangered in any way.

Widdringtonia schwarzii (Figure 1.1c)

W.schwarzii is commonly known as the Willowmore cedar since it is restricted to the low-rainfall areas of the Baviaanskloof and Kouga mountains of the Willowmore district in the eastern Cape, South Africa (Figure 1.2c). It is described as standing 17 to 30m tall in deep rocky ravines or "kloofs" as they are locally known, where these trees can often grow up to 40m tall (van Jaarsveld 1983). The seeds are somewhat flattened and conspicuously winged (Marsh 1966). Despite the similar gross morphology, it is classified separately from *W.cedarbergnsis* on the basis of its seed morphology (being light and conspicuously winged) and its distribution.

W.schwarzii from the Baviaanskloof is very similar to *W.cedarbergensis* in many respects except for seed morphology (Table 1.1). Adults and seedlings are easily killed by fire since they do not resprout and serotiny is only weakly developed (Table 1.1). However, populations are protected from burning by the deep fire-free kloofs of the Baviaanskloof and Kouga mountains. Another redeeming feature of these kloofs is that they are extremely inaccessible to humans. Doringkloof is famous for its forest of enormous cedars which are to this day intact despite their value as timber (Luckhoff 1963). This species is considered in danger of extinction should the causal factors persist (Hall & Veldhuis 1985).

Widdringtonia whytei (Figure 1.1d)

Commonly known the Mulanje Cedar, it is restricted to Mulanje and Zomba in Malawi (Figure 1.2d) and therefore has the northernmost distribution in the genus, along with *W.nodiflora*. It is described as sometimes attaining a height of 45m, with a thick and fibrous bark on the older trees. Branching is symmetrical as opposed to short and regular as in *W.nodiflora* (Chapman 1961). This species is considered in danger of extinction since logging for its timber continues to this day (Burrows & Burrows 1987).

Table 1.1. The Biology and distribution of the four species of *Widdringtonia*

Species	Regeneration Mode	Seed Morphology	Serotiny Level
W.cedarbergensis	Non-sprouter	Large, heavy	Nonserotinous
W.nodiflora	Resprouter	Light, winged	Highly serotinous
W.schwarzii	Non-sprouter	Light, winged	Mildy serotinous
W.whytei	Non-sprouter	Light, winged	Mildly serotinous

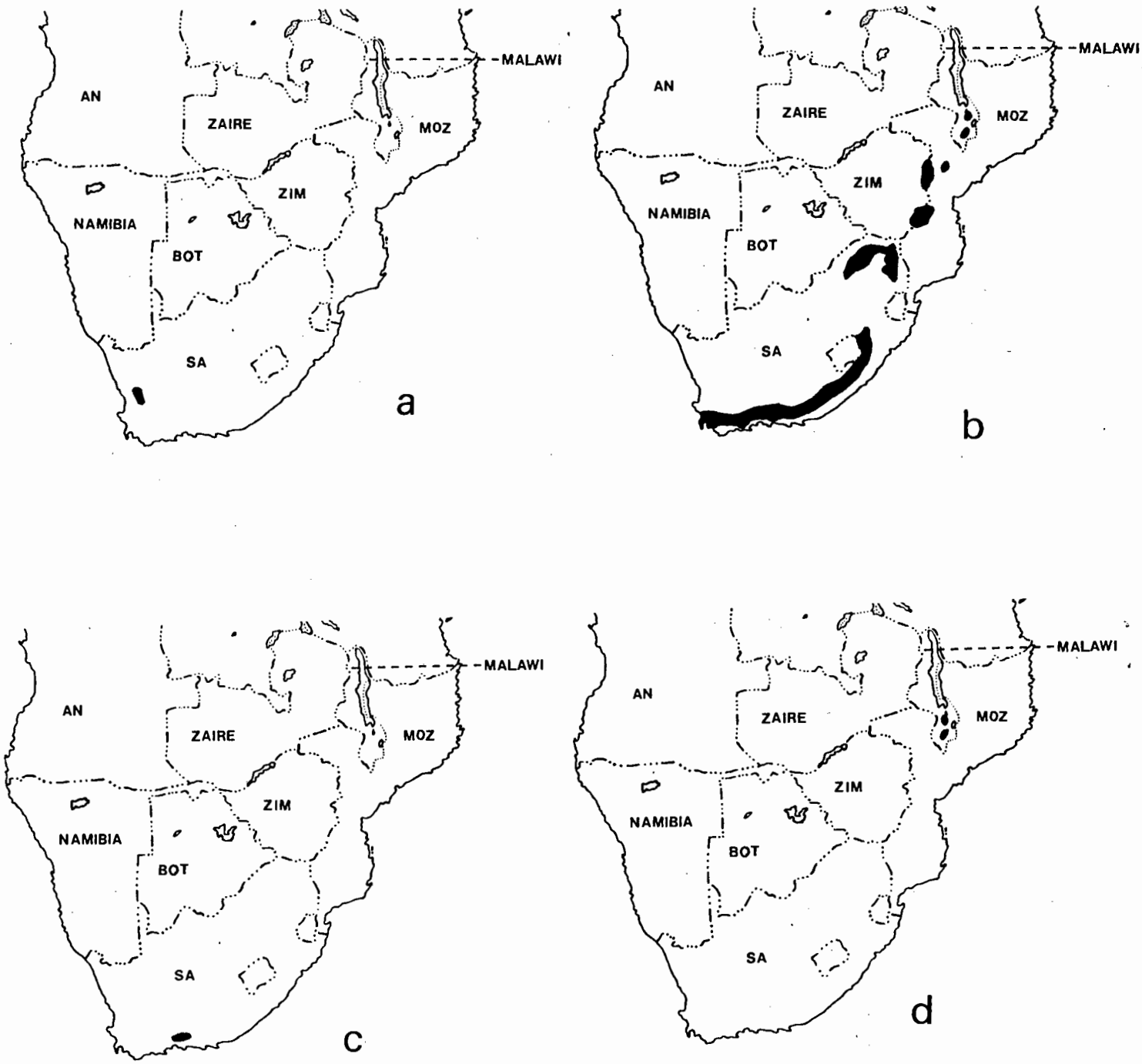


Figure 1.2. Distribution of (a) *W.cedarbergensis*, (b) *W.nodiflora*, (c) *W.schwarzii* and (d) *W.whytei*

Evolution within Widdringtonia and the effect of fire

An attempt to reconstruct the phylogeny of the genus was made by Maze (1993) through the use of molecular techniques. She initially chose a non-coding section of chloroplast DNA for its rapid rate of evolution relative to coding sections of chloroplast DNA. Chloroplast DNA is generally more conserved than is nuclear DNA, since inheritance of chloroplast DNA is uniparental. Maze found no variation between species using this section of chloroplast DNA which suggests the species have evolved recently. The earliest authentic fossil material of *Widdringtonia* is of the Tertiary age from within the present distribution range of *W.nodiflora* (Phillips 1927). The lignite material was identified as *W.nodiflora.*

Temperate floras, such as the Cape Flora (typified by fynbos), and grasslands were established in the Tertiary (Ingrouille 1992). One of the major characteristics of fynbos ecology is the dominant role played by fire. The oldest direct evidence for fire in the region is the Pliocene (Hendey 1983 in Deacon, Jury & Ellis 1992). With the advent of summer dry climates as well as fire stick farming, the frequency of fires is thought to have increased in the Pleistocene (Deacon, Jury & Ellis 1992). Selection for alliances of taxa that have resilience to disturbance by fire began in the Miocene before the Pliocene and intensified in the Pleistocene (Le Maitre & Midgley 1992).

The result of this selection has been to produce a "pyrophilic" vegetation dominated by plants with life strategies adapted to the fire regime. Several characteristics have evolved on more than one occasion in several taxa within the fynbos to enable species to shape their life histories around the effects of frequent fires. Two common characteristics are serotiny and the ability to coppice after fire. Serotiny leads to the accumulation of a seed bank in the canopy, protects seeds from both predispersal predators and fire, and times seed release into the post-fire environment (Le Maitre & Midgely 1992). This ensures that the seeds germinate in a favourable environment, that the resultant seedlings are not likely to be killed by fire and that long range dispersal is possible for some fynbos species (Bond 1988).

Serotiny is a fairly common characteristic in temperate conifers such as *Pinus*. It therefore comes as no surprise that serotiny has evolved in *Widdringtonia* although it is not an ancestral character since serotiny is absent in *W.cedarbergensis*. Serotiny has evolved to a mild degree in *W.schwarzii* and *W.whytei*, and is highly evolved in *W.nodiflora*. Not being serotinous at all, *W.cedarbergensis* releases its seeds into a pre-fire environment where the resultant seedlings are inevitably killed by fire unless they germinate in a protected site such as a rocky enclave.

The advantage of being able to coppice or resprout in a fire prone environment is that adult populations persist through many fires, having longer generation times and thus being less vulnerable to short fire cycles. Resprouters are known to dominate areas where fires are more frequent than elsewhere. In *Widdringtonia*, resprouting is only evident in *W.nodiflora*, the only species within the genus which can be considered a successful member of fynbos communities. Adult individuals of *W.cedarbergensis* are easily killed by fire and may, at most, only withstand cool autumn or winter burns. *W.schwarzii* is able to escape fires by virtue of its habitat - rocky cliff edges or ravines which are inaccessible to fire. Resprouting and serotiny, therefore, are probably derived characters in *Widdringtonia* which have evolved in response to fire.

The ecology of *Widdringtonia cedarbergensis*

Archival documents give plenty of evidence for the presence of large cedar forests in the last two centuries. In 1805 an agricultural commission of De Mist's mentions a cedar forest 24 miles long by 2 miles wide or six hours by half an hour on horseback (Smith 1955). The German traveller von Meyer also mentions how the Cedarberg was covered in the trees. Records of the extent of utilization also give a fair estimate of the extent of the cedar forests. In 1883 approximately 7250 pole stage trees were cut down for the construction of a telegraph line between Calvinia and Piketberg (Andrag 1977). Today it would be impossible to find more than a few hundred pole stage trees (Manders 1985). In the same year the Conservator of Forests wrote "The largest cedar still standing is about 18 ft in girth and about 70 ft in height but it is

a dwarf compared with the big trees whose stumps are still standing as evidence of what they were. These past giants must have been nearly double the girth of any now standing" (Hubbard 1937). Today the largest cedar still standing, which is a unique exception among the majority of trees which are more stunted, is less than 15 ft in girth and about 50 ft in height (pers. obs.).

A pollen core study conducted in the Cedarberg by Sugden and Meadows (1990) disputes the fact that the cedars ever formed a closed canopy forest in the last 4000 years. It is argued that at most, the cedars formed more of a woodland in the recent past. The same paper stresses the importance of climatic changes as the key causal factor controlling the decline of *Widdringtonia cedarbergensis*. It is clear that *W.cedarbergensis* has not evolved in the current climate but the conservation of this enigmatic species has been a priority for many scientists.

W.cedarbergensis is the single most researched species in the Cape fynbos (Richardson 1993). A series of permanent plots were established in the Cedarberg in the late 1960's by officers of the South African Forestry Research Institute, to monitor the apparent decline of the cedar populations and to serve as a basis for detailed demographic studies. Reports written in the 1970's gave preliminary accounts of the demography of the species and focused on the role of fire, reflecting the prevailing view that the key to managing cedar populations lay in understanding the species' response to fire. This view was strengthened after van Wilgen's (1980) study on the mortality of cedars after a large wildfire. Further work was clearly needed to determine how various elements of the fire regime (frequency, intensity and season) affected cedar populations, and whether mammals were contributing to the poor recruitment.

Andrag (1977) conducted a study to determine the status of *W.cedarbergensis*. He concluded that fires were occurring too seldom, when they did burn it was with a high intensity causing a high adult tree mortality. He also found that rodents, hyraxes and baboons were important predators of seeds and seedlings. Seed production may begin when the tree is about 12 years old. Production is minimal, however, and only after 40 years is it significant, with more than 30 clusters of cones per tree (Andrag 1977). Andrag (1977) also found that older trees had a higher survival rate than younger trees because the older trees are more closely associated with rocky

outcrops and are thus more readily protected from fire. Further, the accumulation of fuel around the younger age classes was higher, creating hotter and hence more destructive fires.

Manders (1985) integrated the patterns of mortality and fecundity obtained from the permanent plots in a demographic study to determine whether populations of *Widdringtonia cedarbergensis* are capable of recovery and which management plans serve the best interests of this species. Data from this study suggest that populations of the Clanwilliam cedar are declining today, whether or not they were more abundant in the past. High mortality has occurred in recent wild fires and in some areas there has been an almost complete lack of regeneration. The transition matrix model of Manders (1985) predicts that an interval of 15 to 20 years between fires is short enough to preempt the occurrence of extremely intense wild fires while providing sufficient time to allow the population to recover from the mortality incurred in the burn. Manders (1985) placed much emphasis on the importance of seeds in the growth of populations. However, in a more recent re-analysis of the transition matrix using an elasticity analysis, Privett (1994) found that reproductively mature trees contributed more to population growth than seeds or seedlings. Furthermore, Privett (1994) quantified mortality incurred during fires and incorporated it into the model. Manders (1985) had concentrated heavily on population growth between fires. In this way, it was possible to compare the effects of prescribed versus wild fires on population growth and thus develop a more rigorous fire management plan.

Conservation Genetics

Population genetics is a study of the behaviour of alleles and allele frequencies in space and time. Alleles are alternative forms of genes brought about through mutation (Oldfield 1989). Mutation is the ultimate source of genetic variation and therefore is crucial to evolution at the population scale (Oldfield 1989). Evolution at the scale of the population is called microevolution. Microevolution is brought about through changes in allele frequencies as a result of several processes. These changes can occur if one form is selected over another, or if there is a barrier to gene flow, preventing the spread of a new allele through the entire range of a

population, or if an allele is lost through stochastic processes operating in small populations (genetic drift).

Population genetics is a quantitatively based field which specifies which evolutionary events are possible, how fast genetic change can occur, what forces govern genetic variation, and which evolutionary factors account for various observations. Within the last two decades isozyme electrophoresis has been employed to characterise alleles phenotypically through separation on a starch, polyacrylamide or cellulose acetate gel. Enzymes are nuclear products which reflect the occurrence of mutations when separated electrically on a gel if the mutant isozyme has a different charge to the original isozyme. Population genetics has thus become an empirical study, like many other fields in biology. A wealth of isozymic data has been generated to date, answering many questions that have plagued biologists in the past. The effect of different breeding systems, life histories and biologies can now be linked to evolutionary processes at the population level. This technique has many applications for conservation biology.

Genetic management attempts to maximize the genetically effective population size and to avoid too much inbreeding (Foose 1987) and further to conserve as broad a cross section of the genome as possible. This task is made difficult by diminishing habitats in a developing world as well as by problematic species which are naturally rare. The persistence of threatened species depends on population size since the presence of a small number of individuals in a population through many generations, termed a bottleneck, leads to the depletion of genetic variation (Lande & Barrowclough 1987). Genetic drift and inbreeding are accentuated in small populations. Bottlenecks can be a single generation event during which the population is severely reduced in size (Frankel & Soule 1981). Theoretical (Nei et al. 1975; Lacy 1987) and empirical studies (Leberg 1992) have characterised bottlenecks as having the following effect on genetic diversity:

(1) The loss of heterozygosity is not severe since even two individuals retain 75% of the populations genetic variance.
(2) Loss of alleles becomes a problem, especially if these alleles are important for survival eg. disease resistance.

(3) The total amount of genetic variation lost depends on how long the bottleneck pressures persist.

Billington's (1991) study of *Haplocarpus bidwillii*, a New Zealand podocarp represents a good case study in plant conservation genetics. *Haplocarpus* suffers from a similar problem to that of *Widdringtonia cedarbergensis* where the population has become fragmented after the arrival of Maori and European settlers in New Zealand (between 1200 and 200 years ago). Billington (1991) does suggest, however, that *H.bidwillii* has been fragmented for the last 8000 years though for reasons which she does not explain. For twenty loci, nine were found to be polymorphic. She found that even the most variable populations of *H.bidwillii* showed low levels of genetic variation compared with values reported for northern hemisphere conifers. A strong correlation between genetic variation and population size was found and the three measures of variation used in the study, expected heterozygosity, percentage polymorphic loci and mean number of alleles per locus were found to decline sharply below population sizes of 8000 individuals.

In South Africa, population and conservation genetics are young fields (Grant 1993) and the only example of a plant genetic conservation study in South Africa is given by Dyer and Richardson's (1992) study of the invasive *Hakea sericea*. This thesis is important, therefore, in stimulating interest in the field of conservation genetics with a flagship species such as the Clanwilliam cedar. The problems faced by the Clanwilliam cedar and the active management plan designed to curb these problems both lend themselves to an elegant project. Population sizes in *W.cedarbergensis* are dwindling and subpopulations are becoming increasingly fragmented from each other. By determining the levels of genetic interaction between subpopulations one may be able to predict the effect of further fragmentation caused by fire on the future conservation status of *W.cedarbergensis*. Further, by studying genetic and ecological processes within populations, one may be able to predict the effect of population size on levels of genetic variation. At the same time, the Middelberg plantation seed source for the replanting programme at Welbedacht could be assessed and alternative sources located.

CHAPTER 2. FIELD SAMPLING AND ELECTROPHORETIC PROCEDURES

Introduction

The development of molecular techniques have made the empirical study of population genetics possible in the last three decades. In South Africa, plant biologists have shied away from these advances with the result that technical expertise has not kept apace with this fast-moving field. Genetic aspects of conservation have been vastly neglected as a result. An important motivation for this thesis was to develop a laboratory for enzyme electrophoresis that could be used for a broad spectrum of studies and encourage other workers to use this technique should the need arise.

In this chapter I explain why enzyme electrophoresis was chosen over molecular DNA in studying *Widdringtonia*. The materials and methods are described including a description of the sampling strategies employed.

Characters used to study variation

Three types of characters have been used to estimate levels of genetic variation within species: morphological, allozymes and lastly, DNA sequences (Schaal *et al.* 1991). Each type of character has advantages and disadvantages. It is important to use the right type of character for the question being asked. Morphological assessment of genetic variation is convenient when a rapid estimation of variability is needed or where biochemical surveys are impractical (Schaal *et al.* 1991). Analysing allozyme variation has a broad spectrum of uses, namely, studies of gene flow, studies of breeding systems, and correlations of genetic diversity with various life history characteristics. The disadvantage with this technique is that it samples a small section of the genome, the genes encoding enzymes. These are often selected on the basis of the ease of their products' extraction and ability to migrate through a starch gel (Schaal *et al.* 1991). Nevertheless, allozyme variation often provides the best measure of genetic variation (Schaal *et al.* 1991). Sufficient variablity for population-level

studies in nuclear microsatellites has been found (Mitton 1994) although availability of primers is problematic. Hamrick (1989) has shown that allozyme variation reflects variation of DNA and morphometric traits in organisms. Further, isozymes generally exhibit Mendelian inheritance, codominant expression and the absence of pleiotropic and epistatic interactions (Weeden & Wendel 1989). The advantage of assessing genetic variation through molecular DNA is that phylogenies are relatively easily resolved through this technique since DNA contains historical information (Schaal *et al.* 1991). The disadvantages of using this technique are two-fold: firstly, the technique is expensive and complicated; secondly, it is difficult to study variation within species since it is difficult to find a section of the genome which has adequate levels of nucleotide substitutions. More simply put, it is difficult to find a section of DNA which is evolving rapidly enough to show differences between populations within a species.

The problems addressed in this study involve a population scale study of *W.cedarbergensis* in relation to its congeners. For this reason, we decided that an allozyme study would be the most appropriate means of answering our questions.

What is an isozyme?

The study of genetic variation is made possible largely through the aid of electrophoresis. Electrophoresis is the movement of enzymes through a gel, usually starch, polyacrylamide or cellulose acetate, under the influence of an electric current (Conkle *et al.* 1982). Samples of plant tissue are placed in the gel for a few hours, a voltage applied across the gel for a few hours to separate molecules based on charge and shape, and the gel is removed and stained for specific proteins. Proteins show up as bands on a gel and variants with different mobilities through the gel are easily visualised (Lacy 1992). The different bands that stain up on a gel, therefore, denote functionally related molecules that differ in electric charge. These related molecules are called isozymes. Isozymes tested by determining Mendelian segregation ratios and found to be phenotypic expressions of alleles of a single genetic locus are allozymes (Conkle et al. 1982) Allozymes, therefore, have a simple genetic basis. They are the biochemical consequence of the substitution, deletion, or addition of amino acids in the polypeptides which comprise the enzyme, and they can be distinguished if these changes affect their electrophoretic migration (Gottlieb 1977). Since the amino acid sequence of a

polypeptide is colinear to the nucleotide sequence of its coding structural gene locus, allozymes result from gene mutation (Gottlieb 1977). Changes in amino acid composition will often alter the charge or, less often, the conformation of the enzyme, thereby producing a change in electrophoretic mobility (Weeden & Wendel 1989).

Chapter layout

Isozyme studies are often complicated by factors such as polyploidy. Polyploidy in *Widdringtonia* was therefore explored through chromosome counts in the first phase of the project. The next phase of the chapter describes the pilot study of electrophoresis in *Widdringtonia* which was conducted in order to determine whether the whole project was a worthwhile excercise. Phase three describes the how the genomes of the three species of *Widdringtonia* were sampled once it was decided to proceed with an extensive population genetic study. Lastly, phase four describes the electrophoretic procedures for the bulk of the study and the results are presented.

Phase 1: Determining levels of polyploidy in *Widdringtonia*

Several factors may hamper the interpretation of electrophoretic banding patterns. Enzymes often undergo changes induced by other loci after they have been synthesized (Weeden & Wendel 1989). When this is the case, banding patterns can become difficult to interpret, since the mobilities of the enzymes are affected. In this study, it is assumed that all isozymes have a genetic base.

Alleles that are no longer transcribed or that code for defective polypeptides lacking enzymatic activity are generally referred to as "null alleles" (Weeden & Wendel 1989). The presence of null alleles usually indicates recessive syndromes (Weeden and Wendel 1989) and is another factor to consider in gel interpretation.

Polyploid species can also complicate banding interpretation and it is often important to determine beforehand whether the species shows signs of polyploidy. Polyploids in conifers seem to be rare. One example of a conifer polyploid, however, is *Juniperus chinenesis* where

n=22 instead of n=11 as for the rest of the genus (Vidakovic 1991). It was therefore expected that *Widdringtonia* would not have any polyploid species, with the possible exception of *W.nodiflora*, the most morphologically variable of the three species.

Methods

Germlings of *Widdringtonia cedarbergensis*, *W.nodiflora* and *W.schwarzii* were dissected out and fixed in Carnoy's fixative (Chamberlain 1924) which consists of six parts 100% ethanol: three parts chloroform: one part glacial acetic acid. The material was fixed for 24 hours. The root tips were then placed in 70% ethanol for storage. The root tips were then dissected out and macerated on a slide in a drop of acetic carmine (Gurr 1973). This was then covered with a cover slip and the slide and cover slip squashed between two pieces of filter paper. The slide was then surveyed under a Zeiss Axioskop microscope for any cells in the active process of mitosis and the relevant photographs taken using bright field optics.

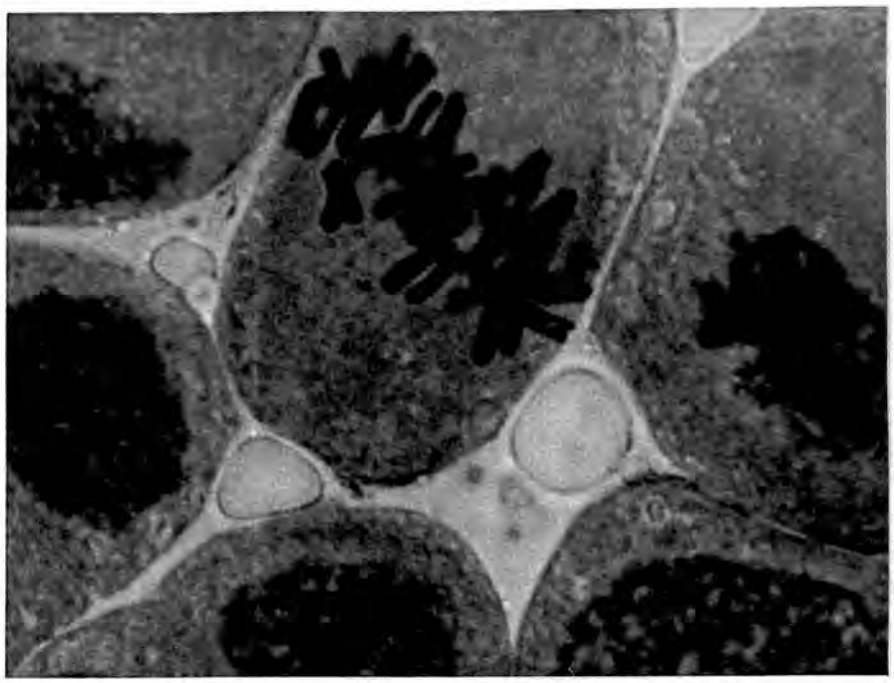


Fig. 2.1. Squashed root tip cells of *W.cedarbergensis* showing chromosomes undergoing mitosis (photo: P.Linder).

Results

A few cells of the root tips appeared to be in an active phase of mitosis (Fig.2.1). There appeared to be no polyploids within the three species of *Widdringtonia*. There are 12 pairs of chromosomes (Fig. 2.1) for all three species. This corresponds with chromosome counts obtained for many other conifer species. In *Abies* (Pinaceae), n=12; in *Juniperus* (Cuppressaceae), n=11; in *Cryptomeria* (Taxodiaceae), n=11 and in *Cephalotaxus* (Cephalotaxaceae), n=12 (Vidakovic 1991).

Phase 2: Pilot study

Before conducting a full scale population genetic study of *Widdringtonia*, it was necessary to be able to isolate sufficient enzyme systems. Techniques were developed on a population of *W.nodiflora* at Orangekloof on Table Mountain.

Materials and methods

Leaves and cones were sampled from 30 trees at regular intervals while walking in a straight line through the population. The samples were temporarily kept cool on crushed ice in an insulated polystyrene box. The cones were separated from the leaves at the lab and the latter placed in a -2°C freezer until the morning. The leaves were crushed in a chilled mortar and pestle using a Tris-maleate buffer. The extract was absorbed onto filter paper wicks (3mm x 7mm) and the wicks inserted into a gel which was 5mm thick. The gels were run in a 4°C room at 200V. The buffers used were Tris-EDTA-Borate pH 8.0 (Gottlieb 1981) and morpholine-citrate Ph 6.1 (Cheliak & Pitel 1987) for both gel and tray buffers. The gels were left to run for 5 hours after which they were sliced 1mm thick and placed in stains. It was usually possible to successfully obtain 3 slices from a gel. The enzyme systems stained for (limited by available chemicals) were as follows: Mdh, Est, Aph, G6pdh, Idh, Me, Per, Sdh. All eight enzyme systems were ran on both morpholine-citrate and Tris-EDTA-Borate gel systems.

The results of the pilot study were reasonable, especially for peroxidase which was highly resolved. Mdh, Aph and Sdh were resolved on morpholine citrate gels and Aph, Idh, Per, Est and Me were resolved on Tris-EDTA-Borate gels. Barring G6pdh, all of the enzyme systems stained gave a successful result. On the strength of this success, it was decided to sample further populations.

Phase 3: Sampling further populations

The Centre for Plant Conservation (CPC) in the United States has a set of guidelines for sampling species for *ex situ* storage. The guidelines are based on decisions centred around how to obtain samples that represent a good cross section of the genome under consideration. The basic questions involved are which species to sample, how many populations to sample within the species, how many individuals to sample within the populations, and how many propagules to sample from each individual (from Guerrant 1992). The CPC recommends sampling from one to five populations within a species. This decision is based on findings from Brown and Briggs (1991). They found that 80% of endangered species surveyed in southwestern Australia are found in five or fewer populations. Further, work by Hamrick and others (Hamrick & Godt 1990) reviewing many published studies of electrophoretic data, showed that 78% of genetic variation is found within populations. This means that most of the genome is represented within one population. The CPC recommends sampling ten to fifty individuals per population. The reason for this is that the allelic content of a sample is proportional to the logarithm of both population size and sample size. This means that the larger the sample size, the smaller the chance of finding rare alleles (Brown & Briggs 1991).

Between February 1992 and March 1993 the populations listed below in Table 2.1 were visited and sampled for leaves and cones. Seven populations were sampled for *W.cedarbergensis* and *W.nodiflora* alike and three populations were sampled for *W.schwarzii*. More populations were sampled for *W.cedarbergensis* and *W.nodiflora* since populations of *W.scharzii* were inaccessble and, further, it was intended that *W.cedarbergensis* be examined for the relationship between population size, genetic variation and fitness. Populations of *W.cedarbergensis* were sampled from the Cedarberg mountains to which the species is

restricted. The entire distribution of *W.cedarbergensis* was well represented from Krakadouw in the north to Welbedacht in the south; the Sneeuberg in the west and Crystal Pools in the east. Populations of *W.nodiflora* were sampled from Hottentot's Holland (SBR and BBAY), the Cape Peninsula (OK and KB), the Witzenberg mountains (BKV and BKR) and the Drakensberg (CPK). The entire distribution of *W.nodiflora* is not represented in this study since populations from Malawi were not sampled. However, an attempt was made to sample more than one population within each area so that patterns of gene flow between nearby populations as well as between mountain ranges could be detected. Populations of *W.schwarzii* were sampled from the Baviaanskloof and Kouga mountains in the eastern Cape to which the species is restricted.

Within these populations, up to thirty trees were sampled, falling within the CPC's recommended limit. In extreme cases such as Doringkloof in *W.schwarzii*, where climbing gear had to be used to reach any of the trees, not more than twenty trees were sampled. This still falls within the recommended limit. Care was taken to sample trees at regular intervals along a crude transect.

Table 2.1. Populations sampled in three species of *Widdringtonia*, their coordinates and the abbreviation codes used in the remainder of this thesis.

Species	Subpopulation	Coordinates	Code
W.cedarbergensis	Sneeuberg/Hoogvertoon	32°28'20"S,19°09'50"E	SB
	Duiwelsgat	32°32'20"S,19°04'00"E	DG
	Welbedacht	32°25'09"S,19°10'05"E	WB
	Crystal Pools	32°20'03"S,19°08'00"E	CPS
	Middelberg	32°21'55"S,19°03'55"E	MB
	Krakadouw/Heuningvlei	32°13'00"S,19°04'54"E	KD
	Krakadouw kloof	32°13'20"S,19°04'00"E	KK
W.schwarzii	Sandvlakte	33°34'50"S,24°09'52"E	SV
	Doringkloof	33°37'00"S,24°03'00"E	DK
	Nuwekloof	33°30'50"S,23°39'00"E	NK
W.nodiflora	Orangekloof	33°59'58"S,18°23'30"E	OK
	Kirstenbosch	33°59'30"S,18°25'30"E	KB
	Bainskloof road	33°36'50"S,19°06'20"E	BKR
	Bainskloof valley	33°37'10"S,19°08'25"E	BKV
	Steenbras River	34°11'40"S,18°49'20"E	SBR
	Betty's Bay	34°21'03"S,18°58'10"E	BBAY
	Cathedral Peak	28°57'55"S,29°12'10"E	CPK

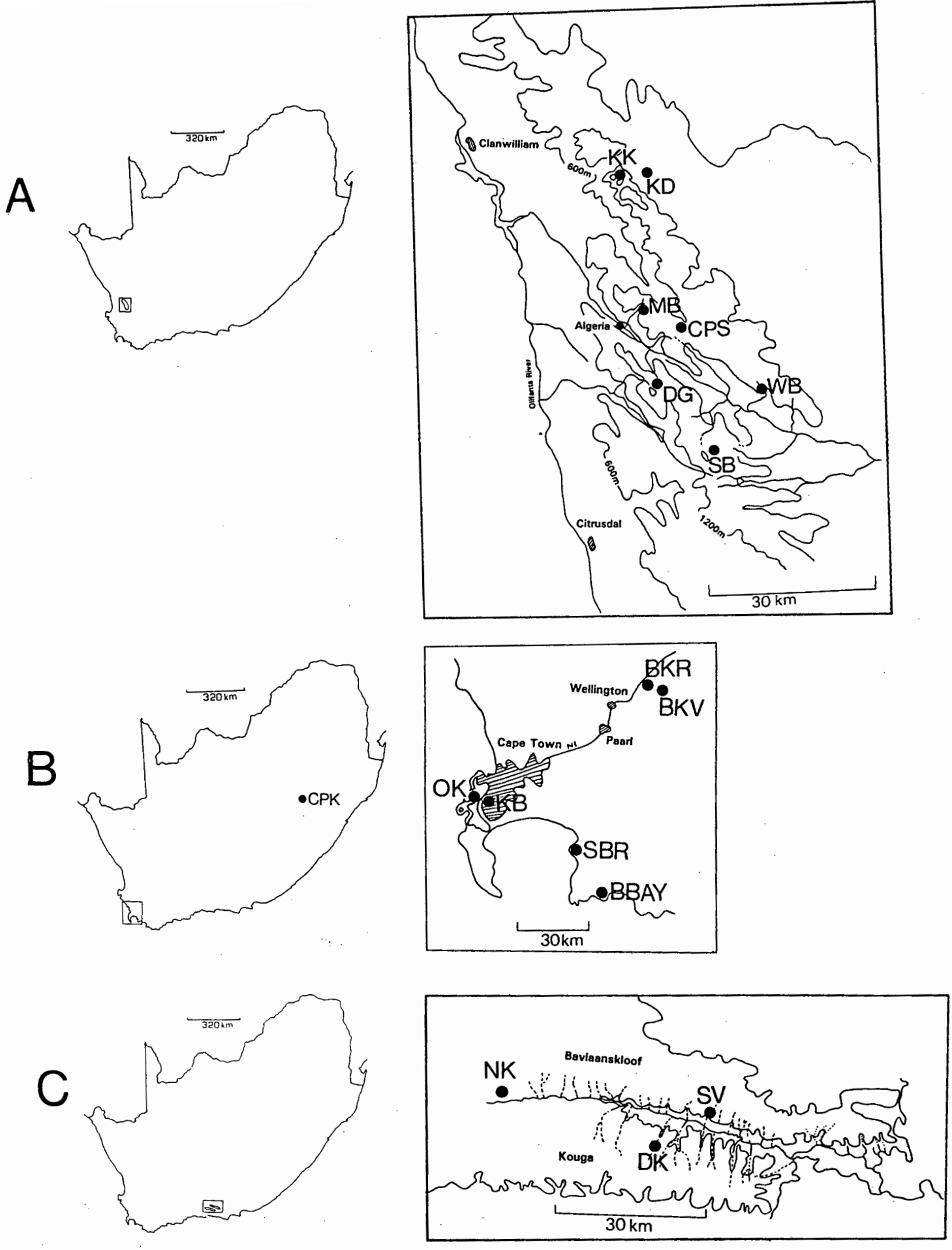


Figure 2.2. Maps showing populations sampled in (a) *W.cedarbergensis*, (b) *W.nodiflora* and (c) *W.schwarzii*

The leaves were kept as cool as possible. Field trips, especially to the Cedarberg, the Baviaanskloof and the Drakensberg were usually longer than one week which made it difficult to keep liquid nitrogen or dry ice in order to keep the leaf samples frozen. Most of the field trips were conducted in the summer with temperatures often over 30°C. In the best facilities it was only possible to keep the samples cool but not frozen in a weak gas or paraffin ice compartment.

Phase 4: Electrophoretic procedure

Leaves and cones were brought back to the lab and leaves frozen immediately at 0^0C. The electrophoretic procedure was carried out as outlined above for the pilot study. The results were poor, however, and it was clear that fresh material was needed in order to obtain satisfactory results. The effects of high temperature were obviously too great for the enzymes to withstand. After consistently bad results after two field trips, it was decided to abandon any further attempts at assaying leaf material and to attempt germling material instead.

Cones from each tree had been collected as a backup and for seedling fitness trials in the final stages of the project. Seeds from each tree from each population were germinated in germination chambers with temperatures alternating between 10°C and 20°C on a 12 hour cycle.

Materials and methods

The germlings were allowed to develop until the secondary shoot bud was detectable. The seedlings were then crushed in a chilled mortar and pestle using a modification of vegetative extraction buffer I from Cheliak and Pitel (1984) outlined below:

8%	PVP (40M)
0.5mM	EDTA
1mM	Dithiothreitol
1mM	Ascorbic acid
0.1%	Bovine Serum Albumin
0.4mM	NAD
0.3mM	NADP
0.2mM	Pyridoxal-5-phosphate

This solution was adjusted to Ph 6.7 with 1M Tris and 0.66ml β-mercaptoethanol added for 100ml of buffer.

Approximately 10 to 14 drops were used per seedling, according on the size of the seedling. Five filter paper wicks, which were cut to the size of 3mm x 12mm from no. 4 Whatmann filter paper prior to extraction, were saturated in the extract and placed in an Eppindorff tube which was directly transferred to a -20°C freezer.

The isozymes were separated on 12% starch gels which were 10mm thick using the buffer systems outlined in Conkle *et al.* (1982) (denoted by *) and Gottlieb (1981) (denoted by $^{+}$) in Table 2.2.

Table 2.2. Gel and electrode buffer formulations used to separate isozymes of Widdringtonia

	Gel Buffer	Electrode Buffer
*	Tris citrate Ph 8.3	Lithium-borate Ph 8.3
*	Tris citrate Ph 8.8	Sodium-borate Ph 8.0
*	Tris citrate Ph 6.3	Tris-citrate Ph 6.3
+	Tris-EDTA-Borate Ph 8.0	Tris-EDTA-Borate Ph 8.0

The gels were left to run for 5 hours and then sliced horizontally 2mm thick. The following enzyme systems were stained using staining procedures outlined in Conkle *et al.* (1982): malate dehydrogenase (Mdh), shikimic acid dehydrogenase (Sdh), isocitrate dehydrogenase (Idh), glucose-6-phosphate dehydrogenase (G6pdh), glutamate dehydrogenase (Gdh), superoxide dismutase (Sod), diaphorase (Dia), aspartate aminotransferase (Aat), acid phosphatase (Aph), menadione reductase (Mnr), peroxidase (Per), malic enzyme (Me), phosphoglucose isomerase (Pgi), phosphoglucomutase (Pgm), leucine amino peptidase (Lap), α-esterase (α-Est), β-esterase (β-Est), fluorescent esterase (Flest) and aconitase (Acon).

The gels were interpreted and scored. Loci were labelled in ascending order from 1 to 3 from fastest (migrating furthest in the gel towards the cathodal end) to slowest (migrating least in the gel towards the anodal end). Alleles were labelled from A to Z in ascending order from fastest to slowest.

Results

The following loci were resolved adequately enough to be interpreted for all three species: Mdh-1, Mdh-2, Sdh-1, Sdh-2, Idh-1, Aph-1, Mnr-1, Mnr-2, Pgi-1, Pgi-2, Lap-1, Est-1, Est-2, Me-1 and Gdh-1. An additional two loci were resolved for *W.cedarbergensis*, Aat-1 and Aat-2. These loci were not adequately resolved for *W.nodiflora* and *W.schwarzii*. However, G6pdh-1 and G6pdh-2 were resolved for the latter two species.

Gel interpretation

Figure 2.3 shows examples of the results obtained for electrophoresis of *Widdringtonia* isozymes.

Sdh - Both Sdh-1 and Sdh-2 were found to be a polymorphic monomer in *W.nodiflora* (Fig. 2.3(a)(i)). Only Sdh-2 was found to be polymorphic in *W.cedarbergensis* (Fig. 2.3(a)(ii)). Both Sdh-1 and Sdh-2 were monomorphic for *W.schwarzii*.

Mdh - Mdh was interpreted as having 2 superimposed loci in *W.nodiflora* (Fig. 2.3(b)). The one locus, Mdh-1 is polymorphic while the other is monomorphic. This interpretation is supported by the fact that these two loci are monomorphic in *W.cedarbergensis* and *W.schwarzii* and show up two clear non-variable zones of enzymatic activity. The fastest locus was not included in the interpretation since, although it appears to be polymorphic, it is poorly resolved. Mdh-1 in *W.nodiflora* stains as a monomer (Figure 2.3(b)) although it is meant to be a dimer (Weeden & Wendel 1989). It is possible that the subunit structure of the missing middle band renders the isozyme inactive. The other possibility is that the fastest or the slowest isozyme are inactive. This is unlikely, however, since homozygotes would not stain at all, increasing the chances of finding lanes empty. However, this was difficult to determine with a superimposed locus.

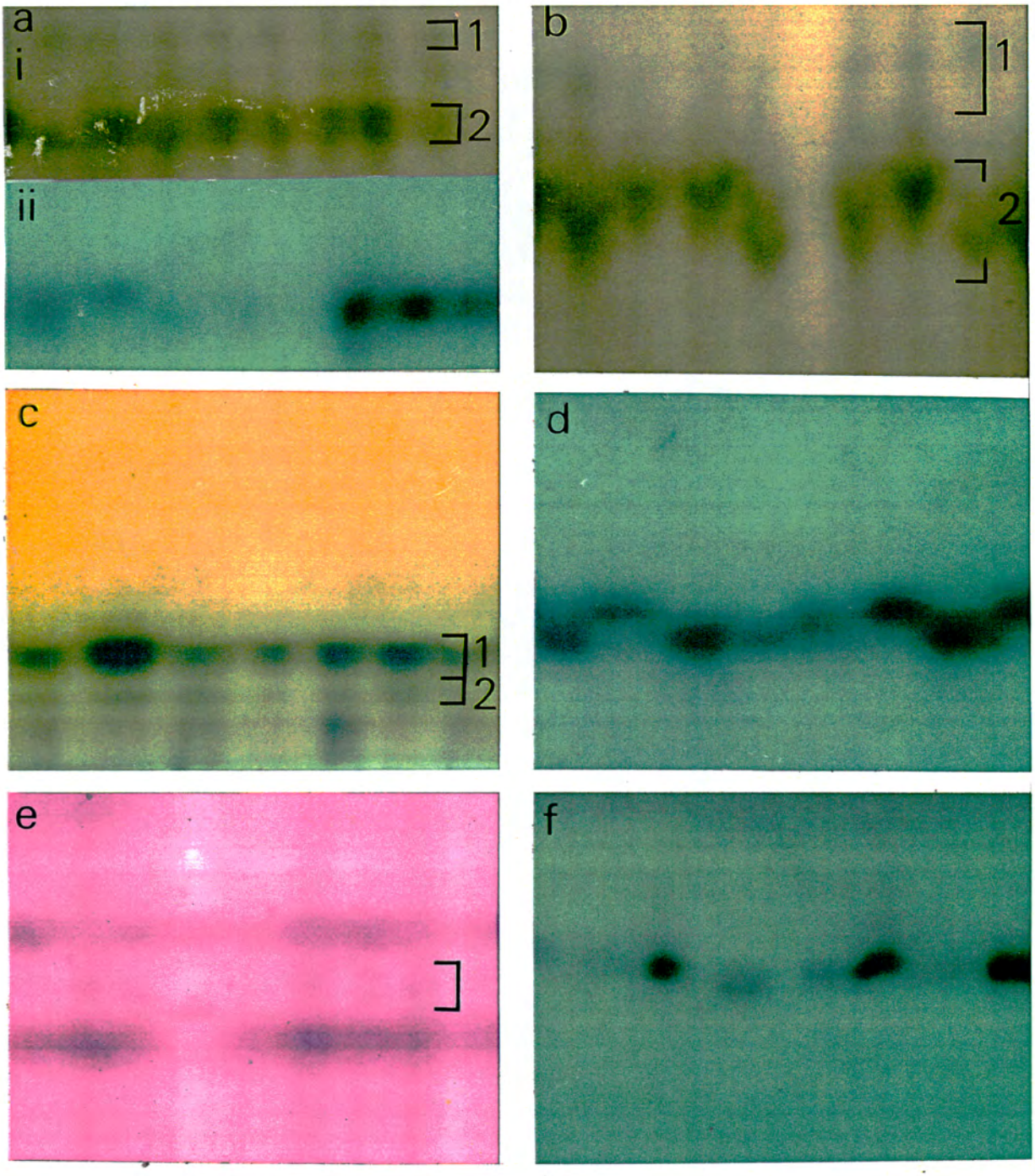


Figure 2.3.(a)(i)Sdh-1 and Sdh-2 in BKV (ii) Sdh-2 in WB and CPS; (b) Mdh-1 and Mdh-2 in OK and KB; (c) Aph-1 and Aph-2 in *W.cedarbergensis*; (d) Pgi-2 in WB; (e) Lap in SBR; (f) Idh in SB.

Pgi - Two loci were resolved for Pgi, only one of which (Pgi-2) was found to be polymorphic monomer for *W.cedarbergensis* (Fig. 2.3(d)) and *W.schwarzii*, and both of which (Pgi-1 and Pgi-2) were found to be polymorphic for *W.nodiflora*.

Lap - Only one locus, which was a polymorphic monomer, was resolved for all three species. The bands were highly resolved although faint in the picture (Fig. 2.3(e)).

Idh - One locus, which was polymorphic, was resolved for Idh. This locus was found to be variable for all three species. Separation of the isozymes was poor (Fig. 2.3(f)), but banding was consistently clear for all gels which made it possible to interpret the patterns observed. The interpretation was compared with results obtained for the same locus separated on a different buffer system. Separation on Tris-citrate Ph 6.3 was good, distinguishing Idh as a dimer in *Widdringtonia*, but not as consistent as the results obtained on Tris-EDTA-borate. The two interpretations were found to be highly similar.

Aat - Two loci were resolved for *W.cedarbergensis* only, one of which was polymorphic (Aat-1). This locus was fairly well resolved as a monomer.

The remainder of the loci resolved were all found to be monomorphic for the three species. Aph-1 and Aph-2 are examples of such loci (Fig. 2.3(c)).

Conclusions

The electrophoretic study of *Widdringtonia* proved successful. There were many technical setbacks during the course of the study, however. Much of this involved becoming familiar with the technique of starch gel electrophoresis and its idiosyncrasies. Another major setback involved having to re-run much of the work since midway through the study it was decided to use germling tissue instead of leaf tissue. Interpretation of the results was not complicated by the effects of polyploidy since a chromosome count beforehand showed no signs of multiple sets of chromosomes. Sampling the genome was problematic only in that populations of *W.schwarzii* were difficult to sample owing ot heir inaccessibility. Further, the entire distribution of *W.nodiflora* was not adequately represented as was intended at the outset. It would have also been preferable to have included *W.whytei* from Malawi in the study since its systematic distinction from *W.nodiflora* is a subject of contention (Pauw 1992). The scope of the project and time constraints, however, did not allow for the inclusion of *W.whytei*.

CHAPTER 3. THE POPULATION GENETICS OF WIDDRINGTONIA CEDARBERGENSIS, W.NODIFLORA & W.SCHWARZII

Introduction

Estimating levels and patterns of genetic diversity within species has become an important aspect of conservation biology. As species become increasingly fragmented, [1]subpopulations become smaller and more isolated and as a result, begin to face demographic and genetic risks (Lacy 1992). *Widdringtonia cedarbergensis*, a member of the Cuppressaceae, is a probable example of a recently fragmented species in southern Africa. There is sufficient documented evidence dating from as far back as the beginning of the 19th century to suggest that *W.cedarbergensis* once had a much more continuous distribution than it has today (see Chapter One). Although the pressures of human exploitation may have been lifted, *W.cedarbergensis* is still under the threat of wild fires to which it is maladapted, unlike the fynbos communities in which it is found.

Aims

The aim of this chapter is to describe genetic variation in *W.cedarbergensis*. In addition, I tested whether the reduction in numbers in *W.cedarbergensis* might have caused a genetic bottleneck by comparing levels of genetic diversity with two congeneric species, *W.nodiflora* and *W.schwarzii*. A third aim was to determine the genetic affinities of the plantation at Middelberg (MB) and Krakadouw (KD), since these plantations have been used as a seed source for a replanting programme.

[1]Subpopulations are referred to as populations relative to the metapopulation in the remainder of this chapter.

The basic premise that a high level of genetic variation can provide insurance against extinction, while a low level may reflect population bottlenecks or indicate an inbred population with greater risk of extinction is followed (Waller et al. 1987). However, *W.cedarbergensis*, *W.nodiflora* and *W.schwarzii*, differ in their biologies, distributions and ecological success. These differences are outlined in Table 1.1, Chapter One. Since each species is different, we expected different population genetic structures and hypotheses were set up accordingly, independent of the effects of population reduction and fragmentation.

Hypotheses

W.cedarbergensis:- Given the fact that this species is a wind-pollinated conifer and has few barriers to gene flow, levels of population differentiation should be minimal, influenced only by the fact that *W.cedarbergensis* is the only species of the three that has very poorly dispersed heavy seeds. *W.cedarbergensis* is a geographically restricted species and therefore relatively low levels of polymorphism are expected. A general tendency has been shown for widespread species to have a higher degree of polymorphism than narrowly restricted species (Karron et al. 1988), although other ecological factors may confound this trend (Loveless & Hamrick 1984).

W.nodiflora:- Since this species is also wind-pollinated and has light, winged seeds, we expect gene flow between populations to be efficient. However, *W.nodiflora* is distributed along most mountain ranges up the east coast and central Africa as far as Malawi. Distances between these mountain ranges present a barrier to gene flow, therefore I expected patterns of population differentiation to be influenced by these distances. Since it is a widespread species and displays a high degree of morphological variation (Pauw 1992), I expected high levels of genetic differentiation in *W.nodiflora*. *W.nodiflora* is a resprouter and therefore adults persist for many generations. This should lead to an accumulation of heterozygotes within population. Resprouting should affect population differentiation in that the establishment of individuals and populations would be a rare event since the probability of colonising seedlings being eliminated by fire are high.

W.schwarzii:- *W.schwarzii* is wind-pollinated with light, winged seeds yet it is confined to the deep narrow kloofs of the Baviaanskloof and Kouga mountains. I therefore expected

relatively high levels of population differentiation since populations are discrete and widely separated by intervening ridges. *W.schwarzii* is also a restricted species and I therefore expected relatively low levels of genetic differentiation.

In effect, *W.cedarbergensis* and *W.schwarzii* should show similar levels of genetic diversity, notwithstanding the effects recent fragmentation, since both have the same limited distribution and similar biologies barring the fact that *W.schwarzii* has lighter seeds with wings. The above hypotheses serve to highlight deviations from expectations. If *W.cedarbergensis* is experiencing a bottleneck effect, then levels of genetic diversity should be lower than those of the other two species.

Methods

To determine levels of genetic diversity in each species of *Widdringtonia*, starch gel electrophoresis was employed. The methods used for this technique as well as the sampling procedure, were outlined in Chapter Two.

Data analysis

For each population for which allozyme data were collected, genotype arrays were analysed using the Biosys-1 package (Swofford & Selander 1989). Allele frequencies were calculated and used in conjunction with the genotype data to calculate a number of mean genetic diversity estimates including: number of alleles per locus (A), % of loci polymorphic (P), mean proportion observed heterozygosity (H_o) and mean proportion expected panmictic heterozygosity (H_e). Levels of inbreeding within populations were estimated through comparing H_o and H_e. H_e is an expected genotypic frequency calculated by using a binomial expansion of the allele frequencies. This binomial expansion is called the Hardy-Weinberg principle (Nei 1987). If observed levels of heterozygosity conform with expected levels of heterozygosity, the population is said to be in Hardy-Weinberg equilibrium. The inbreeding coefficient, F, lends itself to direct comparison of levels of inbreeding between subpopulations. F is defined as the probability that two alleles at a genetic locus in the inbred individual descended from a single gene in a single ancestor shared by the parents (Wright 1969 in Lacy 1992).

The levels and distribution of genetic diversity were calculated using Wright's fixation indices (Wright 1965). These statistics were used to describe three levels of genetic interaction. The basic formula used in Biosys-1 is: $1 - F_{it} = (1 - F_{is})(1 - F_{st})$.

F_{is} represents the level of genetic interaction between individuals within the same deme or cluster. A positive F_{is} is associated with deficiencies of heterozygotes and suggests inbreeding while a negative F_{is} suggests too many heterozygotes relative to Hardy-Weinberg equilibrium (Linhart et al. 1981). F_{st} represents the correlation between random gametes within a given deme relative to gametes within the whole population. This value is used to determine the amount of differentiation between subpopulation. F_{it} represents the correlation between uniting gametes, and therefore the fixation index, within the whole population.

Genetic distance

Nei's (1978) genetic distance (D) is used to describe the extent of genetic relationships between populations of each species. Pairwise comparisons were made and the distance values (D) are presented in a matrix.

Cluster analysis

The Biosys-1 cluster analysis used in this study uses the UPGMA (unweighted pair-group method with arithmetic averaging). This algorithm is described in Sneath and Sokal (1973). The end result was a phenogram showing patterns of genetic relatedness between the different populations within each of the three species.

Results

Gene frequencies

Allele frequencies for 19 enzyme loci were calculated. Five of these loci were found to be polymorphic in *W.cedarbergensis* (Table 3.1a), seven were found to be polymorphic for *W.nodiflora* (Table 3.1b) and four were found to be polymorphic for *W.schwarzii* (Table 3.1c) (see Chapter Two, Table 2.1 for population abbreviations). Allele frequencies among populations of *W.schwarzii* were substantially more uniform than among populations of

W.cedarbergensis and *W.nodiflora*. Major allelic differences between populations of *W.cedarbergensis* included (i) an extra slow allele at the Lap-2 locus in SB and (ii) monomorphism at Idh-1 and Aat-2 loci in KK (Table 3.1a). Allelic differences between populations of *W.nodiflora* included (i) the presence of a unique fast A-allele at Sdh-2 in CPK, (ii) the presence of a unique C-allele at Sdh-1 in SBR, and (iii) the presence of a unique D-allele at Mdh-3 in BKR. Many absences of alleles were found among populations of *W.nodiflora*. Extreme cases were BBAY which had 16 missing alleles in total and CPK which had 14 missing alleles (Table 3.1b). In *W.schwarzii*, only SV was unique with an absent C-allele at Pgi-2 (Table 3.1c).

Estimates of genetic diversity

The number of alleles per locus (A) were calculated for each population within each species (Table 3.2). This calculation included monomorphic loci since the extreme cases where all loci were monomorphic would have A = 1. The mean number of alleles per locus averaged over all populations within each species showed populations of *W.nodiflora* to have a much higher allelic diversity than populations of *W.cedarbergensis* and *W.schwarzii*. *W.schwarzii*, in particular, had the lowest level of allelic variation at each locus. Populations of *W.nodiflora* vary greatly with respect to A. BBAY had the least allelic diversity and BKR had the highest levels of allelic diversity, whereas populations of *W.cedarbergensis* were mostly uniform with respect to A except for the small isolated population, KK, which showed low levels of allelic diversity. One population of *W.schwarzii*, DK, had a higher allelic diversity than the other two. Compared with other studies of gymnosperms, all three species of *Widdringtonia* had low values for A (A = 1.93 for gymnosperms in Hamrick & Godt 1990).

The proportion of polymorphic loci (P) were calculated for each population within each species (Table 3.2). Again, populations of *W.nodiflora* showed a higher percentage of polymorphic loci than *W.cedarbergensis* and *W.schwarzii*. Populations of *W.schwarzii* had the lowest levels of variation. Polymorphism varied greatly between populations of *W.nodiflora* whereas populations of *W.cedarbergensis* and *W.schwarzii* showed greater uniformity although KK in *W.cedarbergensis* was monomorphic at two loci.

Two measures of heterozygosity were given in Table 3.2. H_o gives the mean proportion observed heterozygosity for all polymorphic loci within each population and H_e gives the mean proportion expected heterozygosity within each population. The mean H_o for all populations is highest in *W.schwarzii* and lowest in *W.nodiflora*.

Mean observed and expected levels of heterozygosity varied among populations of all three species although only populations of *W.schwarzii* conformed to Hardy-Weinberg equilibrium (Table 3.2). Populations in both *W.cedarbergensis* and *W.nodiflora* show departure from random mating with few exceptions. The fixation index (F) gives an estimate of the difference between observed and expected heterozygosity. The higher the fixation index, the greater the deviation from Hardy-Weinberg equilibrium. The mean fixation indices of all populations within all species show that populations of *W.nodiflora* deviated from Hardy-Weinberg expectations substantially more than populations of *W.cedarbergensis* and *W.schwarzii* (Table 3.2). In *W.cedarbergensis*, the most problematic populations seem to be WB and SB where fixation indices were fairly high. The plantation, MB, was the only "population" which seems to be outbreeding. KK would also appear to be outbred although this result may be a function of sampling error since KK is monomorphic for two loci. In *W.nodiflora*, critical populations seem to be OK, BKR and SBR where fixation indices were particularly high. BKV was the only population with a low fixation index.

Table 3.1. Allele frequencies obtained at polymorphic loci for (a) *W.cedarbergensis*, (b) *W.nodiflora* and (c) *W.schwarzii* (see Chapter 2 for abbreviation meanings).

(a) W.cedarbergensis

Locus	Allele	WB	SB	DG	CPS	MB	KD	KK
PGI-2	A	0.714	0.859	0.444	0.481	0.632	0.357	0.500
	B	0.286	0.141	0.556	0.519	0.368	0.643	0.500
SDH-2	A	0.637	0.833	0.280	0.578	0.750	0.528	0.813
	B	0.363	0.167	0.720	0.422	0.250	0.472	0.188
IDH-1	A	0.033	0.225	0.206	0.224	0.111	0.125	0.000
	B	0.678	0.650	0.676	0.466	0.833	0.719	1.000
	C	0.289	0.125	0.118	0.310	0.056	0.156	0.000
LAP-2	A	0.838	0.261	0.680	0.774	0.947	0.773	0.983
	B	0.162	0.717	0.320	0.226	0.053	0.227	0.017
	C	0.000	0.022	0.000	0.000	0.000	0.000	0.000
AAT-2	A	0.750	0.235	0.444	0.375	0.900	0.545	1.000
	B	0.250	0.765	0.556	0.625	0.100	0.455	0.000

(b) W.nodiflora

Locus	Allele	KB	OK	BKR	BKV	SBR	BBAY	CPK
PGI-1	A	1.000	0.786	0.094	0.031	1.000	1.000	0.667
	B	0.000	0.214	0.906	0.969	0.000	0.000	0.333
PGI-2	A	0.938	0.778	0.922	0.968	0.932	1.000	0.833
	B	0.063	0.204	0.078	0.032	0.045	0.000	0.167
	C	0.000	0.000	0.000	0.000	0.023	0.000	0.000
	D	0.000	0.019	0.000	0.000	0.000	0.000	0.000
SDH-1	A	0.576	0.707	0.468	0.579	0.774	0.944	0.559
	B	0.424	0.259	0.532	0.421	0.210	0.056	0.441
	C	0.000	0.000	0.000	0.000	0.016	0.000	0.000
	D	0.000	0.034	0.000	0.000	0.000	0.000	0.000
SDH-2	A	0.000	0.000	0.000	0.000	0.000	0.000	1.000
	B	0.076	0.000	0.032	0.105	0.000	0.000	0.000
	C	0.924	1.000	0.968	0.895	1.000	1.000	0.000
IDH-1	A	0.188	0.231	0.188	0.088	0.679	0.778	0.059
	B	0.719	0.692	0.438	0.500	0.321	0.222	0.500
	C	0.094	0.077	0.375	0.412	0.000	0.000	0.441
MDH-3	A	0.200	0.400	0.235	0.286	0.294	0.500	1.000
	B	0.433	0.233	0.559	0.543	0.676	0.500	0.000
	C	0.367	0.367	0.176	0.171	0.029	0.000	0.000
	D	0.000	0.000	0.029	0.000	0.000	0.000	0.000
LAP-1	A	0.061	0.000	0.000	0.000	0.094	0.000	0.000
	B	0.000	0.089	0.071	0.000	0.078	0.000	0.000
	C	0.000	0.000	0.186	0.071	0.000	0.000	0.000
	D	0.742	0.804	0.586	0.914	0.672	1.000	1.000
	E	0.197	0.107	0.114	0.014	0.156	0.000	0.000
	F	0.000	0.000	0.043	0.000	0.000	0.000	0.000

(c) W.schwarzii

Locus	Allele	SV	DK	NKA
PGI-2	A	0.222	0.050	0.000
	B	0.778	0.900	0.850
	C	0.000	0.050	0.150
IDH-1	A	0.350	0.600	0.700
	B	0.650	0.400	0.300
MDH-3	A	0.500	0.600	0.550
	B	0.500	0.400	0.450
LAP-2	A	0.900	0.850	0.700
	B	0.100	0.150	0.300

Table 3.2. Estimates of genetic diversity in populations of (a) *W.cedarbergensis*, (b) *W.nodiflora* and (c) *W.schwarzii* (N = sample size; A = average number of alleles per locus; P = percentage polymorphic loci; H_o = mean observed frequency of heterozygotes; H_e = mean expected frequency of heterozygotes; F = fixation index).

Population	N	A	P	H_o	H_e	F
			(a) *W.cedarbergensis*			
WB	36	1.32	29.4	0.28	0.40	0.302 (0.21)
SB	23	1.36	29.4	0.26	0.37	0.250 (0.34)
DG	23	1.32	29.4	0.37	0.47	0.209 (0.44)
CPS	36	1.32	29.4	0.37	0.50	0.250 (0.15)
MB	14	1.32	29.4	0.27	0.31	0.042 (0.32)
KD	17	1.32	29.4	0.35	0.47	0.217 (0.18)
KK	19	1.16	17.7	0.18	0.17	-0.083 (0.11)
Mean		1.30	27.7	0.30	0.39	0.170
			(b) *W.nodiflora*			
KB	23	1.47	35.3	0.29	0.41	0.233 (0.39)
OK	25	1.57	35.3	0.29	0.44	0.325 (0.12)
BKR	28	1.68	41.2	0.28	0.40	0.301 (0.41)
BKV	23	1.53	41.2	0.21	0.32	0.098 (0.31)
SBR	24	1.52	29.4	0.24	0.39	0.278 (0.30)
BBAY	9	1.16	17.7	0.22	0.34	0.210 (0.19)
CPK	23	1.26	23.5	0.22	0.49	0.245 (0.47)
Mean		1.46	31.9	0.25	0.40	0.241
			(c) *W.schwarzii*			
SV	20	1.21	23.5	0.33	0.40	0.097 (0.25)
DK	10	1.26	23.5	0.38	0.37	0.007 (0.46)
NK	10	1.21	23.5	0.45	0.42	-0.029 (0.61)
Mean		1.23	23.5	0.39	0.40	0.025

F-statistics

The results of Wright's F-statistics are presented in Table 3.3. F_{is} values varied considerably between species. F_{is} was much lower in *W.schwarzii* than in the other two species. F_{is} was high in *W.cedarbergensis*, but especially high in *W.nodiflora*. Similar trends can be seen for F_{it} and F_{st}. Compared with studies of other plants, the F_{st} for *W.nodiflora* approaches that for selfing plants (Gst = 0.510 in Hamrick & Godt 1990). The mean Gst for gymnosperms is 0.068 (Hamrick & Godt 1990). *W.schwarzii* was the only species within the genus that has an F_{st} value close to that found for most gymnosperms.

Genetic Distance

Genetic distances between populations of *W.cedarbergensis*, *W.nodiflora* and *W.schwarzii* are given in Table 3.4 (a,b,c). The mean D value for all pairwise comparisons of populations of *W.cedarbergensis* was 0.907. The mean D value for all pairwise comparisons of populations of *W.nodiflora* was 0.803 and the mean D value for pairwise comparisons between populations of *W.schwarzii* was 0.986. Populations of *W.nodiflora* were therefore less related to each other than were populations of *W.schwarzii*.

Table 3.3. Wright's F-statistics for levels of gene flow within and between populations of (a) *W.cedarbergensis*, (b) *W.nodiflora* and (c) *W.schwarzii*.

Locus	F_{is}	F_{it}	F_{st}
(a) *W.cedarbergensis*			
Pgi-2	0.273	0.350	0.106
Sdh-2	0.079	0.206	0.138
Idh-1	0.390	0.447	0.094
Lap-2	0.426	0.573	0.255
Aat-1	-0.136	0.193	0.289
Mean	0.206	0.354	0.176
(b) *W.nodiflora*			
Pgi-1	0.656	0.890	0.681
Pgi-2	0.066	0.123	0.060
Sdh-1	-0.157	-0.039	0.102
Sdh-2	0.468	0.899	0.811
Idh-1	0.364	0.500	0.214
Lap-2	0.519	0.571	0.109
Mdh-3	0.315	0.461	0.213
Mean	0.364	0.498	0.313
(c) *W.schwarzii*			
Pgi-2	0.335	0.372	0.056
Idh-1	0.336	0.394	0.088
Lap-2	-0.053	-0.002	0.048
Mdh-3	-0.559	-0.549	0.007
Mean	-0.015	0.054	0.050

Cluster Analysis

The results of the pairwise comparisons of genetic distance between populations using Nei's unbiased genetic distance are presented graphically using a UPGMA cluster analysis (Fig 3.1, a,b,c). In *W.cedarbergensis*, there was no relationship between geographic proximity of populations and genetic relatedness (see Figure 2.1a Chapter 2). SB was the most unique population. Further, the Middelberg plantation, MB, was most related to WB and the KD plantation was most related to DG and CPS.

In *W.nodiflora*, there was a strong relationship between genetic relatedness and geographic proximity of populations (see Figure 2.1b, Chapter 2). The cluster analysis shows that populations of *W.nodiflora* situated close together, for example BKV and BKR, are as similar to each other as are populations of *W.schwarzii* and of *W.cedarbergensis*. Disjunctions in genetic similarity between mountain ranges were found in *W.nodiflora*.

In *W.schwarzii*, all three populations were genetically very similar although NK and DK form a separate cluster. With such a small sample size it was difficult to relate this pattern to geographic proximity.

Table 3.4. Matrices of Nei's (1978) unbiased genetic distance (D) between populations of (a) *W.cedarbergensis*, (b) *W.nodiflora* and (c) *W.schwarzii*.

(a) *W.cedarbergensis*

	WB	MB	KD	SB	DG	CPS	KK
WB	1.000						
MB	0.974	1.000					
KD	0.913	0.913	1.000				
SB	0.819	0.819	0.819	1.000			
DG	0.913	0.913	0.993	0.819	1.000		
CPS	0.913	0.913	0.984	0.819	0.984	1.000	
KK	0.974	1.000	0.913	0.819	0.913	0.913	1.000

(b) *W.nodiflora*

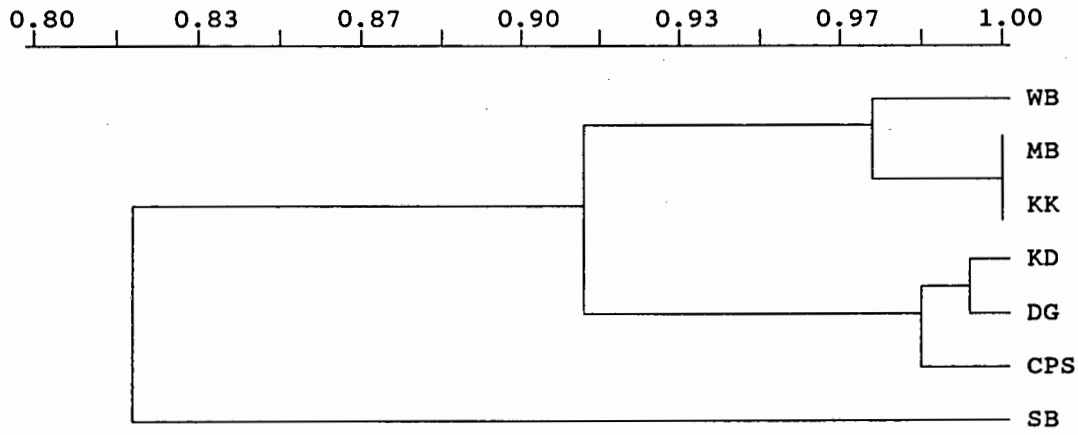
	KB	BKR	SBR	OK	CPK	BKV	BBAY
KB	1.000						
BKR	0.779	1.000					
SBR	0.922	0.779	1.000				
OK	0.982	0.779	0.922	1.000			
CPK	0.664	0.664	0.664	0.664	1.000		
BKV	0.779	0.993	0.779	0.779	0.664	1.000	
BBAY	0.922	0.779	0.986	0.922	0.664	0.779	1.000

(c) *W.schwarzii*

	SV	DK	NK
SV	1.000		
DK	0.979	1.000	
NK	0.979	1.000	1.000

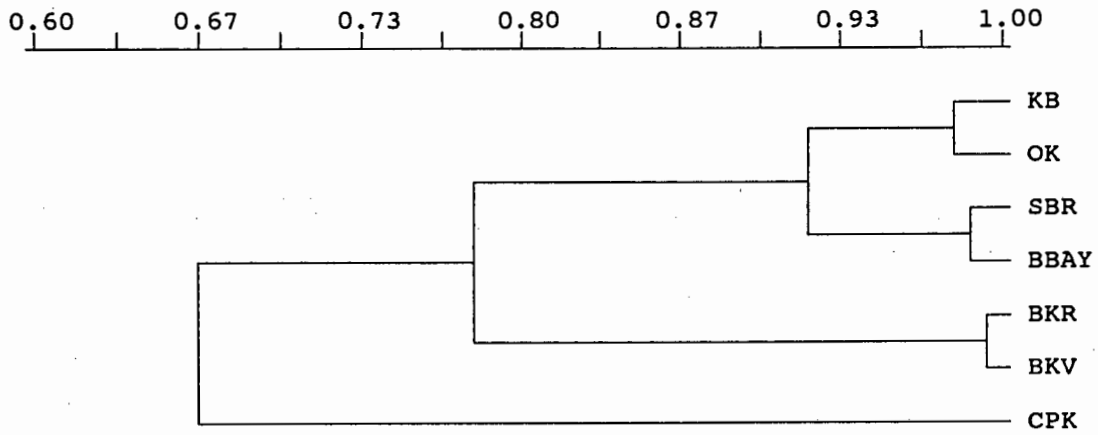
Figure 3.1. Dendrogram based on a UPGMA cluster analysis using Nei's (1978) genetic distance (D) calculated from the allelic frequencies of populations, showing relationships between populations of (a) *W.cedarbergensis*, (b) *W.nodiflora* and (c) *W.schwarzii*.

(a) *W.cedarbergensis*



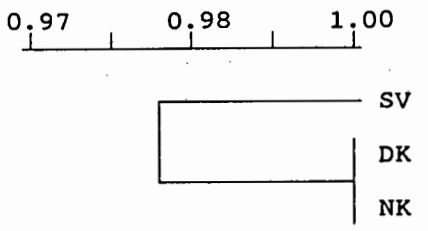
Cophenetic correlation = 0.865

(b) *W.nodiflora*



Cophenetic correlation = 0.964

(c) *W.schwarzii*



Cophenetic correlation = 0.725

Discussion

Levels of genetic polymorphism

Narrowly endemic species are generally known to be genetically depauperate. This has been shown in case studies for plants such as the narrowly endemic *Bensoniella oregona* Saxifragaceae) (Soltis et al. 1992), and the rare *Eucalyptus pulverulenta* (Peters, Lonie & Moran 1990). Two extreme endemics, *Pedicularis furbishae* (Waller, O'Malley & Gawler 1987) and *Pinus torreyana* (Ledig & Conkle 1983) have been shown to have no variation at the loci examined (although one population of *Pinus torreyana* was polymorphic at 3.4% of the loci). Conversely, widespread species are generally known to be more variable than their more endemic congeners (Loveless & Hamrick 1984; Hamrick & Godt 1990; Karron et al. 1988). This general trend holds true for *Widdringtonia*. *W.nodiflora* was the most widespread species of all and had the highest number of alleles per locus as well as a higher percentage of polymorphic loci than the other two more restricted species. The differences between *W.nodiflora* and *W.cedarbergensis* are not as marked as they are between *W.nodiflora* and *W.schwarzii*, however, which suggests that *W.cedarbergensis* might once have had a wider or more continuous distribution than it has today. Further, *W.schwarzii* may have experienced a more intense and sustained bottleneck in the past than did *W.cedarbergensis* which has led to a greater loss of polymorphism and allelic diversity. This argument has been invoked for the difference in genetic diversity between two narrowly endemic species of *Astragalus* (Karron et al. 1988). *W.cedarbergensis* and *W.schwarzii* certainly have higher levels of polymorphism and allelic diversity than has been found for the rare *Pinus torreyana*, (Ledig & Conkle 1983).

There is sufficient evidence to suggest that these two components of diversity, polymorphism and allelic diversity, are more reliable indicators of the effects of population bottlenecks than are mean observed and expected proportions of heterozygosity. This has been shown mathematically by Nei, Maruyama and Chakraborty (1975), in computer simulations by Lacy (1987) and by Leberg (1992, 1993) in a semi-natural experiment on mosquitofishes. The life history of *Widdringtonia* is very different to that of a mosquitofish which has a generation time of 56 days (Leberg 1993). However, average heterozygosity depends not only on the size of the bottleneck but also on the rate of population growth (Nei, Maruyama & Chakraborty 1975). Average number of alleles per locus, on the other hand, is "profoundly

affected by bottleneck size but not so much by population growth" (Nei, Maruyama & Chakraborty 1975). The finding that *W.nodiflora*, the most abundant and widespread species within the genus has the lowest levels heterozygosity, suggests that, in this case at least, heterozygosity is an unreliable indicator of the effects of bottlenecks in a between-species comparison.

Organisation of genetic variation

The high fixation index in *W.cedarbergensis* was surprising since inbreeding within populations was thought to be unlikely since the species is wind-pollinated. Therefore even if the population has been dramatically reduced, it is unlikely that the adults left are related. In retrospect, inbreeding In *W.cedarbergensis* can be attributed to two factors considering isozyme loci were resolved for seedlings rather than adults. Firstly, individuals could be selfing due to lowered tree densities. Evidence for lowered outcrossing rates has been found in low density stands of ponderosa pine as a result of inefficient pollen movement (Farris & Mitton 1984). Subpopulations of *W.cedarbergensis* were possibly more continuous in the past, promoting outcrossing. Alternatively, neighbouring adult trees could originate from seeds from the same parent since the seeds of *W.cedarbergensis* are very poorly dispersed. The low level of inbreeding in the plantation, MB, supports both explanations since trees in the plantation are regularly spaced, promoting turbulence for enhanced pollen dispersal. Trees standing close together are not likely to be related since they were planted presumably from random parents albeit possibly from the same population. Therefore progeny from the plantation trees are not likely to be inbred.

Of all three species, *W.nodiflora* showed the greatest departure from predictions made at the outset of the study. The large scale morphological variation found within this species led to a prediction of high levels of genetic diversity. My expectations were met to some extent in that *W.nodiflora* shows the most polymorphism and number of alleles per locus than the other two species. However, the morphological variation witnessed was probably a direct result of a high degree of genetic differentiation indicated by extremely poor levels of gene flow within and between populations.

W.nodiflora invests much of its life history into vegetative growth during resprouting after fires. Sexual reproduction is not an uncommon feature of *W.nodiflora*, however, although phenological patterns appear to be quite complex (per.obs, H.Nieuwmeyer pers.comm.). The isozyme analyses have been conducted on germlings from sampled seed in this study, and therefore do not necessarily reflect the patterns of diversity of the adult populations. It is likely that a high amount of selfing occurs between ramets of the same genet within populations of *W.nodiflora*. It then follows that the generation of seeds used for isozyme analysis in this study are likely to be inbred, hence the departure from Hardy-Weinberg equilibrium. Evidence for lowered fitness (inbreeding depression) in seed set has been found in low density stands *W.nodiflora* (Gibson unpubl.) and is discussed in Chapter 4. Autogamy (the ability to self-fertilize in the absence of foreign pollen) has been associated with high levels of inbreeding depression as measured by seed set before in *Kalmia latifolia* (Ericaceae) (Rathcke & Real 1993). The high level of differentiation between populations of *W.nodiflora* is probably aided by the effects of resprouting and fire. The chances of establishing a population are slight since seedlings of *W.nodiflora* are easily killed by fire. Once a population is established, however, it persists for a very long time, since *W.nodiflora* resprouts after fire although the cluster analysis disputes this explanation. Neighbouring populations were more related than were distant populations showing that distance may presents a major barrier to gene flow, causing a high level of population differentiation.

The low level of genetic differentiation among populations in *W.schwarzii* was contrary to expectations. The kloofs in which populations of *W.schwarzii* are found, therefore, present no effective barrier to gene flow. The low level of fixation within the populations contradicts the fact that *W.schwarzii* has such low levels of polymorphism and allelic diversity. However, it does help explain why *W.schwarzii* is still an ecologically successful species. The low level of fixation within populations may be attributed to the fact that seeds of *W.schwarzii* are dispersed further than those of *W.cedarbergensis* so that individuals in the same stand are less likely to be related than are individuals of *W.cedarbergensis*. It seems that all populations of *W.schwarzii* are part of a panmictic population and that the effects of bottlenecks act on the metapopulation as a whole. For this reason, *W.schwarzii* is possibly less susceptible to the effects of fragmentation than is *W.cedarbergensis*. Further, this

evidence serves to highlight the importance of seed dispersal in patterns of genetic variation in *Widdringtonia*.

The genetic relationships between populations points to further evidence for a once more widespread distribution of *W.cedarbergensis*. Geographic proximity of populations does not determine genetic relatedness which implies that all the populations of *W.cedarbergensis* were once part of a greater panmictic population with a high degree of relatedness. As the metapopulation became increasingly fragmented, insufficient levels of gene flow between the various subpopulations were sustained, rendering the smaller subpopulations susceptible to genetic drift.

In contrast, patterns of population relatedness correspond with geographic proximity in *W.nodiflora* (discussed above) and *W.schwarzii*. In *W.schwarzii*, DK and NK are situated on opposite sides off the wide valley floor that separates the Kouga and the Baviaanskloof mountains. These two populations are highly related suggesting that gene flow across the valley floor is highly efficient. SV, on the other hand, is situated on the same side of the valley as NK, but is somewhat genetically distinct from the other two subpopulations. This suggests that gene flow between kloofs within the same mountain range is inefficient. However, it is not as inefficient as predicted at the outset of the study.

Conclusion

With the aid of isozymes, there is evidence to suggest that *W.cedarbergensis* once had a wider or rather more continuous distribution than it has today. Two components of diversity, allelic diversity and the number of polymorphic loci, indicated that *W.cedarbergensis* has lost variation as a result of population reduction and fragmentation. Further, high levels of inbreeding are evident in most populations which is likely to be a result of poorly dispersed seed or large distances between trees leading to poor pollen transfer. As a result, populations were found to be highly substructured, a striking deviation from predictions at the outset. This, compounded by the fact that populations of *W.cedarbergensis* are highly differentiated from each other, renders *W.cedarbergensis* especially vulnerable to fragmentation. The MB plantation is a potentially outbred seed source for the replanting programme. The lack of

inbreeding in MB was attributed to small inter-tree distances and unrelated neighbours sharing pollen. *W.schwarzii* showed the least allelic diversity and number of polymorphic loci although populations of *W.schwarzii* were panmictic and highly outbred. According to the these findings, therefore, *W.schwarzii* should not be very susceptible to fragmentation but rather to a reduction in the metapopulation as a whole. The estimates of diversity in *W.nodiflora* were high but populations are highly differentiated. This was attributed to the sprouting behaviour of the species and distance as a barrier to gene flow. Evidence for substantial levels of inbreeding within populations of *W.nodiflora* were probably a result of selfing between ramets of the same genet.

CHAPTER 4: ECOLOGICAL AND REPRODUCTIVE FITNESS DIFFERENCES BETWEEN POPULATIONS OF WIDDRINGTONIA CEDARBERGENSIS

Introduction

The effort to conserve *Widdringtonia cedarbergensis* by Cape Nature Conservation has concentrated on a replanting scheme in a limited area of the Cedarberg called Welbedacht. This has involved collecting seeds predominantly from the Middelberg plantation and germinating and cultivating seedlings in a nursery until they are ready for replanting in the field. Seed collections from natural populations for the replanting scheme have begun recently, however. Not much is known about the original seed source of the plantation. Further, there is little or no record of the vigour of the trees in the plantation or of any of the natural populations. This should be an important consideration for the replanting scheme since differences in vigour between populations might reflect genetic differentiation. It would be best if the seed source for the replanting scheme originated from the fittest stock so that the chances for survival and high growth rate of the seedlings are high. In this chapter, various reproductive fitness differences between six populations are examined to determine whether there are any consistent trends in fitness. Only *W.cedarbergensis* is included in this study since this is the only species being actively managed for conservation.

The relationship between levels of inbreeding and fitness of the various populations is dealt with in Chapter Six. However, it is necessary to explain why the reproductive attributes were used as measures of fitness. Reproductive characters are considered ideal "fitness characters" (Frankel & Soule 1981) because they are typically characters which are the expression of dominant alleles. Dominant alleles are readily phenotypically exposed during the process of inbreeding and, if they are deleterious, are eliminated from the population by natural

selection (Frankel & Soule 1981). Recessive alleles, on the other hand, can persist within a population for longer periods of time at a low frequency since they are seldom phenotypically exposed. For this reason, traits determined by genes with a significant amount of dominance or overdominance will change the most as a result of inbreeding. Dominance is often observed in traits related to reproduction which is why inbreeding depression can be expected to be most prevalent in characters such as fecundity, fertility, developmental rate and sexual maturity, litter size and related traits (Frankel & Soule 1981). In this chapter, five characters have been chosen as indicators of fitness: population size structure, seedling:parent ratio, cone production, seed set, abortion fraction and seed mass. Population size structure and population growth are not really fitness traits but rather an indication of overall well-being within populations. The term "ecological fitness" is therefore used rather loosely in this chapter and elsewhere in this thesis.

Methods

In March 1993, six populations (described in Chapter 2, Table 2.1) of *Widdringtonia cedarbergensis* were visited to record differences in reproductive attributes. These attributes were population growth, cone production, seed set, seed embryo abortion fraction and seed mass. The plantation, KD, was only censused for three fitness components: seed set, seed mass and abortion fraction. These components were statistically analysed for differences between populations. Where necessary, data were transformed to normalise the distribution. Where appropriate, a multiple range test, using confidence intervals, was used to determine patterns of differences between populations.

Population Growth

Approximately thirty adult (reproductively mature) trees were sampled within each population. The circumference of each tree was measured at 1.5m above the ground. The number of seedlings and saplings were counted and their heights recorded within a 5m radius of each tree. In the analysis trunk circumferences were converted to diameters using the equation for the circumference of a circle ($c = 2\pi r$) and individuals were grouped into classes according to the size classes used by Manders (1985) in his transition matrix. These classes are given in Table 4.1.

Table 4.1. Population size classes from Manders (1985) for *Widdringtonia cedarbergensis*. Classes 1 to 6 are measured by shoot height (h); Class 6 is measured by a combination of height and trunk diameter; Classes 1 to 6 are measured by trunk diameter at 1.5m above the ground.

class	size
1	<=25cm
2	25-50cm
3	50-75cm
4	75-100cm
5	100-125cm
6	125cm-150cm h
7	d<=5cmh, h>150cm
8	5cm-10cm
9	10cm-20cm
10	20cm-40cm
11	40cm-60cm
12	>60cm d

Frequencies of each size class were obtained for each population and plotted. In this analysis, seedlings were considered to be those individuals in class 1, pre-reproductive individuals were considered those in classes 2 to 7, and juveniles and adults were considered to be those in classes 8 to 12. Since the method of sampling was biased towards adult trees, a seedling:parent ratio was also obtained for each population. In this analysis, seedlings were considered to be those individuals found in size class 1 to 7, and adults were considered to be those individuals found in size classes 8 to 12.

Cone Production

Estimates of numbers of cones on each adult tree that was sampled were obtained by averaging the counts made by two people. This method was consistent for each population. An estimate of canopy damage was obtained in the same manner. In the analysis, a scatter plot of log number of cones against log trunk circumference was obtained to determine whether there was a relationship between tree size and fecundity. Plots of log number of cones against log trunk circumference were then obtained for each population. Regression lines of these plots were then compared for differences in regression coefficients, slopes and intercepts in order to assess reproductive fitness differences between populations. The number of cones for each individual was corrected for percentage canopy damage and regression lines

of corrected values were obtained for each population. These were also compared for differences in regression coefficients, slopes and intercepts.

Seed Set and Abortion Fraction

Estimates of seed set were obtained by randomly selecting 20 cones from each population in the laboratory and counting the total number of seed scars within each cone. The differences in seed set between populations were analysed by a one-way ANOVA using a log transformation to normalise the data. The methodology used here was not entirely conducive to obtaining a true estimate of seed set within each population, however. Ideally, variation within individual trees should have been compared with variation between individuals of each population before variation between populations was compared. It was also possible to obtain an estimate of embryo abortion fraction from each cone since aborted seeds are detectable by the small scars left behind in the cone compared with the obviously large scars left by full seed.

Seed Mass

Estimates of seed mass were obtained from the growth experiment described in Chapter Five. As a result, mean seed masses of 10 seeds were obtained for each population and replicated 25 times, giving a mean seed mass for 250 seeds per population. A one-way ANOVA was used to test for differences in seed mass between populations.

Results

Population Growth

Graphs showing size structure of the seven populations of *W.cedarbergensis* highlight three major trends (Figure 4.1). The first trend was found in CPS, DG and SB, where between 50 - 65% of the individuals fell into the seedling class (class 1), with proportionately much fewer of the individuals falling into the pre-reproductive and juvenile classes. The second trend was found in KK and WB where the distribution of sizes was polarized fairly evenly into the seedling and adult size classes with a noticeable absence of pre-reproductive individuals.

The most striking feature of all the graphs was the poor graduation of seedlings to class 2. Only in WB did class 2 individuals exceed 10%. The third trend was found in MB, the plantation, there was a marked absence of adult trees but juveniles were strongly represented.

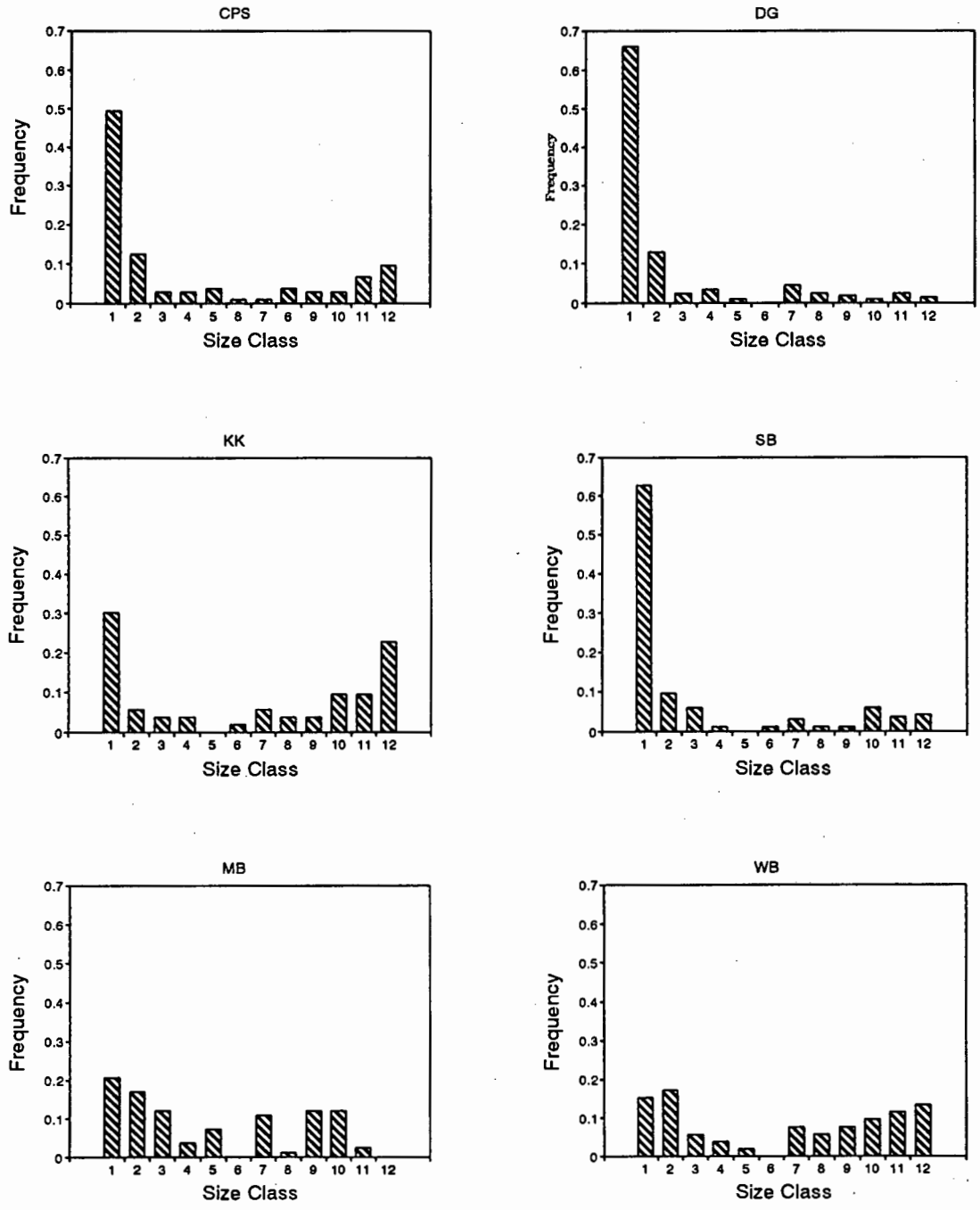


Figure 4.1. Size structure in populations of *W.cedarbergensis*

A more appropriate measure of population growth rate was the seedling to parent ratio (Figure 4.2). This ratio was highest in the populations SB and DG, two populations situated close together. The seedling to parent ratio was also high in CPS although this value is probably an underestimate. The lowest seedling to parent ratios were found in MB, KK and WB.

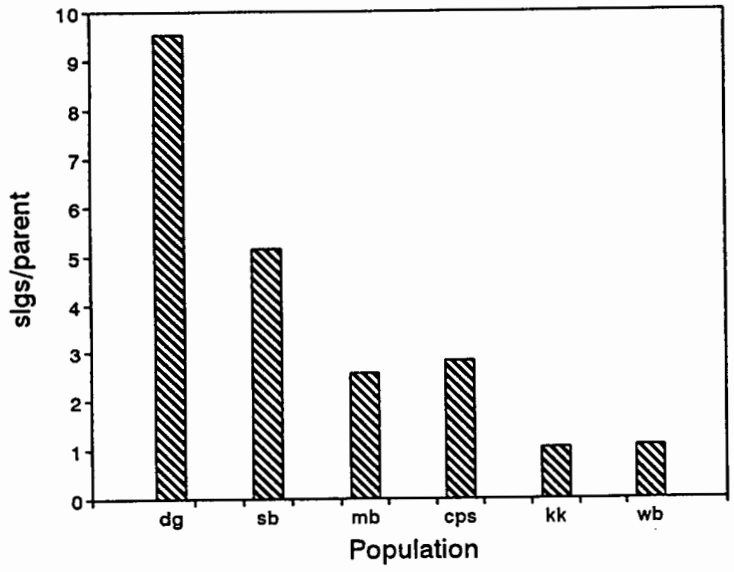


Figure 4.2. Seedling to parent ratio in six populations of *W.cedarbergensis*. Seedlings constitute individuals found in size classes 1 to 7, and adults constitute individuals found in size classes 8 to 12.

Cone Production

The scatter plot showed that above a trunk diameter of 101.86cm, trees became senescent with reduced cone production relative to trunk size (Figure 4.3). Individuals with trunk diameters above 100cm were therefore omitted from the regression analyses below. Many individuals remained non-productive throughout the juvenile phase but few were found to be non-productive within the reproductively optimal size range.

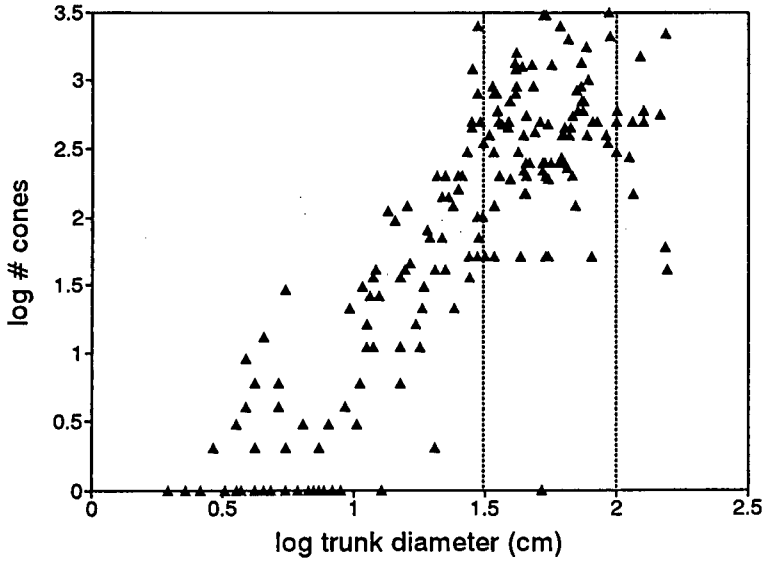


Figure 4.3. Scatter plot of log number of cones against log trunk diameter. Vertical lines indicate a reproductive optimum between 32cm to 100cm trunk diameter and reduced cone production relative to size above 100cm trunk diameter.

There was a positive relationship between log # cones and log trunk diameter in all populations of *W.cedarbergensis* (Figure 4.4). This relationship is significant for all populations and for both adjusted (for canopy damage) and unadjusted data sets (Table 4.2). There was little difference between regressions obtained for adjusted and unadjusted data. The biggest differences between adjusted and unadjusted data sets were found in DG and CPS where damage caused by baboons (who eat the seeds) was possibly greater than in other populations. Slopes for the regressions between log number of cones and log trunk diameter differed very slightly between populations. For unadjusted data, SB had the steepest slope although the slopes of KK and WB are almost as steep. The strength of the relationship in WB, however, was weakest of all. Therefore, the y-intercept for the relationship in WB cannot be compared with SB and KK. The y-intercept was highest in MB suggesting that the rate of accumulation of cones begins at a much earlier age in the plantation than in the natural populations.

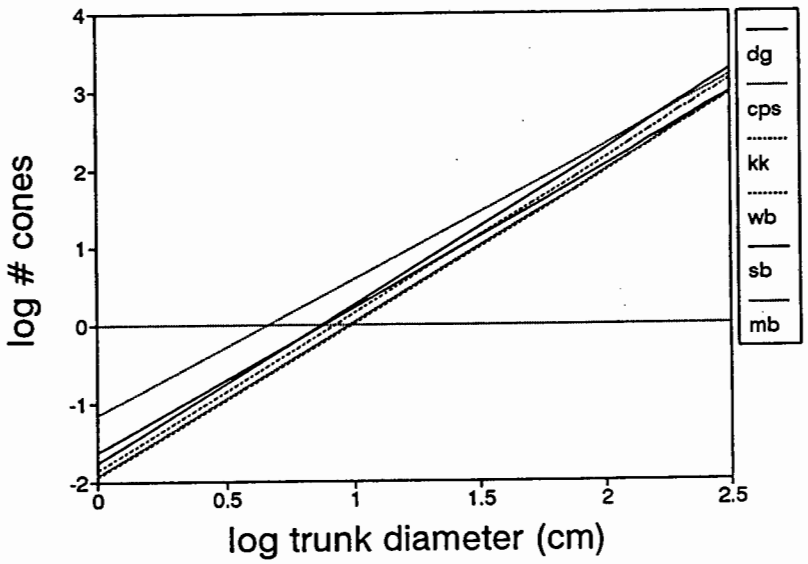


Figure 4.4. The relationship between cone production (unadjusted for canopy damage) and trunk size in populations of *W.cedarbergensis*. Solid lines indicate regression lines.

Table 4.2. Regressions between log number of cones and log trunk diameter for six populations of *W.cedarbergensis*. Two data sets are used: a) represents data adjusted for percentage canopy damage and b) represents unadjusted data. (= P < 0.005).**

Population	Data	df	Slope	R^2	Y-intercept	P
DG	a	27	1.92	0.88	-1.70	**
	b	27	1.84	0.87	-1.63	**
CPS	a	22	2.06	0.54	-2.06	**
	b	22	1.95	0.53	-1.92	**
KK	a	19	2.12	0.75	-1.97	**
	b	19	1.99	0.72	-1.85	**
WB	a	21	2.03	0.73	-2.02	**
	b	21	1.96	0.70	-1.95	**
SB	a	29	2.07	0.79	-1.82	**
	b	29	2.01	0.79	-1.76	**
MB	a	30	1.75	0.73	-1.14	**
	b	30	1.74	0.73	-1.16	**

Seed Mass

Significant differences were found in seed mass between populations (Table 4.3). A multiple range test revealed that KD was the only population whose seeds were larger than the rest of the populations. This difference was significant at the 0.005 level.

Table 4.3. Means table for mean seed mass for populations of *W.cedarbergensis*. N = sample size, SE = standard error. Significant differences were found using a one-way ANOVA (F = 13.205, p < 0.005; MRT = confidence interval multiple range test).

Population	N	Mean seed mass x 10^{-2} (g)	SE x 10^{-2}	Log mean seed mass (g)	MRT
DG	25	10.19	0.42	-2.346	*
CPS	25	9.58	0.20	-2.351	*
KK	25	9.64	0.16	-2.343	*
WB	25	8.78	0.16	-2.437	*
SB	23	9.48	0.14	-2.359	*
MB	25	9.00	0.12	-2.410	*
KD	25	13.47	0.16	-2.006	*
Mean		10.02	0.20	-2.321	

Seed Set

There were significant differences in log mean seed set between populations (Table 4.4). The seed set data reveals two major findings. Firstly, KK has the lowest seed set out of the six populations. Secondly, CPS and DG have the most variable seed set shown by a multiple range test.

Table 4.4. Mean seed set per cone in six populations of *W.cedarbergensis*. Significant differences were found between populations using a one-way ANOVA (F = 3.071, 0.005 > p < 0.001; MRT = confidence interval multiple range test).

Population	N	Log mean seed set	SE	MRT
DG	20	2.548	0.013	**
CPS	20	2.476	0.022	**
KK	20	2.286	0.176	*
WB	25	2.617	0.020	*
SB	25	2.607	0.031	*
MB	26	2.563	0.025	*
KD	24	2.562	0.030	*
Mean		2.531	0.024	

To detect differences in abortion rates between populations, it was necessary to use a Kruskal-Wallis one-way analysis by ranks since it was impossible to normalise the data

through log or square root transformations. There appeared to be no significant difference between populations (Table 4.5).

Table 4.5. Mean embryo abortion fraction within populations of *W.cedarbergensis*. No significant differences were found between populations using a Kruskal-Wallis one-way analysis by ranks (F = 8.651, p = 0.194).

Population	N	Mean abortion fraction	SE
DG	20	0.784	0.052
CPS	20	0.832	0.040
KK	20	0.934	0.934
WB	25	0.841	0.841
SB	25	0.878	0.878
MB	26	0.901	0.024
KD	24	0.887	0.022
Mean		0.867	0.012

Discussion

Reproductive and ecological fitness components seems to differ between populations of *W.cedarbergensis*. Since fitness is a relative concept, all variables are expressed as percentages relative to one the fittest population in the table below (Table 4.6). SB and DG are the two consistently fit populations with regard to the reproductive components examined in this chapter although the trend is rather weak. The lack of consistency in this regard suggests that differences in adult fecundity between populations are subject to environmental heterogeneity rather than genetic differences.

Population growth and size structure

Of considerable importance is population growth since it is the ultimate indication of productivity, fecundity, fertility, seed viability, survival and mortality. Of all the populations, four (DG, SB, CPS and MB) show evidence for a positive growth rate while WB and KK show signs of stagnation. Poor regeneration in KK and WB was possibly a result of lowered cone production above an optimal tree size. Although statistical evidence is only presented in Chapter 6 (Table 6.1), S:P was not correlated with seed set, seed mass or cone

production. This suggests that S:P is determined by growth and mortality patterns rather than fecundity. Senescence could also be a major factor determining population growth rate and is possibly indicated by a high proportion of individuals residing in class 12 (characteristic in WB and KK) since individuals in this size class may exceed 100cm trunk diameter which is the upper limit of the reproductive optimum. The findings of Privett's (1994) elasticity analysis on Manders' (1985) transition matrix supports this since he found that reproductive adults contribute more to population growth than any other size class. Poor transition of seedlings to class 2 was not necessarily a result of high seedling mortality. Manders (1985) transition matrix showed that only 6.2% of seedlings die within one year. 74.3% of the seedlings remain in class 1, 17.9% of the seedlings moved up to class 2 and 1.6% of the seedlings moved up to class 3. This provides a good explanation as to why there is such a disjunction between proportionate representation of class 1 and class 2 individuals in most of the populations.

Table 4.6. Summary table of reproductive fitness in *W.cedarbergensis*. Values are expressed as percentages relative to the fittest population for each variable (indicated by 1.00). Slope and Y-intercept refer to the regression analyses of cone production against trunk diameter; S:P refers to the seedling to parent ratio; abortion refers to seed embryo abortion.

Variable	Population						
	DG	CPS	KK	WB	SB	MB	KD
Slope	0.92	0.97	0.99	0.98	**1.00**	0.87	-
Y-intercept	0.33	0.17	0.20	0.16	0.25	**1.00**	-
S:P ratio	**1.00**	0.23	0.11	0.11	0.54	0.27	-
Seed Set	0.93	0.87	0.84	**1.00**	**1.00**	0.95	0.95
Seed Mass	0.46	0.45	0.46	0.37	0.44	0.40	**1.00**
Abortion	**1.00**	0.78	0.31	0.74	0.57	0.46	0.57
Mean	0.77	0.58	0.48	0.56	0.63	0.66	-

Seed set and seed size

Variation in seed set and seed size can be attributed to varying tree density. Gibson (unpubl.) found evidence for lowered seed set and increased seed size in low density stands of *W.nodiflora*. However, seed set and seed size do not co-vary between populations of *W.cedarbergensis* (Chapter 6, Table 6.1). The results of Chapter 3 indicate that populations are further substructured as a result of limited gene flow (indicated by extremely high F_{is} values). Seed set could therefore be limited by availability of outcrossed pollen in populations of *W.cedarbergensis*. Gymnosperms exercise "mate choice" through polyembryony (Sorenson 1982). Embryos produced through outcrossing survive in preference to embryos produced through selfing in the same seed (Willson & Burley 1983; Sorenson 1982). A general decline in seed viability may therefore indicate high levels of selfing. Gibson (unpubl.) found evidence for reduced seed set in low density stands of *W.nodiflora*. This may be a result of poor pollen transfer as a result of large inter-tree distances in low density stands. This suggestion is supported by lowered levels of outcrossing found in low density stands of ponderosa pine (Farris & Mitton 1984).

Cone production

The rate of cone accumulation (indicated by the slope of the regressions) varied very slightly among populations as did size at which trees began to produce cones (indicated by y-intercept). The plantation MB was an exception, however, producing cones at a much smaller tree size than other populations. This is possibly because the plantation is still young and has proportionately more juveniles and less senescent trees than the natural populations.

Conclusion

Reproductive and ecological fitness components were found to vary, albeit slightly in many cases, between populations of *W.cedarbergensis*. Three broad types of population structures were found where (i) some populations had between 50-65% of individuals falling into the seedling class, (ii) other populations lacked adequate representation in the seedling class and (iii) the MB plantation, being a young plantation, which lacked adults. These population structures were reflected by seedling:parent ratios. Measures of adult fecundity did not point to any one population being fitter than others in this respect. This was attributed to the effects of environmental heterogeneity. This finding strongly indicated the lack of genetic

influence on adult fecundity. Differences in vegetative characteristics of seedlings between populations are examined in Chapter 5. Chapter 6 links levels of genetic variation to levels of fitness, showing that differences in adult fecundity are not determined by genetic variation. DG, SB and, to some extent, CPS, can be considered the three populations showing no negative demographic effects. Low seedling numbers in the MB plantation were attributed to the fact that it is a young "population" with very few large trees and lack of regeneration in KK and WB could be attributed to lowered cone production above an optimal tree size. This was a strong indication that some populations may be senescent.

CHAPTER 5: SEEDLING FITNESS DIFFERENCES BETWEEN POPULATIONS OF WIDDRINGTONIA CEDARBERGENSIS.

Introduction

The effort to conserve *Widdringtonia cedarbergensis* by Cape Nature Conservation has concentrated on a replanting scheme in a demarcated area in the Cedarberg called Welbedacht (van der Merwe & Wessels 1993). This has involved collecting seeds predominantly from the Middelberg plantation (abbreviated as MB in this thesis) which is easily accessible from the forest station, and germinating and rearing the seedlings in a nursery until they are ready for replanting. More recently, however, seed collections from natural populations for the replanting scheme have begun. Little, if anything, is known about the original seed source of the plantations since no records are in existence. This chapter reports a screening of natural populations and plantations directly for seedling vigour in the form of germination success, chlorotic seedling frequency, dry biomass and drought survival.

Chapter Aims

In this chapter, several practical and theoretical considerations for the implementation of the replanting scheme are addressed. It aims to determine (i) whether there are differences in seedling fitness components between populations; (ii) whether the differences, if any, are consistent among populations; (iii) which populations are more consistent with regard to seedling fitness; and (iv) levels of seedling vigour in the plantations.

Seedling vigour is important in the replanting scheme since seedlings have to face at least four months of summer drought each year. Mustart (1993) has found that survival success of the replanted seedlings varied markedly between planting years according to the severity of the

ensuing annual drought. Further, the replanting programme operates on a limited budget and, for effective results, it would be desirable to have seeds with high germination success, rapid growth rate and high survival success.

The fitness of the plantation seed stock is an important aspect of this chapter. The plantations represent unstructured populations with no familial groupings unlike the natural populations. Determining the fitness of the resultant seeds and seedlings is important firstly because the plantation is a convenient seed source; secondly because the seeds of the plantation should be outbred, and thirdly because the replanting area will ultimately be a plantation itself and the existing plantations are therefore natural experiments.

Materials and Methods

In order to find differences in seedling fitness between populations of *W.cedarbergensis*, a growth experiment was set up in a glasshouse at U.C.T. in June 1993. The populations studied were the seven populations of *W.cedarbergensis* described in Chapter 2, Table 2.1. The same seed collection was used in this experiment as for that described in Chapter 2.

Seeds of each subpopulation were sown in pipes which were 0.5m in length and 7cm in diameter. The pipes were open-ended but had a double layer of gauze taped onto one end for drainage purposes. The pipes were filled with a 50:50 mixture of acid-washed sand and with peat. Ten seeds were sown in each pipe and these were replicated 25 times for each subpopulation. The pipes were arranged in a randomized block design of 7 blocks with 25 pipes in each, situated in a glasshouse.

Initially, the pipes were watered daily. Germination rate was recorded twice weekly for 11 weeks. Seedlings were thinned until one seedling was left in each pipe. This was done in such a way that the seedling left to grow in the pipe was the first seed to germinate. Peculiarities such

as chlorotic effects were recorded. After six months all seedling shoots were measured for canopy height and width. Ten seedlings per subpopulation were harvested at this stage, oven-dried at 60°C and their above- and below-ground biomass determined.

The remainder of the seedlings were left in their pipes in order to test for differences in drought survival. Initially, the pipes were transferred into plastic troughs which were filled with water to 20cm. The plants were not watered from this point until the termination of the experiment. Survival of the seedlings was recorded once a month. The object of the troughs was to ensure that some of the more etiolated seedlings did not die as a result of a higher transpiration rate than the less etiolated seedlings. Survival at this point was therefore determined by root length. After 3 weeks, the water level in the troughs was lowered to 5cm. This was done so to simulate the gradual lowering effect of a water table during a summer drought. After 5 weeks the pipes were removed from the troughs completely until the completion of the experiment. The droughted seedlings were accidentally watered in January 1994. The survival experiment was terminated in July 1994, 13 months after the growth experiment was initiated.

Data Analysis

Seedling size was estimated as volume calculated by using the equation for the volume of a cylinder (πr^2 x height). Differences in germination percentage and seedling volume between populations of *W.cedarbergensis* and blocks were determined using a two-way ANOVA.

Peto and Peto's Logrank Test outlined in Pyke and Thompson (1986) was used to compute pairwise differences in germination rate and survival between populations of *W.cedarbergensis*. This is a goodness of fit method which computes expected germination or survival values for each interval using the proportion of seeds germinated or seedlings dead, in the case of survival rate, at each interval in each population relative to the sum of germination or mortality events for all the populations. The logrank statistic was then computed which gives a chi-square value. Differences in frequencies of chlorotic seedlings were determined by a Chi-squared contingency analysis.

One-way ANOVAs were used to find differences in root, shoot and total biomass of seedlings between populations and between experiment blocks. A two-way ANOVA was not possible since the initial balanced design became unbalanced through random selection of seedlings. Homogeneity of variance was tested using a Cochran's C test and a multiple range test, using confidence limits, was used to determine patterns of variation between populations. Appropriate transformations or non-parametric tests were used where data was not normally distributed.

Results

Germination

Germination percentages were remarkably high over all populations. Differences in germination percentage between populations were found to be significant, however, without interactions with block positions (Table 5.1, here and elsewhere, * = $P<0.01$, ** = $P<0.05$, *** = $P<0.001$, NS = non-significant; MRT = confidence limit multiple range test). Higher germination percentages were found in DG, CPS while WB, MB and to a lesser extent, KK were found to have lower germination percentages (Table 5.2).

Germination rates are depicted by the curves in Figure 5.1. Germination of seeds began after 3.5 weeks in all populations. After this point the curves showed considerable variation indicating differences in germination rate. The logrank test showed that differences between the curves were significant for most pairwise comparisons except for the comparison between DG and KD and between DG and WB (Table 5.3a). An attempt was made to quantify the differences in germination rate in Table 5.3b by counting the number of germination events in each population between the period when 1/3 of the running time of the experiment was completed and 2/3 of the running time of the experiment was completed. DG and CPS were found to have the highest germination rates while KK and WB had the lowest germination rates.

Table 5.1. Results of a two-way ANOVA on the germination percentage between populations and experiment blocks of *W.cedarbergensis* (NS = non significant).

Level	F	d.f.	P
Population	5.60	6	**
Block	2.13	6	NS
P x B	1.35	36	NS

Table 5.2. Germination percentage means for *W.cedarbergensis* (values in parentheses are standard errors; MRT = confidence limit multiple range test.

Population	N	Germination %	MRT
WB	25	78.4 (2.9)	*
MB	25	78.4 (4.3)	*
KK	25	82.4 (4.5)	**
KD	25	87.2 (2.5)	***
SB	25	88.8 (2.5)	***
CPS	25	91.6 (2.9)	**
DG	25	96.4 (1.4)	*

Table 5.3. (a) Logrank values for pairwise comparisons of germination rates between populations of *W.cedarbergensis* (NS = non significant); (b) Germination rate calculated as number of germination events between 1/3 of the total running time of the experiment and 2/3 of the total running time of the experiment.

(a) Logrank analysis

	DG	KK	WB	SB	MB	KD	CPS
DG							
KK	6.16						
WB	NS	5.59					
SB	12.40	17.40	11.83				
MB	7.91	12.91	7.34	19.16			
KD	NS	8.34	NS	14.58	10.09		
CPS	11.36	16.36	10.79	22.60	18.11	13.54	

(b) Germination rate

	DG	KK	WB	SB	MB	KD	CPS
Number of individuals	236	177	183	211	191	206	216

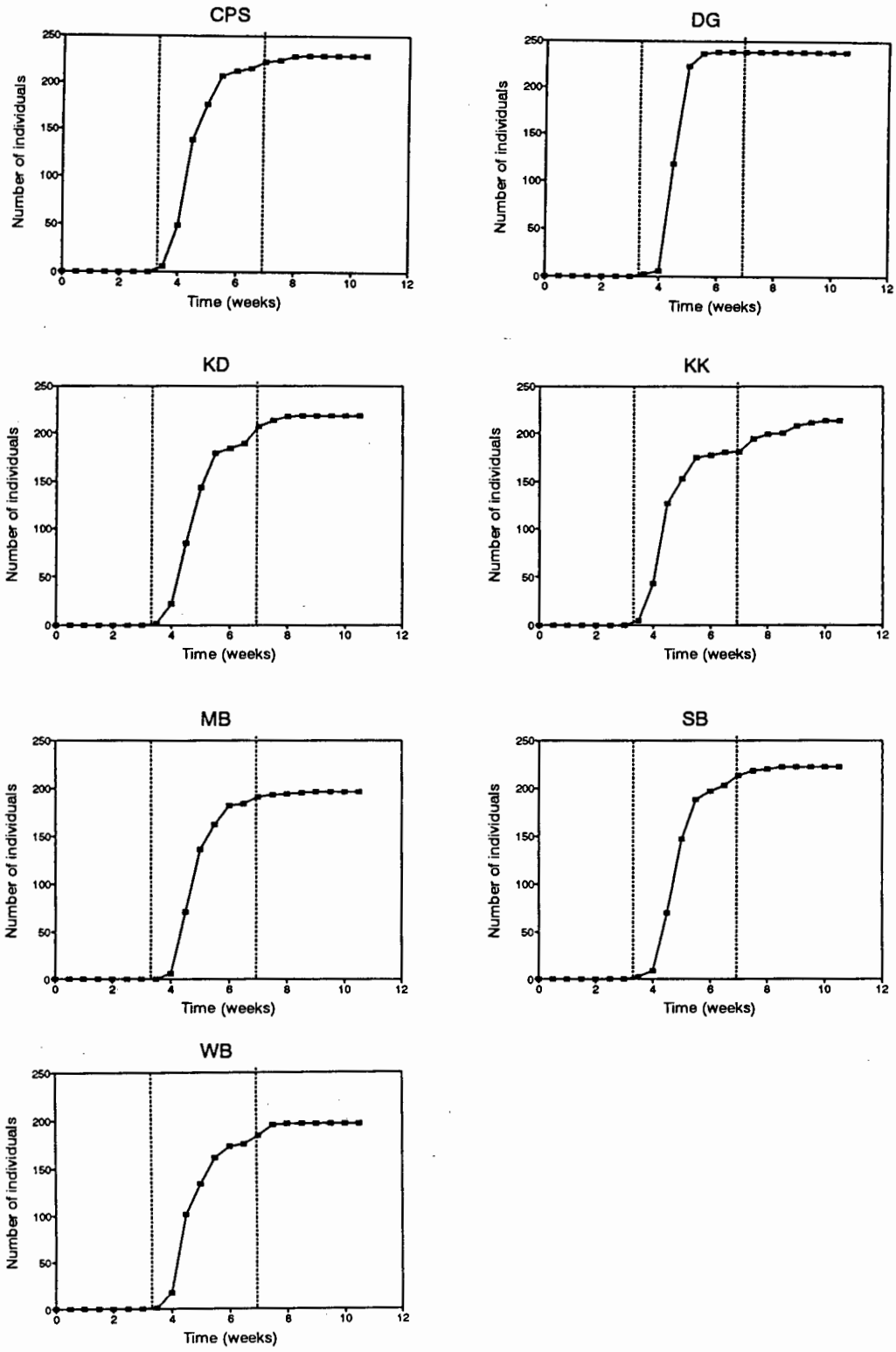


Figure 5.2. Germination rate of seedlings in different populations of *W.cedarbergensis*. Vertical dotted lines indicate 1/3 to 2/3 total experiment running time interval.

Chlorotic seedling frequency

A significant difference in chlorotic seedling frequency between populations was found (Table 5.4). Chlorotic seedlings were found to be dramatically more frequent in the two plantations, MB and especially KD, the frequencies of which account for as much as 21% and 52% of the variation found between populations respectively (Table 5.4). The incidence of chlorosis is markedly absent in the natural populations.

Seedling Size

Seedling volume differed significantly between populations but there was no significant block effect (Table 5.5). A table of means (Table 5.6) showed that seedlings in DG were more than double the volume of seedlings in WB, MB and KK.

Table 5.4. Differences in frequency of chlorotic seedlings in populations of *W.cedarbergensis*. $\chi^2 = 159.66$, d.f. = 6, $P < 0.001$.

Population	CPS	DG	KD	KK	MB	SB	WB	Total
Number Germinated	229	238	218	213	196	222	196	1512
Number Chlorotic Seedlngs	2	1	40	0	27	1	1	72
Expected Chlorotic Seedlings	11.0	11.4	10.5	10.2	9.4	10.7	9.4	
χ^2	7.35	9.51	83.42	10.22	32.88	8.75	7.52	159.6

Table 5.5.Results of a two-way ANOVA on seedling volume (πr^2 x height) between populations and experiment blocks.

Level	F	d.f.	P
Population	20.17	6	***
Block	1.69	6	NS
Pop x Block	1.15	36	NS

Table 5.6. Mean seedling volume (πr^2 x height) for populations of *W.cedarbergensis*. Values in parentheses indicate standard error.

Population	N	Volume (mm^3)	MRT
WB	25	131730 (12848)	*
MB	25	145473 (12734)	*
KK	25	157853 (12597)	*
SB	25	180986 (16016)	**
CPS	25	212982 (13722)	**
KD	25	253185 (14973)	**
DG	25	301104 (15130)	*

Table 5.7. Results of two one-way ANOVAs of populations and experiment blocks on (a) root biomass (b) shoot biomass and (c) total biomass (NS = non significant).

Level	F	d.f.	P
(a) Shoot biomass			
Population	6.32	6	**
Block	1.48	6	NS
(b) Root biomass			
Population	3.52	6	*
Block	0.53	6	NS
(c) Total biomass			
Population	5.26	6	**
Block	0.99	6	NS

Table 5.8. (a) Shoot, (b) root and (c) total dry biomass of seedlings of populations of *W.cedarbergensis* harvested after six months.

Population	N	Mean dry mass (g) (S.E.)	MRT
		(a) Shoot biomass	
MB	11	0.109 (0.008)	*
KK	10	0.109 (0.012)	*
WB	10	0.119 (0.008)	**
SB	10	0.140 (0.007)	***
DG	9	0.150 (0.005)	**
KD	9	0.156 (0.008)	*
CPS	8	0.158 (0.010)	*
		(b) Root biomass	
KK	10	0.103 (0.015)	*
MB	11	0.109 (0.011)	**
WB	10	0.135 (0.015)	***
DG	9	0.142 (0.009)	***
SB	10	0.146 (0.015)	***
CPS	8	0.157 (0.014)	**
KD	9	0.169 (0.010)	*
		(c) Total biomass	
KK	10	0.212 (0.026)	*
MB	11	0.218 (0.018)	*
WB	10	0.254 (0.022)	**
SB	10	0.286 (0.018)	***
DG	9	0.292 (0.010)	***
CPS	8	0.315 (0.022)	**
KD	9	0.325 (0.016)	*

Seedling Biomass

Major differences in root, shoot and total biomass between populations were found but there were no significant block effects (Table 5.7). In total, seedlings from KD had 63% more dry biomass than seedlings in KK and MB (Table 5.8c). Differences in root and shoot biomass followed the same trend between populations and a distinction can be made between the populations MB, KK and WB which all had light seedlings, and the populations SB, DG, KD and CPS which had much heavier seedlings.

Drought Survival

Seedling mortality rates recorded during the drought experiment showed considerable variation in shape (Figure 5.2). The logrank analysis showed that most pairwise comparisons of the curves were significantly different with the comparisons between SB and KK, and SB and MB (Table 5.8a). Lethal dose average, indicated on the curves, show that survival of seedlings under droughted conditions was more pronounced in WB and MB. An attempt was made to further quantify mortality rate differences by counting the number of deaths occurring between 1/3 of the running time of the experiment and 2/3 of the running time. Sample sizes were possibly too small for a realistic estimate of mortality rate using this method.

Table 5.9. (a) Logrank values of pairwise comparisons of survival rates between populations of *W.cedarbergensis*. Values below a Chi-square of 3.84 were insignificant at the 0.05 level; (b) Mortality rate measured by number of deaths between 1/3 of the total running time of the experiment and 2/3 of the total running time of the experiment.

(a) Logrank analysis

	DG	KK	WB	SB	MB	KD	CPS
DG							
KK	22.30						
WB	30.40	8.88					
SB	22.05	NS	8.63				
MB	25.38	3.86	11.96	NS			
KD	27.58	6.06	14.16	5.81	9.14		
CPS	62.21	40.69	48.79	40.44	43.77	45.97	

(b) Mortality rate

Population	DG	KK	WB	SB	MB	KD	CPS
Number of individuals	7	10	8	9	10	12	8

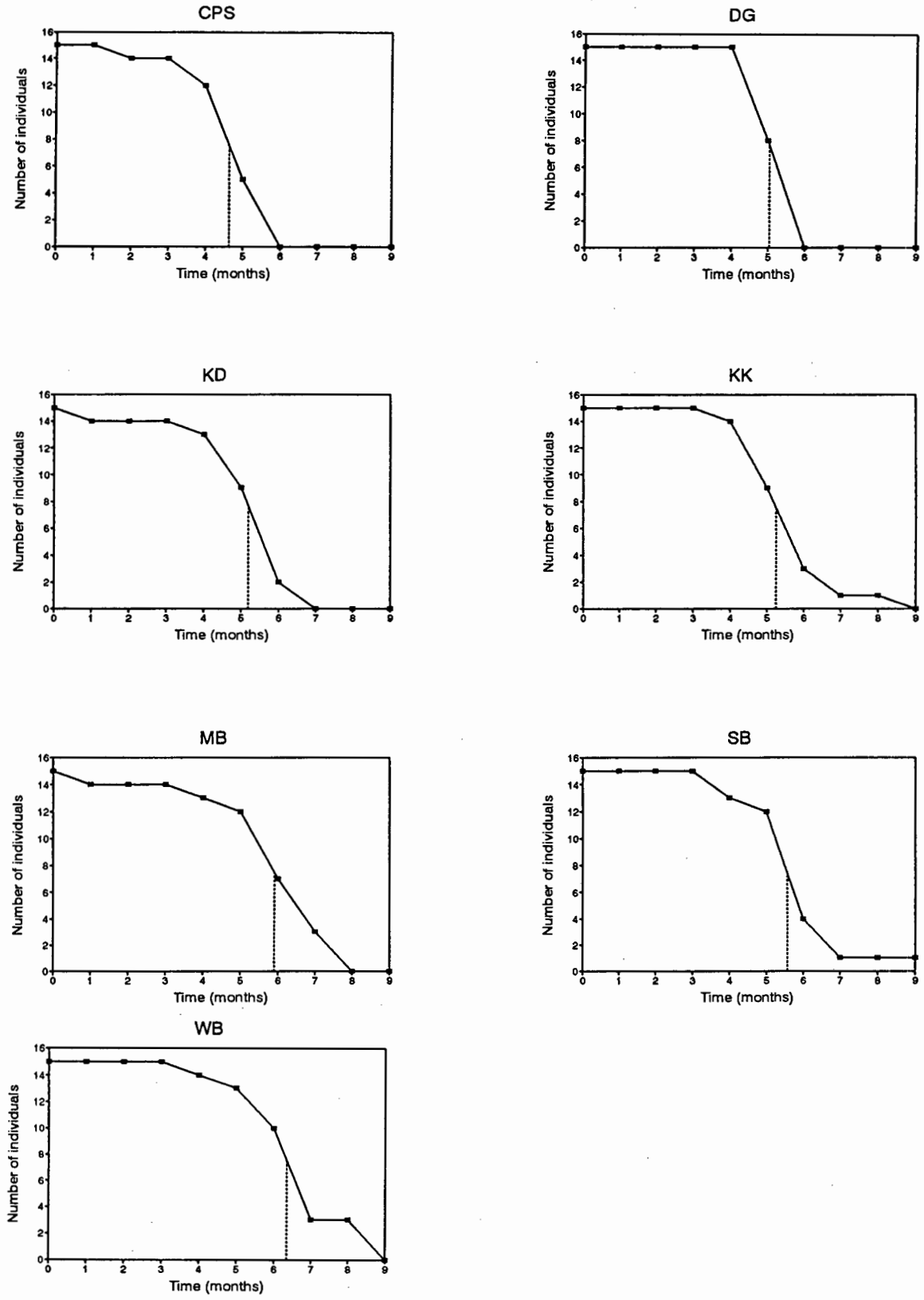


Figure 5.2. Mortality rate of seedlings subjected to drought in populations of *W.cedarbergensis*. The vertical dotted line indicates date by which 50% of individuals died.

Discussion

Seedling Vigour

Fitness components are summarised in Table 5.9 as a proportion relative to the maximum estimate for each variable. A strong trend in consistency between populations was found where populations such as DG, KD and CPS (KD being a plantation) were found to have the fittest seedlings and where KK and WB were found to have seedlings lacking in vigour. Since the growth experiment was in effect a common garden experiment, the differences in seedling fitness components between populations of *W.cedarbergensis* are likely to have a genetic base and unlikely to be a result of environmental heterogeneity.

Table 5.9. Summary table of seed and seedling fitness in *W.cedarbergensis*. Fitness values are expressed relative to the population with the maximum estimate for each variable (indicated by 1.00) for each variable.

Variable	Population						
	CPS	DG	KD	KK	MB	SB	WB
Germination %	0.95	**1.00**	0.90	0.86	0.81	0.92	0.81
Germination rate	0.92	**1.00**	0.87	0.75	0.81	0.89	0.78
Chlorosis	0.95	0.98	0.00	**1.00**	0.33	0.98	0.98
Volume	0.71	**1.00**	0.84	0.52	0.48	0.60	0.44
Shoot biomass	**1.00**	0.95	0.99	0.69	0.69	0.89	0.74
Root biomass	0.93	0.84	**1.00**	0.61	0.65	0.86	0.80
Total biomass	0.97	0.90	**1.00**	0.65	0.67	0.88	0.78
Mortality rate	0.86	**1.00**	0.29	0.75	0.57	0.71	0.86
LDA	0.69	0.77	0.77	0.77	0.92	0.85	**1.00**

The general differences in seedling vigour may be a reflection of inbreeding depression (i) as a result of population size or (ii) pollen movement within populations as a result of adult tree density. The effect of these two factors on fitness components are examined in Chapter 6.

Seedlings, however, would be more susceptible to these two factors since they represent the next generation.

The results presented in this chapter have implications for the replanting scheme at Welbedacht. Populations whose seedlings have high germination success, rapid growth rate and high drought survival rate would be ideal as a seed source for the replanting programme. Populations which ranked high in all three of these aspects were CPS, DG and KD.

Germination is an important component of fitness in plant species (Menges 1991a) since the earliest seeds to germinate can capitalise on resources soonest and thereby outcompete other plants more effectively. Other studies of plant species have found significant differences in germination percentage between populations (Menges 1991a). Shea (1987) mentions that several studies of conifers have reported a reduction in germination percentage in selfed seeds.

Numerous studies have shown that survival of seedlings is related to heterozygosity and thus a good measure of fitness (Farris & Mitton 1984; Shea 1987). Survival rate in *Widdringtonia* is interesting in that it took nine months for all the seedlings to die. This may be an overestimate of how long seedlings would ordinarily survive in the field since the seedlings were accidentally watered early on in the survival experiment. On average, however, half the seedlings died after 5 months in all populations. The tail-enders would therefore definitely survive a summer drought, assuming that this experiment simulates of conditions in the field. Populations with more tail-enders, such as WB, MB and SB, therefore are more likely to have more seedlings at the end of summer droughts.

The interpretation of seedling growth differences was potentially complicated by differences in seedling emergence time. However, because late emerging seedlings were thinned in each pipe, size differences represent seedlings that germinated within days of each other. Growth rate is a good surrogate measure of fitness in pines (Bush et al. 1987). Differences in growth rate between populations of *Pinus rigida* were found although they were to some extent attributed to environmental differences between sites (Bush et al. 1987).

Patterns of chlorotic seedling frequency were at variance with trends of fitness for other variables. Hardly any chlorotic seedlings were found in the natural populations, although high incidences were found in the two plantations, KD and MB. Albinism has also been reported for *Pinus ponderosa*, the Ponderosa pine (Mitton et al. 1981). Frequency of albinism was used to measure rates of outcrossing in *Pinus ponderosa* since albinism represents a single recessive allele rendering homozygous albinos easily visible. Outcrossing rates estimated in this way were found to approximate outcrossing rates estimated from protein polymorphisms. This is contrary to the findings of this study where levels of outcrossing found in MB, one of the plantations of *W.cedarbergensis* were higher than most of the natural populations, although levels of outcrossing were low in the other plantation, KD. Patterns of chlorosis do not follow the trends of other fitness values although the differences in chlorotic frequency between populations were so striking as to be included in this study. However, chlorosis in *Widdringtonia* is likely to have a genetic basis, i.e. the expression of a single lethal recessive allele, although this has by no means been determined. Possible causes of high chlorotic seedling frequency in plantations of *W.cedarbergensis* include (i) outbreeding depression as a result of the plantations being established from a varied seed source or (ii) the founder effect as a result of the plantation being established from seeds from a small number of trees.

The plantations generally show reasonable to high levels of seedling fitness apart from chlorotic effects. These results, therefore, cast the future of the replanting programme at Welbedacht in a favourable light since the area will ultimately be a plantation.

Conclusion

Populations of *W.cedarbergensis* differed markedly with regard to seedling vigour. These differences are important considerations for the replanting scheme at Welbedacht since it is necessary to utilise the fittest seed source for effective results. The differences between populations were surprisingly consistent across fitness variables with the result that the populations MB, WB and KK could be isolated as having weak seedlings and DG, CPS, SB and KD as having more vigourous seedlings. DG and KD could be singled out especially as the two

most consistently fit populations. Although the plantations have been a convenient seed source in the past for replanting owing to their accessibility and proximity to the forest station (in the case of MB), seed collection for the replanting programme should be supplemented by seeds collected from vigorous natural populations such as DG and CPS. These two populations represent the western and eastern Cedarberg respectively and are therefore ideal as a seed source combination to buffer the possible effects of local adaptation in the replanting scheme. Chlorotic seedling frequency was highest in the plantations, especially KD. Seed collection from this plantation should be avoided to circumvent wasted effort.

CHAPTER 6. INBREEDING DEPRESSION AND POPULATION SIZE IN WIDDRINGTONIA CEDARBERGENSIS

Introduction

The concept of inbreeding depression was developed through crop studies where outbred strains were found to be more healthy and productive than inbred strains. This observation was eventually explained in terms of heterozygosity where outbreeding led to increased levels of heterozygosity and subsquently increased fitness. The apparent relationship between fitness and heterozygosity was termed heterosis. Conversely, the lack of fitness found in inbred strains was termed inbreeding depression. The precise mechanism by which homozygosity is related to decreases in viability and fecundity is disputed (Charlesworth & Charlesworth 1987; Lacy 1992). Evidence for and against the relationship between fitness and heterozygosity in naturally-occurring species has been widely reported (reviewed in Ellstrand & Elam 1993; Charlesworth & Charlesworth 1987; Mitton & Grant 1984), surrounding the topic with controversy.

With the development of modern conservation science, much emphasis has been placed on the demographic and genetic fates of small populations which are particularly subject to environmental and genetic stochasticity (Menges 1991b) and to genetic drift and inbreeding (Ellstrand & Elam 1993). The erosion of genetic variation in small populations has led to studies of fitness in relation to population size. Only two studies for plant species (Menges 1991a; van Treuren et al. 1993), of which I am aware, link fitness to population size. The present study is the only one of which I am aware that links levels of genetic variation to both genetic variation and to population size. One such study (Lacy 1992) exists for an animal species.

Chapter Aims

Populations of *W.cedarbergensis* are highly differentiated with regard to population genetic structure (see Chapter 3). As such, they are particularly vulnerable to genetic drift and inbreeding. Further, there is an ever-present threat of an intensifying bottleneck and extensive population fragmentation. The major cause of this threat is the damaging effect of fire to which the Clanwilliam cedar is poorly adapted; compounded by intensive logging practices in the last two centuries. In this chapter, the following questions are asked:

1. Is there a relationship between fitness and heterozygosity in populations of *W.cedarbergensis*? An attempt to answer this question is made by determining whether the fitness components described in Chapters 4 and 5 co-vary with levels of observed heterozygosity reported in Chapter 3.
2. Which components, if any, co-vary with heterozygosity?
3. Do fitness components and heterozygosity co-vary with population size?
4. Is there a critical population size in *W.cedarbergensis*?

Materials and Methods

The populations sampled in this chapter are described in Chapter 2. Estimates of relative reproductive and ecological fitness components for each population were obtained from the results of Chapter 4 and estimates of seedling fitness components were likewise obtained from the results of Chapter 5. Levels of heterozygosity for each population were estimated using protein electrophoresis, the methods and results of which are given in Chapter 3. Fitness component estimates were incorporated into a correlation matrix with levels of heterozygosity reported in Chapter 3 for each population. Significant trends between fitness components and heterozygosity were determined. Relative measures were used for all fitness variables. The correlation matrix was analysed using Statgraphics software.

Population size was estimated by categorising each population with respect to number of individuals, distribution of trees, and degree of isolation from other populations. Populations were rated on a scale of 1 to 5 accordingly (Table 6.1).

Table 6.1. Size scale ratings for populations of *W.cedarbergensis* according to tree numbers, tree distribution and degree of isolation.

Population	Description	Scale
KK	≤ 100 individuals sparse distribution > 5km to next population	1
KD	≤ 150 individuals dense distribution >5km to next population	1.5
WB	≤ 250 individuals clumped distribution < 2km to next population	2
MB	≤ 1500 individuals dense distribution < 1km to next population	3
SB	≤ 2000 individuals clumped distribution < 1km to next population	4
DG	≤ 2500 individuals clumped/continuous distribution < 1km to next population	5
CPS	≤ 2500 individuals clumped/ continuous distribution < 1km to next population	5

The relationship between population size and heterozygosity was plotted in order to determine whether there was a minimum viable population size for *W.cedarbergensis*. The effect of very recent fires were disregarded in estimating population size. For example, the population DG which was once an enormous population of cedars, was reduced in size by about 90% by a wild fire in 1991. Since the fire was recent enough not to have had any effect on the seed stock used in this study (cones take several years to mature), the effect of this fire on population size was ignored.

Table 6.2. Results of a correlation matrix analysis showing significant and insignificant relationships between fitness traits and heterozygosity (* = P<0.05; ** = P<0.01; *** = P<0.001; (+) = positive relationship; (-) = negative relationship)

	slope	smass	sset	abort	S:P	y-int	germ%	grate	chlor	svol	shoot	root	total	srate	LDA	Het
[a] slope																
[a]smass	-															
[a]sset	-	-														
[a]abort	-	-	-													
[a]S:P	-	-	-	-												
[a]y-int	*(-)	-	-	-	-											
[b]germ%	-	-	-	-	-	-										
[b]grate	-	-	-	*(+)	*(+)	-	*(+)									
[b]chlor	*(+)	-	-	-	-	***(-)	-	-								
[b]svol	-	-	-	-	*(+)	-	**(+)	**(+)	-							
[b]shoot	-	-	-	-	-	-	*(+)	*(+)	-	-						
[b]root	-	-	-	-	-	-	-	-	-	-	*(+)					
[b]total	-	-	-	-	-	-	-	*(-)	-	-	**(+)	***(+)				
[b]srate	-	-	-	*(+)	-	-	-	-	-	-	-	-	-			
[b]LDA	-	-	-	-	-	-	*(+)	-	-	-	-	-	-	-		
[c]Het	-	-	-	*(+)	-	-	-	*(+)	-	-	*(+)	-	*(+)	-	-	

a denotes reproductive and ecological fitness components described in Chapter 4; slope and y-int refer to slope and y-intercept of regressions of cone production against trunk diameter; abort refers to seed abortion fraction; S:P refers to seedling to parent ratio.

b denotes seedling fitness components described in Chapter 5: germ% and grate refer to germination percentage and germination rate; chlorosis refers to chlorotic seedling frequency; svolume refers to seedling canopy volume; shoot, root and total refer to seedling dry biomass values; srate refers to drought survival rate; LDA refers to lethal dose average.

c denotes percentage observed heterozygosity reported in Chapter 3.

Results

The results of the correlation matrix are shown in Table 6.2. 18% (21/120) of the correlations were significant. Only four fitness components were found to co-vary with heterozygosity. These components were abortion fraction, germination rate, shoot biomass and total biomass. These four variables, however, co-varied with other measures of fitness. Abortion fraction was positively related to germination rate and survival rate. Germination rate was positively associated with seedling volume, shoot biomass, total biomass, abortion fraction and seedling:parent ratio. Shoot mass was positively related to root biomass, total biomass, germination % and germination rate. Total biomass was positively related to germination rate, shoot biomass and root biomass.

Figure 6.1 depicts the relationship between heterozygosity and (a) abortion fraction, (b) germination rate, (c) shoot biomass, (d) total biomass, and (e) population size. The populations DG and CPS and to a lesser extent, KD, are consistently at the upper end of the scale in all of the plots. WB, KK, MB and to some extent, SB, are consistently at the lower end of the scale in plots (a) to (e). Figure 6.1(e) shows a similar trend in that DG and CPS which have the highest levels of heterozygosity are found at the upper end of the population size scale and KK, WB and MB which all have lower levels of heterozygosity are found at the lower end of the population scale. There are two noticeable outliers in this plot, however. These are KD and SB. In all the plots there was a noticeable drop in fitness or population size below 30% heterozygosity. This level of heterozygosity occurred below a population size scale of 4. Levels of fitness were critically low below 25% heterozygosity at a population size scale of 1.

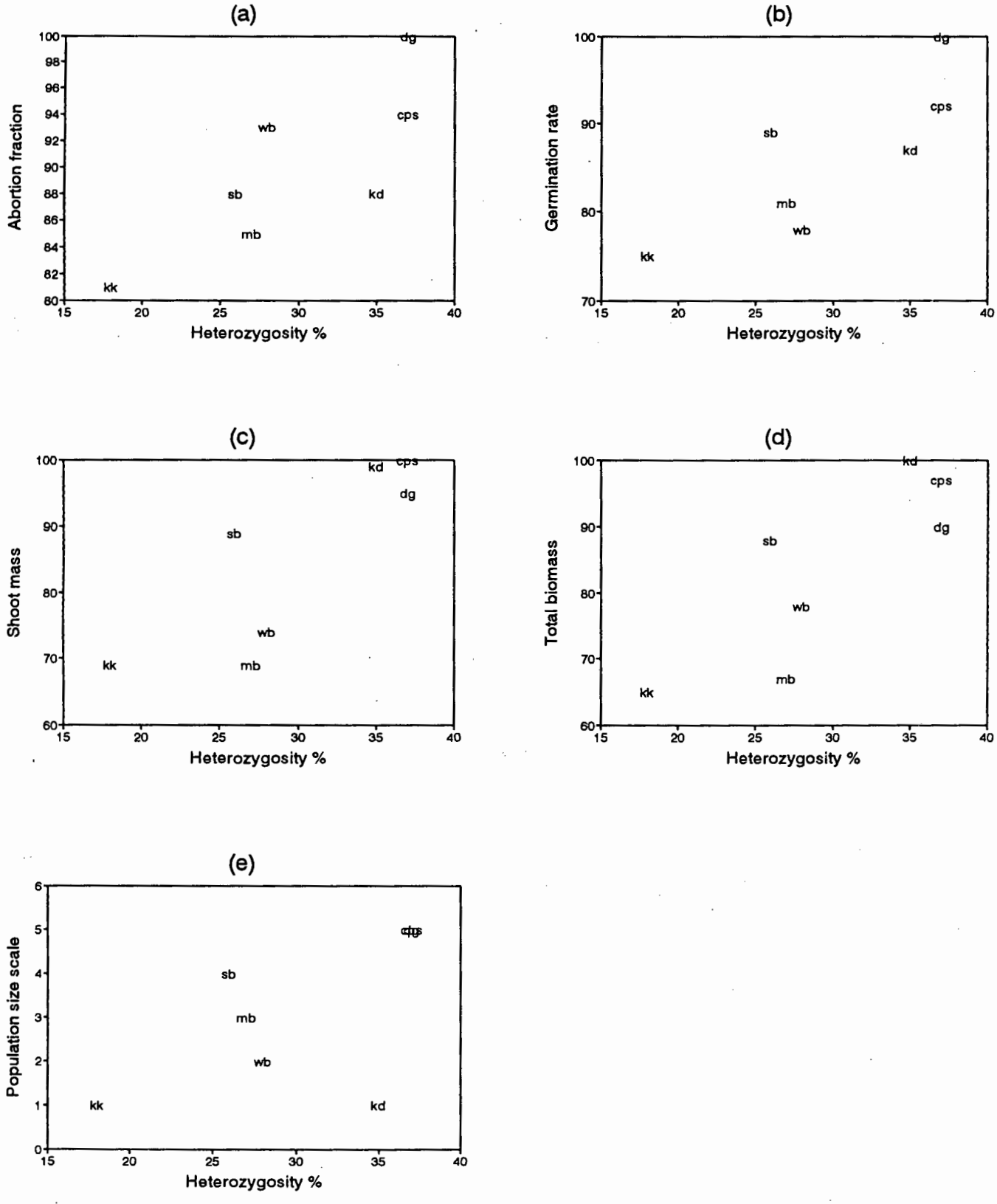


Figure 6.1. Plots showing the relationship between heterozygosity and (a) abortion fraction, (b) germination rate, (c) shoot biomass, (d) total biomass, and (e) population size.

Discussion

Evidence for inbreeding depression

Genetic quality of populations of *W.cedarbergensis* appears to have a direct impact on four out of fifteen measures of fitness. This implies that even though so many traits are significantly variable among populations, the variation in these traits is not genetically determined. The traits that were found to share a significant relationship with heterozugosity were abortion fraction, germination rate, seedling shoot dry mass, total dry mass and survival rate, all of which are commonly associated with levels of heterozygosity (see van Treuren et al. 1993).

1. EMBRYO ABORTION.- Levels of embryo abortion have sometimes been negatively associated with levels of genetic variation in other studies. Wiens et al. (1987) found that outcrossed flowers of *Epilobium angustifolium* aborted more embryos than did selfed flowers and they attributed this to the effect of genetic load and developmental lethals. Levels of embryo abortion in *W.cedarbergensis*, however, are clearly positively influenced by increased levels of genetic variation. This suggests that *W.cedarbergensis* is reliant on the availability of outcrossed pollen for reproductive success. Pollen movement has been found to be limited by plant density in animal-pollinated rain-forest species (House 1992) and in wind-pollinated conifers (Farris & Mitton 1984). The fine-scale fragmentation of tree clumps within subpopulations of *W.cedarbergensis* as a result of fire may play a major role in determining levels of embryo abortion.

2. GERMINATION.- The correlation between germination *rate* and protein heterozygosity in populations of *W.cedarbergensis* implies increased levels of developmental stability in more genetically variable seedling populations of *W.cedarbergensis*. Differences in developmental stability *among* populations have been reported for a side-blotched lizard (Soule 1979). The Higher germination rates are advantageous in that seedling establishment is quick, increasing the chances of escaping early size-dependent mortality. Reports of germination rates in other conifer species are rare although several studies in conifers have reported a reduction in germination percentage, which co-varies with germination rate here, in selfed seeds (Ellstrand & Elam 1993). Menges (1991a) found a marked decrease in germination

percentage in small populations of *Silene regia*, a fragmented prairie species, and attributed these differences to inbreeding depression.

3. GROWTH RATE AND PLANT SIZE.- The association between protein heterozygosity has been widely reported in animal studies and was first reported in plants for quaking aspen (*Populus tremuloides*) (Mitton & Grant 1984). Growing to a large size quickly decreases the chances of early size-dependent mortality in seedlings (McGraw & Garbutt 1990). Much interest has been given to growth rate in adult trees as a fitness trait in pine species. In *Pinus ponderosa* and *Pinus contorta*, heterozygosity was related to the variability in growth rate but not to the mean growth rates of mature trees. Very few studies have studied variation in growth rate of seedlings as a fitness trait in conifers although differences in seedling growth rates have been found between genotypes within populations for *Pinus ponderosa* (Farris & Mitton 1984 reviewed in Mitton & Grant 1984) and between populations of *Pinus rigida* (Ledig, Guries & Bonefeld 1983). The findings indicate that growth rate may be a useful fitness trait in conifers.

Traits not linked to heterozygosity in this study have been determined as fitness traits in other studies. Chlorosis, for example, should give a good indication of inbreeding depression if it represents a single lethal recessive allele. Mitton et al. (1981) derived similar estimates of outcrossing levels using either seedling albino frequencies or allozyme data in ponderosa pine. Albinism in *W.cedarbergensis* is a poor measure of fitness yet has a fatal effect on seedlings. Mortality induced by a lethal recessive gene is classified under hard selection (Beardmore 1983) where selection on the phenotype is an absolute criterion dependent on the genotype and independent of all other factors. Hard selection in *W.cedarbergensis* may therefore play a minor role in its evolution compared with environmental effects.

Most traits that were found to co-vary with heterozygosity in *W.cedarbergensis* were seed- or seedling-linked traits. This finding is not unusual since there is usually a large disadvantage to the products of selfing in conifers compared with outcrossed progeny (reviewed in Charlesworth & Charlesworth 1987). These results imply that current population dynamics are being impacted by differences in progeny fitness among populations of *W.cedarbergensis*.

Other complexities such as masting years and energy allocation may further confound the relationship between adult fitness and heterozygosity. In adults, all surplus energy is divided between reproduction and growth, whereas in seedlings, all surplus energy is allocated to growth alone (see Mitton & Grant 1984). Masting seems to occur in populations of *W.cedarbergensis* (pers. obs). Cones are frequently eaten by baboons and masting may have evolved to reduce seed reduction (Harper 1977). Linhart and Mitton (1985) looked at the role played by genetic variation in this complexity. They found that groups of trees that deviate from their predicted productivity of female cones differ from one another not only in growth rates but also genetically. More genetically variable trees were clustered more tightly around the mean cone production. It is possible that differences in heterozygosity levels among populations of *W.cedarbergensis* affect variation in cone production rather mean cone production.

Genetic effects on population ecology

Traits, such as germination rate, which are affected by genetic quality appear to have an impact on the ecology of populations of *W.cedarbergensis.* The sample size for all correlations was 7 (6 in some cases), since there were only seven populations in the study. Sampling error was therefore a problem in this study, and for many correlations between fitness traits and heterozygosity, probabilities were close to significance. However, where such a relationship with heterozygosity was obscured by sampling error, it was picked up through co-variation with another variable. In this way, germination %, seedling root biomass, seedling volume and seedling:parent ratio were indirectly related to heterozygosity. Germination %, root biomass and seedling volume represent some circularity in the data since germination co-varies with germination rate and root biomass and seedling volume co-vary with other seedling size and growth estimates. Co-variation between germination rate and seedling:parent ratio, on the other hand, is of particular interest since it implies that the demography of the Clanwilliam cedar is indirectly impacted by genetic quality. These findings therefore implicate poor genetic quality in extinction vortices (Gilpin & Soule 1986) in populations of *W.cedarbergensis*.

Population Size and Fitness

The majority of inbreeding depression studies have focused on individual genotypes. I am aware of only two studies of plant species to date which have looked at the effect of population size on inbreeding depression. These are Menges (1991a) and van Treuren et al. (1993). Lacy (1992) tested the hypothesis that small, isolated populations of *Peromyscus* mice would show less depression in fitness than would large, central populations. He found that remnant, insular populations had one quarter to one-third the genetic diversity of large central populations. Loss of fitness measured as infant viability, did not correlate with initial genetic diversity. The present study is the only study of which I am aware that links levels of genetic variation to both inbreeding depression and population size. The evidence presented here suggests that smaller populations of *W.cedarbergensis* suffer a deficiency of heterozygotes which leads to inbreeding depression. This in turn influences seed and seedling performance which influences population structure in the field. In *Haplocarpus bidwillii*, a dioecious conifer, a strong positive relationship was found between population size and genetic variation (Billington 1991) which is depleted in small populations through genetic drift and inbreeding followed by intensified selection on homozygotes.

In *Widdringtonia*, the two outliers in this analysis highlight the problem of small sample size in this study. It would have been preferable to have a much larger sample size in order to obtain a more rigorous result. The one outlier, KD is a small plantation with high levels of heterozygosity. A plausible explanation for this is that KD may have been established from seeds collected from a large population or several populations. SB, on the other hand is a large natural population whose seedlings are suffering from low levels of heterozygosity. This is possibly a result of a high level of population substructuring as a consequence of adult mortality during recent fires. Pollen movement among trees at different densities and genetic interaction between groups of trees on rocky outcrop refuges is an area which needs further investigation.

Implications for Conservation

The results presented here show that critically small populations of *W.cedarbergensis* suffer a major reduction in genetic variation. Critically small populations can be considered those

populations below 250 individuals such as KK and WB. To boost population fitness levels, several priorities for the conservation of the Clanwilliam cedar should be encouraged:
(1) progeny fitness could be boosted by possible hand pollination from diverse sources (a dubious practicality); (2) replanted seedlings should originate from a variety of sources to avoid familial grouping and thus inbreeding when seedlings reach reproductive maturity; (3) seedling replanting should be implemented in all critically small or highly structured populations to boost the seedling:parent ratio; and (4) further fragmentation *within* populations should be prevented as much as possible to minimise inter-tree distances.

Conclusion

Evidence for inbreeding depression in embryo abortion levels, germination rate, seedling shoot dry mass and seedling total dry mass was found for populations of *W.cedarbergensis*. Fitness seems to be dictated by population size to a large extent although the effect of inter-tree distances on fitness traits needs to be examined. Smaller populations of *W.cedarbergensis* showed a tendency for low performance with regard to fitness components while larger populations were noticeably more vigorous. The effects of genetic quality were extended to other traits through intercorrelation. Furthermore, genetic quality and seedling fitness were correlated with the demogarphic structure of populations (seedling:parent ratio). This implies a possible negative feedback system across generations or what has been termed as an "extinction vortex" (Gilpin & Soule 1986). These findings have several implications for conservation. A striking feature of this study was the impact of genetic erosion on seedling fitness traits more so than the genetic imapct on adult fecundity. This implies that the cedar is undergoing a genetic bottleneck and is at a critical stage where the next generation will be genetically depauperate if measures are not taken. Furthermore, it highlights the importance of the prevention of further adult mortality.

CHAPTER 7. CONCLUSION

I initially set out to answer several questions in this thesis. In this chapter I consolidate my findings and consider the extent to which my questions have been answered. Future directions for research are given.

1. Is there evidence for genetic erosion in the Clanwilliam cedar?

I showed that *W.cedarbergensis* had higher levels of genetic polymorphism than *W.schwarzii* and suggest that this was a result of a more sustained and prolonged bottleneck in *W.schwarzii* in the past. Populations of *W.schwarzii* were panmictic and outbred, perhaps partly explaining why this species is still ecologically successful despite the fact that it has been subjected to a bottleneck. Populations of *W.cedarbergensis*, on the other hand, were genetically isolated from each other and generally inbred which indicated that gene flow problems begin within populations, possibly as a result of trees being restricted to rocky outcrops. In this way it can be seen how susceptible *W.cedarbergensis* is to fragmentation on a fine scale.

W.nodiflora had much higher levels of polymorphism than *W.cedarbergensis* yet also had highly differentiated and inbred populations. I attributed levels of inbreeding in *W.nodiflora* to the effects of selfing between ramets of the same genet as a result of the sprouting behaviour of this species, and population differentiation to distance between populations as a barrier to gene flow. Neighbouring populations of *W.nodiflora* were more related to each other than were distant populations. This indicated the importance of distance as a barrier to gene flow rather than the effects of resprouting which seems to have an important influence on gene flow within populations.

One problem area remains unresolved in this question and deserves further attention, namely the fine-scale genetic structure within populations of the Clanwilliam cedar. This is important

since we need to know patterns of genetic interaction between tree clumps situated on rocky outcrops of *within* populations in order to determine the exact scale at which fragmentation becomes problematic. This could be established by (i) comparing levels of gene flow between rocky outcrops using seedling isozymes and (ii) comparing adult differentiation between rocky outcrops to determine whether neighbouring trees are related. Resolving this problem would essentially be determing whether inbreeding is more important than genetic drift in populations of *W.cedarbergensis*. The emphasis in conservation genetics has fallen heavily on population size in preserving genetic variation. However, in the cedar, this may be less important than population structure. If trees are becoming isolated from one another, the risk of self-pollination will increase, and with it inbreeding depression. Erosion of genetic diversity by a change in population structure causing increased inbreeding will be much more rapid than erosion due to genetic drift in long-lived organisms such as trees. Far more emphasis needs to be placed on population structures and its effect on breeding systems in the conservation genetics of plants.

2. **Does genetic variation have an impact on fitness components in the Clanwilliam cedar?**

Evidence which supports the relationship between heterozygosity and fitness was found for a select number of traits in this thesis. These traits were all seed- or seedling-related indicating the impact of levels of genetic variation on the seedling generation. A few other traits were found to co-vary with these fitness traits, indicating a syndrome of genetic effects on regeneration. The relationship between heterozygosity and seedling:parent ratio was a strong indication that the impact of genetic variation has a domino-effect from individual seedling fitness through to the ecology of *W.cedarbergensis* populations. The fact that many other traits were not correlated with genetic variation even though they were significantly different between populatons suggests that populations are subject to a considerable amount of environmental heterogeneity.

3. **Does population size have an impact on genetic variation and fitness?**

I showed that population size was correlated with levels of genetic variation in populations of *W.cedarbergensis* and also with a few select fitness traits. The findings here contribute to a very small body of evidence showing the effect of population size on fitness. Populations of *W.cedarbergensis* are subject to an extinction vortex below a critical population size which is around 250 individuals.

The effect of tree density and substructuring within populations on levels of genetic variation and fitness need to be investigated since estimates of population size included density to some extent with the result that density effects on genetic variation and fitness have not been clearly isolated.

Implications for management

The findings of this thesis are directly applicable to the active conservation of the Clanwilliam cedar. **Firstly**, the results showed which seeds should be used in the replanting programme. The plantation, MB, is an adequate seed source since it was found to be outbred for the loci examined in Chapter 3, and seedlings showed adequate signs of vigour. However, the seed source should be supplemented by seeds from DG (Duiwelsgat) and CPS (Crystal Pools) since these two populations showed the highest levels of seedling vigour. Furthermore, these two populations represent the eastern and western sections of the Cedarberg respectively, which would possibly counter the effects of local adpatation, if any, once seedlings are trans-located.

Secondly, the results of this thesis give information on where replanting is important. Small populations below 250 individuals, such as KK (Klein Krakadouw Kloof) and WB (Welbedacht), suffered from population growth rates which could be boosted by increasing seedling:parent ratios with replanting. Welbedacht is a population which is already part of the replanting scheme, but perhaps all small and isolated populations should be incorporated into the active management plan. Highly substuctured populations which have large inter-tree distances, and where trees are restricted exclusively to rocky outcrops are also a source of

concern from a pollen movement point of view. The seedling replanting scheme should therefore also aim at closing gaps between trees, making populations more continuous to enable the movement of pollen and reduce the risk of selfing.

Thirdly, the results of this thesis showed that the bottleneck currently being experienced by the Clanwilliam cedar is at a critical stage where seedling vigour is profoundly affected by inbreeding and which is subsequently impacting the demography of populations.

Fourthly, the results of this thesis showed that adult tree survival is extremely important from a genetics point of view the since more pollen-producing individuals there are, the more progeny are likely to be outbred. These findings support those of Privett (1994), who found that adults contribute the most to population growth above any other size class, and contradict those of Manders (1986), who emphasised the importance of seedlings. Evidence for senescence was found since cone production was reduced above an optimal tree size. Supposedly senescent trees cannot be neglected from conservation priorities, however, since they may still produce large amounts of pollen necessary to increase the chances of outcrossing within populations.

From a general conservation genetics point of view, this study is a good example of how important genetics is for the ecology of populations and species. It also raises the issue of population structure versus population size in their importance in maintaining genetic diversity.

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