DETERMINING GENE FLOW, LINKAGE AND PARENTAL CONTRIBUTION IN *PINUS ELLIOTTII* X *PINUS CARIBAEA* PINE HYBRIDS.

JACQUELINE HEIDI DOYLE

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Supervisor: Prof. A-M. Botha-Oberholster

Co-Supervisor: Prof. B. D. Wingfield

Dedicated to

Chris

PREFACE

The need to understand how variations in survivorship, fertility and gene flow contribute to changes in allele frequencies within and amongst populations has been the driving force behind the research of many population biologists. The rapid development of molecular techniques has provided these population biologists with a new range of technical tools with which to approach a wide range of questions.

In this dissertation the results of a study, which was carried out in the Department of Genetics, University of Pretoria, Pretoria, under the supervision of Professor Anna-Maria Botha-Oberholster and the co-supervision of Professor Brenda D. Wingfield, are presented. The results are original and have not been submitted in any form to another University.

Chapter 1 focuses primarily on the background and history of *P. elliottii x P. caribaea* hybrid pine. The chapter addresses where the hybrid fits in and why it is of economic importance to the forestry industry. The chapter also focuses on molecular biology studies that have been done on conifers and more importantly on members of the family Pinaceae.

In chapter 2 the role of DNA fingerprinting using microsatellite markers was investigated. In this chapter microsatellite markers developed for use in related pine

species were tested for their ability to produce fingerprints in representative populations of the *P. elliottii x P. caribaea* hybrid. The microsatellite markers were also tested for their ability to identify linkage, gene flow and parental contribution in the hybrid. The development of microsatellite markers that are more specific to the *P. elliottii x P. caribaea* hybrid pine was also addressed. Using Randomly Amplified Microsatellites (RAMs), primers specific to microsatellite loci distributed throughout the *P. elliottii* genome were designed. These microsatellite markers were then tested for their ability to detect polymorphisms in the *P. elliottii x P. caribaea* hybrid pine.

The final chapter, chapter 3, focuses on the viability and morphology of *P. caribaea* pollen. In this chapter the influence of storage and collection conditions on pollen viability was investigated. The chapter also investigates the correlation between germination rate, viability and pollen morphology.

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INTRODUCTION

One of the driving factors behind forestry, as with any other commercial crop, is the optimization of the plant with its growing environment, thereby ensuring maximum yield per square meter. One of the major draw backs to forest tree improvement is the timespan involved from seedling to mature adult (anything from 20 to 40 years). This lengthy maturation period means that many years need to be invested in research and breeding and that progress towards the optimization of the forest trees with their environment is a slow and ongoing process.

One of the main foci in forest tree breeding is the creation of hybrids between promising parents with the objective of creating genetically superior offspring, which display hybrid vigor. An example of one such promising hybrid is the *Pinus elliottii x Pinus caribaea* hybrid. In 1955, the first crosses of the *P. elliottii x P. caribaea* hybrid were made in Australia (Hinze, 2000). The *P. elliottii x P. caribaea* hybrid, which displayed extraordinary growth characteristics (such as rapid growth combined with excellent wood quality), attracted widespread attention and extensive testing was initiated in South Africa during the late 1960's and early 1970's (Denison and Kietzka, 1993; Malan, 1995; Stanger *et al.*, 1999).

Molecular techniques offer many tools that can be employed by breeders to severely reduce the time scale of field trials. The aim of this study was to investigate the potential of microsatellite markers for determining gene flow, linkage and parental contribution in

the *P. elliottii x P. caribaea* hybrid. To this end the cross-species amplification of microsatellite markers was investigated, as this technique holds important implications on a large scale in the forestry industry. The ability to use markers developed in closely related *Pinus* species would not only greatly reduce the time and costs involved in marker development, but also the time and costs involved during the nursery phase and the selection of superior clones for future plantations. For example, once superior *P. elliottii x P. caribaea* hybrids are identified, based on field trial performance, it would be possible to screen the seedlings produced in order to verify that they really are the progeny of the controlled pollination between the superior parents. This procedure would allow seedlings that do not conform to the microsatellite fingerprints expected for the hybrid to be discarded early on in the screening phase and ensure that only the correct genotypes enter into the bulking-up phase.

A number of obstacles have been encountered that complicate the large-scale planting of the *P. elliottii x P. caribaea* hybrid. Firstly, the *P. caribaea* pollen ripens approximately three months before the *P. elliottii* ovules are receptive (Mather, 2000); and secondly a degree of incompatibility seems to exist between *P. elliottii* and *P. caribaea,* which results in low seed set and a low number of viable hybrid seeds (SAFCOL, 1996).

The second aim of this study was therefore to investigate the association between *P. caribaea* pollen morphology and viability and to try and link this association to viability. The presence of a significant association would make it possible to incorporate pollen morphology as a screening step. This would be beneficial during the selection of the

pollen parent by reducing the chances of inferior *P. caribaea* pollen parents from being used in crosses during hybrid production and ultimately from entering into the hybrid performance trials in general. The correct storage of the *P. caribaea* pollen would ensure the viability of the pollen used in the cross and therefore result in increased pollination that should in turn result in increased fertilization and thus higher seed set and ultimately more offspring.

CHAPTER 1

LITERATURE REVIEW

1.1 The genus *Pinus*

1.1.1 *Pinus* taxonomy and morphology

The Pinaceae is the largest family of the order Coniferales and includes the firs, spruces, cedars and larches in addition to the actual genus *Pinus* (Johnson, 1978). The genus *Pinus* consists of approximately 100 taxonomically distinct species and many named hybrids, varieties, forms and cultivars (Poynton, 1979; Wakamiya *et al.,* 1993). The natural range of the pines extends from above the polar circle southwards to the subtropics, with pines occurring in Europe, North Africa, Asia, Malaysia, North and Central America (Poynton, 1979; Van Gelderen, 1989).

All species of the genus *Pinus* display long evergreen needles in tight bundles of between two and five, each bundle wrapped at its base in a papery sheath (Van Gelderen, 1989). These needle bundles are referred to as fascicles and the number of needles per fascicle is species-specific. All the species are monoecious, evergreen, resinous trees, with the pollen-bearing microsporangia found axillary on the lower branches, while the macrosporangia are found on the upper branches (Den Ouden and Boom, 1978). The microsporangia are produced in catkin-like inflorescences, which are composed of spirally arranged scales containing two pollen sacs each. The macrosporangia are produced in cone-like inflorescences, with the fertile scales arranged in spirals and a small bract scale attached to each fertile scale (Vidaković, 1991). The cones are ovate, cylindrical or globose and are composed of spirally arranged, thick, woody cone scales.

The cones usually contain two seeds per cone scale and the seeds tend to be nut-like or ovoid, winged or wingless structures, the kernel surrounded by a shell and containing between four and fifteen cotyledons (Den Ouden and Boom, 1978; Vidaković, 1991). Table 1.1 shows a comparison of *P. elliottii* and *P. caribaea* morphological characteristics.

Table 1.1. Comparison of *P. elliottii* and *P. caribaea* morphological characteristics (Johnson, 1978; Vidaković, 1991).

Morphological characteristics	***P. elliottii***	***P. caribaea* var. *hondurensis***
Tree height	• 20 –30 m high	• 25 – 35 m high
Needle morphology	• 2 needles per fascicle • 17 – 25 cm long • Dark green with tufted ends	• 3 needles per fascicle • 12 – 18 cm long • Stiff, light green with serrate margin
Cone morphology	• Ovate to conical • 7 – 15 cm long • 3 – 5 cm in diameter	• Elongated-oblong • 6 – 13 cm long • 4 – 7.5 cm in diameter
Seed morphology	• Ovate to triangular • Black or mottled grey • 6 mm long • Wing 15 – 30 mm long	• Triangular • Black • 6 mm long • Wing 25 mm long and loosely attached

The *P. elliottii x P. caribaea* hybrid tends to display intermediate morphological characteristics of both parents, but according to Bester (2001) no data pertaining to its morphological characteristics have been published to date.

1.1.2 Reproduction in *Pinus*

The pine tree produces two kinds of spores (Robbins *et al.*, 1964). The haploid (n) microspores or pollen grains develop in microsporangiate, male, staminate cones, while the megaspores develop in megasporangiate, female, ovate cones (Sinnott and Wilson, 1957). According to Singh (1978) the 'One-year type reproductive cycle' is commonly exhibited by most members of the Pinaceae. During pollination the wind borne pollen grains come to rest in the pollination droplet produced on the micropyl of the ripe ovule (Wright, 1976). As the pollination droplet dries it contracts, thereby carrying the pollen grain into the pollen chamber, where it germinates and subsequently requires up to four months to fertilize the ovule (Singh, 1978; Willemse, 1968). The fertilization results in the formation of a zygote, which develops into a mature embryo (Robbins *et al.*, 1964; Singh, 1978). The differentiated ovule with its enclosed megagametophyte and embryo constitutes the seed, which is released from the ripe female cone and dispersed by the wind (Hufford, 1978; Moore *et al.*, 1995; Sinnott and Wilson, 1957). Figure 1.1 shows the life cycle of the pine.

1.1.3 *Pinus* pollen studies

The fitness of male gametophytes depends on both the phenology of the male sporangia as well as the amount of pollen produced and on pollen grain traits (Nakamura and Wheeler, 1992; Nikkanen *et al.*, 2000). These pollen grain traits include germination percentage, germination rate, pollen tube growth rate and selective fertilization.

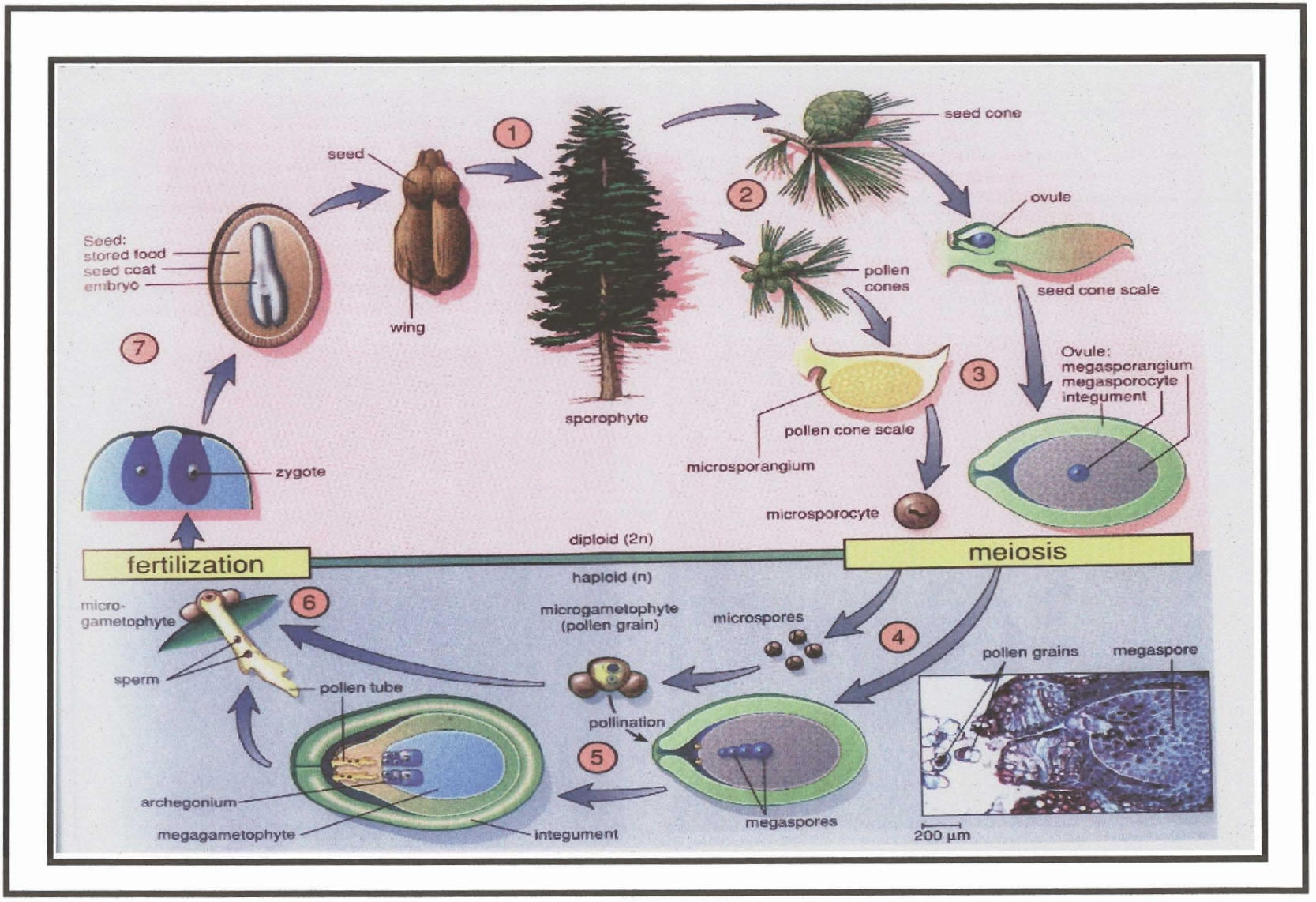


Figure 1.1. Life cycle of the pines (Mader, 1998).

The majority of pollen studies have focused on pollen developmental stages (De Win *et al.*, 1996; Rowley *et al.*, 1999; Willemse, 1971 a, b and c), morphological descriptions (Erdtman, 1952; Pacini *et al.*, 1999) and chemical changes within pollen grains during development and germination (Pardi *et al.*, 1996; Piffanelli *et al.*, 1998). The high-powered resolution provided by the electron microscope has also been widely employed for morphological studies in *Pinus*. Scanning electron microscopy has been used to investigate the surface structure and pollination of many *Pinus* species (Owens *et al.*, 1998; Tomlinson, 1994), while transmission electron microscopy has been used to scrutinize the ultrastructure of the pollen grains (Kurmann, 1991; Ting and Tseng, 1965; Tomlinson, 1994).

Recently Nikkanen *et al.* (2000) investigated the role which variation plays in pollen viability among genotypes of *Picea abies*. Nikkanen *et al.* (2000) also showed that there is a correlation between the variation in pollen viability among pollen donors and the potential for male gametophyte competition.

1.2 The *Pinus elliottii x Pinus caribaea* hybrid

1.2.1 Background and history

The history of the *P. elliottii x P. caribaea* hybrid pine begins in 1955, when the first crosses between *Pinus elliottii* and *Pinus caribaea* were made in Australia (Hinze, 2000). The *P. elliottii x P. caribaea* hybrid, which displayed extraordinary growth characteristics due to hybrid vigor, attracted widespread attention, but it was not until the late 1960's

early 1970's that extensive testing was initiated in South Africa (Malan, 1995). The greatest obstacle facing this promising hybrid was its low seed set, which made commercial planting unviable (Slee and Abbott, 1990). This obstacle was partially over come with the development of an effective vegetative propagation technique. The vegetative propagation involved the bulking up of the hybrid material in hedges from which thousands of cuttings were then made. With enough material available SAFCOL started its first commercial plantings of the hybrid in 1997 and has increased the area from 2 136 ha in 1998 to 6 000 ha by June 2000 (Hinze, 2000).

1.2.2 Breeding Programme

The breeding of the *P. elliottii x P. caribaea* hybrid involves the pollination of *P. elliottii* ovules with *P. caribaea* var. *hondurensis* pollen. Although *P. caribaea* var. *hondurensis* displays the lowest wood density when compared with *P. caribaea* var. *bahamensis* and *P. caribaea* var. *caribaea,* it also exhibits the fastest growth and was therefore selected as the pollen parent (Bester, 2000). Due to hybrid vigor the resultant *P. elliottii x P. caribaea* hybrid displays the good properties of both parents; namely the fast growth of *P. caribaea* and the excellent wood density of *P. elliottii* (Denison and Kietzka, 1993; Stanger *et al.,* 1999). It is due to this hybrid vigor that the *P. elliottii x P. caribaea* hybrids are favored above improved parent species for commercial planting (Bester, 2000). Table 1.2 shows a comparison of *P. elliottii, P. caribaea* and the *P. elliottii x P. caribaea* hybrids growth characteristics, while in Figure 1.2 the growth rate of the *P. elliottii x P. caribaea* hybrid is compared with that of its parents and other pine species.

Table 1.2. Comparison of *P. elliottii, P. caribaea* and the *P. elliottii x P. caribaea* hybrids growth characteristics (Bester, 2000; Vidaković, 1991).

P. elliottii	***P. elliottii x P. caribaea* hybrid**	***P. caribaea***
Slower growing	Faster growing	Fast growing
Lower yielding	Higher yielding	High yielding
Strong, heavy and hard wood	Stronger, heavier and harder wood	Light wood
Excellent wood quality	Good wood quality	Low to intermediate wood quality

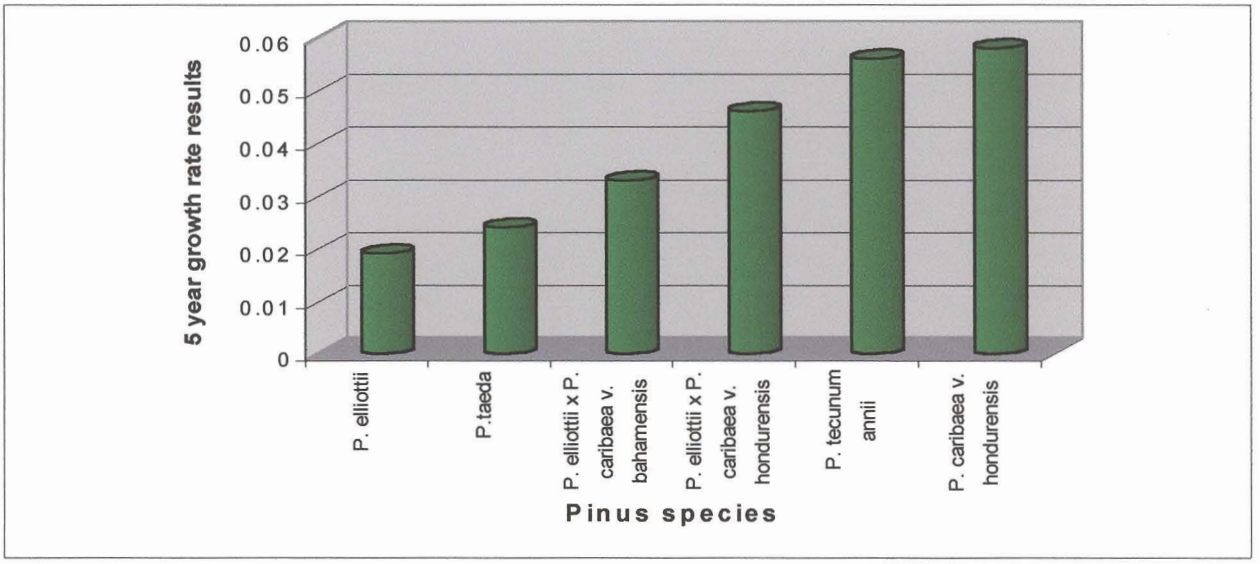


Figure 1.2. Comparison of *P. elliottii x P. caribaea* hybrid growth rate with the growth rates of the parent species and other pine species. (Bester, 2000)

Generally a low number of mature cones are harvested from the original macrosporangia pollinated (Table 1.3). This low success rate is partially due to the natural abortion phenomenon, which occurs in many pine species, but can also be attributed to mechanical damage, which occurs in the seed orchards and tree banks during controlled pollination

(Aronen *et al.*, 1998). According to Aronen *et al.* (1998), controlled pollination can result in as much as a 50% reduction in seeds per cone when compared with open pollination. Parental incompatibility between the *P. elliottii* and *P. caribaea*, as well as the fact that the *P. caribaea* pollen ripens three months before the *P. elliottii* ovules are ripe and ready for pollination also plays a major role in the low success rate (Slee and Abbott, 1990; Mather, 2000). This low success rate results in an average seed set between 2 and 15 full, viable seeds per *P. elliottii x P. caribaea* hybrid cone (Van der Merwe and Wentzel, 2000). To combat this phenomenon improved pollen management strategies were devised and more compatible parents, based on previous experience, were selected for the hybrid breeding programme. Using these strategies the number of full seeds harvested increased from 7 427 in 1996 to 18 439 in 2000 (Van der Merwe and Wentzel, 2000). Table 1.3 shows the results of SAFCOL's controlled pollination programme for the pollination seasons from 1994 to 1998 and the number of cones harvested from 1996 to 2000.

Table 1.3. Results of SAFCOL's controlled pollination programme (Van der Merwe and Wentzel, 2000).

Year	**1996**	**1997**	**1998**	**1999**	**2000**
Total no. of flowers pollinated	2689	4349	2696	2178	2866
Total no. of cones harvested	1472	2408	1173	1476	1227
Total no. of seeds	22 620	24 355	18 136	56 765	43 220
Total no. of full seeds	7427	4124	3147	17 680	18 439
Average no. of full seeds per cone	5.0	1.7	2.7	12.0	15.0

1.2.3 Nursery Practice

The lifeblood of the *P. elliottii x P. caribaea* hybrid programme is the bulking up of viable plant material to be used in the commercial plantings (Denison and Kietzka, 1993). To this end the hybrid seeds harvested are used to establish hedges. From these hedges up to 3.5 million cuttings can be produced per annum (Van der Merwe and Wentzel, 2000). Strict nursery practices, which involve keeping hedge plants around 15 to 20 cm above ground level, ensure that juvenile cuttings of 5 to 10 cm long with an active growing tip of 3 to 5 mm can be harvested. After storage in water containing fungicide, the cuttings are set in special medium before being transferred to special climate controlled rooting tunnels. Rooting takes between 2 and 3 months after which the cuttings are hardened off for 5 months before being made available for commercial planting (Wentzel and Olivier, 2000).

1.2.4 Wood quality and performance of the *P. elliottii x P. caribaea* hybrid

Studies performed on 20-year-old *P. elliottii x P. caribaea* hybrid trees showed that the hybrids performed extremely well with regard to its growth characteristics and wood quality when compared with either *P. elliottii* or *P. caribaea* parent (Malan, 2000). The hybrids are characterized by rapid growth in the first few years, which results in wide growth rings and therefore wood of lower density when compared with *P. elliottii* (Malan, 1995). However, the quality of the *P. elliottii x P. caribaea* hybrid timber was found to be strong enough to ensure that sawn boards of higher strength grades were cut from it (Malan, 2000). The exceptional growth characteristics of the *P. elliottii x P.*

caribaea hybrid will allow the current SAFCOL plantation rotation period to be shortened from 30 years, for current *P. patula* growing stock, to between 20 to 25 years (Truter, 2000). Furthermore, the annual profits per hectare are estimated at between R 2 744 and R 4 700 per ha, in comparison with R 1 801 per ha currently obtained for *P. patula* (Truter, 2000). The combination of these savings is a theoretical increase of between 1.5 and 2.6 times the revenue in comparison with existing plantations.

1.3 Polymorphic markers

According to Butcher *et al.* (1999), reliable information on the distribution of genetic variation is a prerequisite for sound selection, breeding and conservation programmes for forest trees. There are two ways in which the genetic variation of a species can be assessed; either by measuring morphological and metric characters in the field or by studying molecular markers in the laboratory. The development of DNA markers such as RAPDs (Random Amplified Polymorphic DNA), RFLPs (Restriction Fragment Length Polymorphisms) and microsatellites has provided the molecular tools required for the study of variation in coding, non-coding and highly variable regions of both nuclear and organelle genomes (Butcher *et al.,* 1999; Helentjaris *et al.,* 1985; Hicks *et al.,* 1998; Nkongolo, 1999; Strauss *et al.,* 1992). Table 1.4 shows a brief comparison of the molecular techniques currently available. The fact that microsatellite markers are co-dominant and can be amplified from small amounts of DNA by polymerase chain reaction (Echt and May-Marquardt, 1997) makes this technique more desirable than

RAPDs, which are dominant markers (Parker *et al.*, 1998), or RFLPs, which require large amounts of DNA (Winter and Kahl, 1995).

Table 1.4. Comparison of properties of molecular techniques currently available for generating molecular markers (Rafalski and Tingey, 1993).

	RFLP	**RAPD**	**Microsatellites**
Principle	Endonuclease restriction Southern Blotting Hybridization	DNA amplification with random primers	PCR of simple sequence repeats
Type of polymorphism	Single base changes Insertions Deletions	Single base changes Insertions Deletions	Changes in length of repeats
Genomic abundance	High	Very High	Medium
Level of polymorphism	Medium	Medium	High
Dominance	Co-dominant	Dominant	Co-dominant
Amount of DNA required	2 – 10 µg	10 – 25 ng	50 – 100 ng
Sequence information	No	No	Yes
Radioactive detection	Yes / No	No	No
Development costs	Medium	Low	High
Start-up costs	Medium / High	Low	High

1.3.1 Microsatellites

Microsatellites, also called short tandem repeats (STRs) or simple sequence repeats (SSRs), consist of tandemly repeated units of between one and six base pairs. These tandem repeats are often highly polymorphic due to variation in the number of repeat units (Dayanandan *et al.*, 1998). According to Jarne and Lagoda (1996), microsatellite variation is the result of errors that occur during DNA replication. These errors are caused by the DNA polymerase that "slips" when copying the repeat region, thereby changing the repeat number (Kashi *et al.*, 1997). Microsatellites occur on average every 30 to 50 kilobases in eukaryotic genomes (Lehn and Davis, 1999). This abundance and wide spread distribution makes them very valuable markers for molecular studies.

Several properties of microsatellites make them favorable genetic markers when compared with other genetic methods such as minisatellites, RAPD's, RFLP's and allozymes. These desirable properties include: (i) that they can be used to identify highly polymorphic sequences with allele sizes smaller than 500 bp (Bruford, 1999); (ii) that they have a high mutation rate and vary over a narrow size range (Queller *et al.*, 1993); (iii) that the variability in these loci can be assayed by PCR in combination with gel electrophoresis (Bruford, 1999); (iv) that they can be used to assess variation from minute amounts of material that might contain highly degraded DNA, i.e. forensic or ancient DNA samples, (Ellegren, 1991; Hagelberg *et al.*, 1991); and (v) that they display a Mendelian co-dominant mode of inheritance (Jarne and Lagoda, 1996). Furthermore the fact that these markers are hypervariable, co-dominant and highly reproducible make

them ideal for genome mapping and population genetic studies (Dayanandan *et al.*, 1998). Another major advantage of microsatellites is that once microsatellite markers have been developed in one species, they can be successfully used for cross-species amplification with some success (Maurizio *et al.*, 1999; White and Powell, 1997).

Microsatellites can be grouped into three types, namely pure $(AG)_n$, compound $(A)_n(AG)_n$ and interrupted $(AG)_nGTCT(AG)_n$. The compound and interrupted types of microsatellites have been found to be less polymorphic than pure microsatellites (Depeiges *et al.*, 1995). The pure microsatellites can be divided further into three subgroups, namely dinucleotide $(AG)_n$, trinucleotide $(AGA)_n$ and tetranucleotide $(AGAT)_n$ repeats (Jarne and Lagoda, 1996). According to Estoup *et al.* (1993) dinucleotide repeats, which occur approximately every 30 – 50 kb, with a repeat number of less than 30, are the most frequently used loci for population biology studies. Generally the animal kingdom tends to be rich in the CA dinucleotide repeats (Dib *et al.*, 1996; Dietrich *et al.*, 1996), while TA or GA dinucleotide repeats are more commonly encountered in plants (Depeiges *et al.*, 1995; Lagerkrantz *et al.*, 1993). Trinucleotide repeats, which occur in both plants and animals and which are found mainly within the exon regions, are generally studied in connection with human diseases and cancers (Charlesworth *et al.*, 1994). Tetranucleotide repeats, which occur in many higher organisms, are rarely used for population studies and often occur as compound or interrupted stretches (Jarne and Lagoda, 1996).

According to Robinson and Harris (1999), as the taxonomic distance between taxa increases, the incidence of null alleles is likely to increase as well. Null alleles in microsatellites are recognized by the non-inheritance of parental alleles in some of the offspring (Bruford, 1999). Callen *et al.* (1993) demonstrated that null alleles were the result of a mutation within the priming site of the DNA flanking the microsatellite, thereby inhibiting the primers from binding, and subsequently resulting in complete loss of the amplification product. Where null alleles are transmitted vertically through apparent homozygotes, they can result in an individual's genotype being inconsistent with classical Mendelian inheritance (Callen *et al.,* 1993). Null alleles can also result in the loss of informativeness, where failure to detect the allele results in the individual being scored as a homozygote (Pemberton *et al.,* 1995).

1.3.2 Applications of microsatellites in forestry

Microsatellites have been used for a variety of applications ranging from population, parentage and kinship studies (Hokanson *et al.*, 1998; Lathrop *et al.,* 1985), to forensics (Hagelberg *et al.,* 1991) and gene mapping (Dib *et al.*, 1996; Nakamura *et al.*, 1987). Microsatellites have also played an important role in forestry where they have been used for a variety of applications. The first microsatellites specific to forest trees were developed by Smith and Devey (1994) in *Pinus radiata*. Since then microsatellites have been developed for a range of temperate and tropical forest trees including various *Abies* species (Vendramin and Ziegenhagen, 1997), *Eucalyptus grandis* and *Eucalyptus urophylla* (Brodani *et al.,* 1998; Van der Nest *et al.,* 2000), *Fagus crenata* (Tanaka *et al.,*

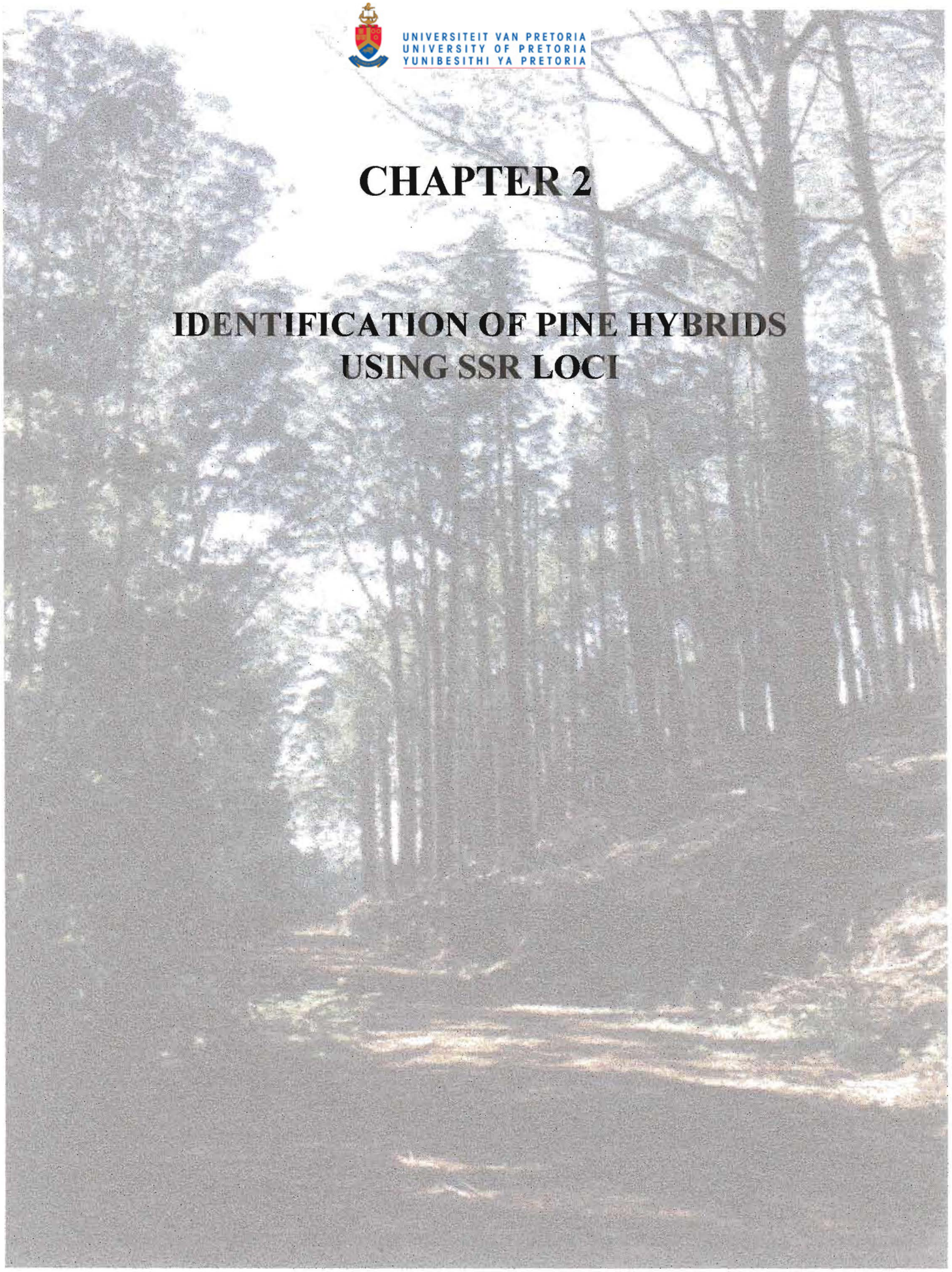
1999), *Melaleuca alternifolia* (Rossetto *et al.*, 1999), *Picea abies* (Pfeiffer *et al.*, 1997), *Picea sitchensis* (Van de Ven and McNicol, 1996), various *Pinus* species (Table 1.5), *Quercus macrocarpa* (Dow *et al.*, 1995) and *Shorea curtissii* (Ujino *et al.*, 1998).

Table 1.5. Microsatellites developed in various *Pinus* species.

Application	Species	Reference
Gene flow	*Pinus radiata*	Smith and Devey, 1994
Linkage	*Pinus radiata*	Devey *et al.*, 1996
	Pinus radiata and *Pinus taeda*	Devey *et al.*, 1999
	Pinus strobus	Echt and Nelson, 1997
Marker development	*Pinus contorta*	Hicks *et al.*, 1998
	Pinus radiata	Fisher *et al.*, 1998
	Pinus strobus	Echt *et al.*, 1996
	Pinus sylvestris	Kostia *et al.*, 1995
Phylogeny	Various *Pinus sp.*	Soranzo *et al.*, 1999
	Pinus taeda	Williams *et al.*, 2000
Population studies	Various *Pinus sp.*	Schmidt *et al.*, 2000
	Pinus contorta	Thomas *et al.*, 1999
	Pinus pinaster	Vendramin *et al.*, 1998

The main areas were microsatellite markers are being applied in forest trees include studies on genetic diversity in natural and breeding populations, gene flow, pollen and seed dispersal and mating systems (Butcher *et al.*, 1999). These parameters impact on the conservation of forest genetic resources, by using microsatellites to monitor forest management practices. Microsatellites can also be used for germplasm identification and

for the construction of genetic linkage maps, with marker assisted selection as the eventual goal.



CHAPTER 2

IDENTIFICATION OF PINE HYBRIDS USING SSR LOCI

2.1 CROSS-SPECIES AMPLIFICATION OF SSR LOCI IN THE *PINACEAE* FAMILY

2.1.1 Introduction

Microsatellites, also called "simple sequence repeats", consist of tandemly repeated, short DNA motifs that provide an attractive source of genetic polymorphisms for both mammals and plants (Wang *et al.,* 1994; Weber and May, 1989). They have been found and used for genetic analysis in many mammalian species (Mellersh *et al.,* 1997; Moore *et al.,* 1991; Ostrander *et al.,* 1992) and in other eukaryotes, such as insects (Colson and Goldstein, 1999; Strassmann, 1998), birds (Groenen *et al.*, 1998; Moran, 1993), fish (Rico *et al.,* 1993) and plants (Akkaya *et al.,* 1992; Hokanson *et al.,* 1998; Lavi *et al.,* 1994; Thomas and Scott, 1993; Wang *et al.,* 1994).

In 1989 the first reports of microsatellite isolation and characterization of allelic variability at these loci using PCR were reported. In these early studies microsatellites were either cloned and sequenced (Litt and Luty, 1989; Tautz, 1989), or were identified from sequence databases (Weber and May, 1989). Based on this information primers were designed to recognize sequences flanking the microsatellite region. The polymorphic amplification products obtained after PCR with these specifically designed primers were then separated on polyacrylamide gels (Wu *et al.,* 1994). Using this approach the resolution of alleles differing by as little as one base pair was possible, and several loci could be analyzed together on the same gel (Rafalski and Tingey, 1993).

Given the long and expensive procedures involved in the isolation of SSR loci, it would be advantageous to be able to utilize primer sequences developed in one species in other closely related species. The problem is that while cross-species amplification is not uncommon, it does not always occur and amplification across genus boundaries tends to be unusual. Examples of cross-species amplification have been displayed in both the animal and plant kingdoms. Moore *et al.* (1991) found that high levels of sequence conservation exist within the primer regions of mammalian genomes in closely related species, while White and Powell (1997) found similar sequence conservation among *Meliaceae* species.

The aim of this study was to determine the conservation of microsatellite sequences between species and to use these heterologous PCR primers for the analysis of microsatellites in closely related species. The usefulness of these cross-species microsatellite markers was also investigated with regard to DNA profiling, gene flow and parental contribution determinations in the *P. elliottii x P. caribaea* hybrid.

2.1.2 Materials and Methods

2.1.2.1 Plant material

For the production of the *P. elliottii x P. caribaea* hybrids the *P. elliottii* tree E 503 was used as the female parent. Various *P. caribaea* tree types (i.e. Ach 33, Ach 49, Ach 93, Ach 271 and Pch 23) were used as the pollen donors. All plant material was obtained

from SAFCOL, South Africa. Diploid needle and zygotic embryo tissues, as well as haploid megagametophyte and pollen tissues were used to obtain genomic DNA.

2.1.2.2 DNA preparation

The extraction methods used to obtain genomic DNA's required for this study are summarized in Table 2.1.

Table 2.1. Plant Material and extraction methods used in the study.

Plant Material	Extraction Method	Reference
P. elliottii needle tissue	CTAB	Murray and Thompson, 1980
P. elliottii megagametophyte tissue	Lee – Binelli modification	Lee and Taylor, 1990 Binelli and Bucci, 1994
P. elliottii x P. caribaea hybrid zygotic embryo tissue	Lee – Binelli modification	Lee and Taylor, 1990 Binelli and Bucci, 1994
P. caribaea needle tissue	CTAB	Murray and Thompson, 1980
P. caribaea pollen	PCR directly	Petersen *et al.*, 1996

The extraction of the *P. elliottii* and *P. caribaea* needle tissue DNA was done using a modified CTAB method (Murray and Thompson, 1980). Bulked needle samples of approximately 3 g were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. Thereafter, 3 ml of heated CTAB extraction buffer (5% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, pH 8.0, 100 mM Tris-HCl, pH 8.0,

1% (w/v) PVP) was added to the frozen tissue, which was then ground further to form a paste. This tissue paste was transferred to a large test-tube, and the mortar was rinsed with a further 3 ml of CTAB extraction buffer. The mixture was incubated at 65°C for one hour, after which an equal volume of phenol / chloroform / isoamyl alcohol (25:24:1) was added and the contents mixed by inverting. The mixture was centrifuged (12 000 x *g* for 15 min) at room temperature, and the aqueous phase was transferred to a new tube. Two and a half (v/v) times ice cold, 100% ethanol was added to the aqueous phase and gently mixed by inverting. The mixture was left on ice for up to 5 min to allow for complete DNA precipitation. After 5 min the precipitated DNA was spooled using a sterile Pasteur pipette. The DNA was washed twice in 70% ethanol; vacuum dried and resuspended in 50 to 100 µl of sterile water. The DNA concentration was determined spectrophotometrically and all samples were subsequently diluted to 50 ng/µl.

The extraction of DNA from *P. elliottii* megagametophyte tissue and *P. elliottii x P. caribaea* hybrid zygotic embryo tissue was performed using a modification of the methods described by Lee and Taylor (1990) and Binelli and Bucci (1994). The procedure is similar to the CTAB extraction method previously described, with the following exceptions. Firstly, the individual zygotic embryos and megagametophytes were ground to a fine powder, in eppendorf tubes, in liquid nitrogen using sterile glass pestles, as opposed to the bulked needle samples ground in a mortar and pestle. Subsequently 200 µl of heated extraction buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA, pH 8.0, 3% (w/v) SDS, 1% (v/v) 2-mercaptoethanol), as opposed to 3 ml CTAB extraction buffer, was added and the tubes were vortexed briefly prior to incubation at

65°C for one hour. Secondly 5 µl of 3 M NaOAc, pH 8.0, was added to the aqueous phase prior to the addition of 2.5 (v/v) 100% ice-cold ethanol. Finally, the vacuum dried DNA was resuspended in a maximum of 50 µl of sterile water and was quantified spectrophotometrically.

2.1.2.3 Primers

Eleven different primer pairs specific to microsatellite loci identified in other *Pinus* species were selected for the DNA profiling of the *P. elliottii / P. caribaea* population (Table 2.2). Of the eleven microsatellite markers screened, two were developed in *Pinus radiata* (PR 4.6 and PR 9.3), while six of the eleven were developed in *Pinus taeda* (RPTest 2, RPTest 5, RPTest 6, RPTest 9, RPTest 15 and RPTest 20). The remaining three of the eleven microsatellite markers were developed in *Pinus strobus* (RPS 105, RPS 150 and RPS 160), which is a soft pine of the subgenus *Strobus,* while both *Pinus radiata* and *Pinus taeda* are hard pines of the subgenus *Pinus* (Echt *et al.,* 1999).

2.1.2.4 PCR conditions

PCR conditions were based on a modification of conditions suggested by Smith and Devey (1994). All amplification reactions were carried out in 25 µl reaction volumes. The reaction mixture contained 50 ng/µl template DNA, 1% (v/v) Promega magnesium free, thermophilic DNA polymerase 10X buffer, (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% (v/v) Triton®X-100), 300 µM dNTPs, 3 mM $MgCl_2$, 0.25 µM of each primer, and

Table 2.2. List of microsatellite primers used in the study.

Primer	Sequence (5' – 3')	Reference
PR 4.6 (forward)	GAAAAAAAGGCAAAAAAAAGGAG	Smith and Devey, 1994
PR 4.6 (reverse)	ACCCAAGGTACATAACTCG	
PR 9.3 (forward)	GAAATTTAACACCACACCGTTG	Smith and Devey, 1994
PR 9.3 (reverse)	TGGGGCTTAAAGTGAAATGG	
RPTest 2 (forward)	AATCCTTGTGCCAGGAACAC	Echt and Burns, 1999
RPTest 2 (reverse)	ATTTTATCAGCGCCACTGCT	
RPTest 5 (forward)	ACAACAATAATAACGGGGGC	Echt and Burns, 1999
RPTest 5 (reverse)	ACGCTTTAGATCCTCCTGCA	
RPTest 6 (forward	AGGATTCCAACAGCATCACC	Echt and Burns, 1999
RPTest 6 (reverse)	CTGAACATGAAGCGCAGTGT	
RPTest 9 (forward)	CCAGACAACCCAAATGAAGG	Echt and Burns, 1999
RPTest 9 (reverse)	GCCTGCTATCGAATCCAGAA	
RPTest 15 (forward)	GAACGTGGTTATGGCGGTAG	Echt and Burns, 1999
RPTest 15 (reverse)	CCAGGGACAGTTACCAGCAT	
RPTest 20 (forward)	GTTCCCACTCAAGGGTTGAA	Echt and Burns, 1999
RPTest 20 (reverse)	ACATCATTTGTTGCCGCATA	
RPS 105 (forward)	TGGACATCCTAGTCGGAACC	Karhu *et al.*, 2000
RPS 105 (reverse)	AAAATCATTTCTGTATCAGAACAA	
RPS 150 (forward)	TCCATCAGTGAGCAGTGG	Karhu *et al.*, 2000
RPS 150 (reverse)	CACTTGGGCTTCCTCTTC	
RPS 160 (forward)	ACTAAGAACTCTCCCTCTCACC	Karhu *et al.*, 2000
RPS 160 (reverse)	TCATTGTTCCCCAAATCAT	

1 U Promega *Taq* DNA polymerase in storage buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol, 0.5% (v/v) Tween®20, 1% (v/v) Triton®X-100). Temperature cycling was done using a Perkin Elmer GeneAmp® PCR system 9700 (Applied Biosystems) thermal cycler programmed for an initial step of 4 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C. The amplification cycles were followed by a final elongation step of 5 min at 72°C.

A modification of the protocol described by Petersen *et al.* (1996), in combination with PCR conditions described by Smith and Devey (1994), were used to obtain amplification products directly from *P. caribaea* pollen grains, without prior DNA isolation. The procedure involved the suspension of 50 to 100 pollen grains in distilled water, in an eppendorf tube, and grinding briefly with a glass pestle to facilitate the release of the DNA from the pollen grains. This suspension was then mixed with the Promega thermophilic DNA polymerase 10X buffer, magnesium free, and heated to 95°C for 10 min to destroy all enzymatic activity and to burst any intact pollen grains which might still be present. The PCR amplifications were subsequently performed in 25 µl reactions as described previously.

2.1.2.5 PAGE analysis

The amplification products were analyzed by separation on 7.5% non-denaturing polyacrylamide gels (3.75 % (v/v) FMC® Long Ranger Gel Solution, 1% (v/v) TBE buffer, 0.1% (w/v) APS, 0.004% (v/v) TEMED) in 1 X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 26.6 V/cm for one and a half hours. After separation the amplification products were detected by staining with (1:10 000) Sybr®GreenI Nucleic Acid Gel stain (Boehringer Mannheim). The Amplification products were visualized by UV illumination. Boehringer Mannheim 100 bp DNA ladder was included as the molecular size marker in all electrophoresis profiles.

2.1.3 Results

Of the eleven microsatellite markers included in this study two primer pairs were found to produce informative polymorphisms (primer PR 4.6 and primer RPTest 9), five were found to be monomorphic (primer RPTest 5, primer RPTest 15, primer RPTest 20, primer RPS 105 and primer RPS 150) for the entire *P. elliottii* and *P. caribaea* population. Four primer pairs did not result in any amplification products (primer PR 9.3, primer RPTest 2, primer RPTest 6 and primer RPS 160). The results of the eleven SSR markers that were screened are shown in Table 2.3.

The polymorphic microsatellite markers, PR 4.6 and RPTest 9, produced amplification products that could be used to clearly distinguish between the *P. elliottii* and *P. caribaea*

(Figure 2.1, Figure 2.2 and Figure 2.3). These polymorphic amplification products could also be used to determine which parent contributed which allele to the *P. elliottii x P. caribaea* hybrid of a specific cross (Figure 2.1). In Figure 2.1 the 250 bp fragment in lane 4 of the microsatellite fingerprints, of the E 503 x Pch 23 hybrid population, clearly suggest that Pch 23 was definitely used as the pollen donor during the controlled pollination.

Table 2.3. Results of SSR cross-species amplification.

SSR marker	***Pinus* species for which marker was developed**	**Number of loci**	**Polymorphism**
PR 4.6	*Pinus radiata*	2	Polymorphic
PR 9.3	*Pinus radiata*	0	None
RPTest 2	*Pinus taeda*	0	None
RPTest 5	*Pinus taeda*	1	Monomorphic
RPTest 6	*Pinus taeda*	0	None
RPTest 9	*Pinus taeda*	2	Polymorphic
RPTest 15	*Pinus taeda*	1	Monomorphic
RPTest 20	*Pinus taeda*	1	Monomorphic
RPS 105	*Pinus strobus*	1	Monomorphic
RPS 150	*Pinus strobus*	1	Monomorphic
RPS 160	*Pinus strobus*	0	None

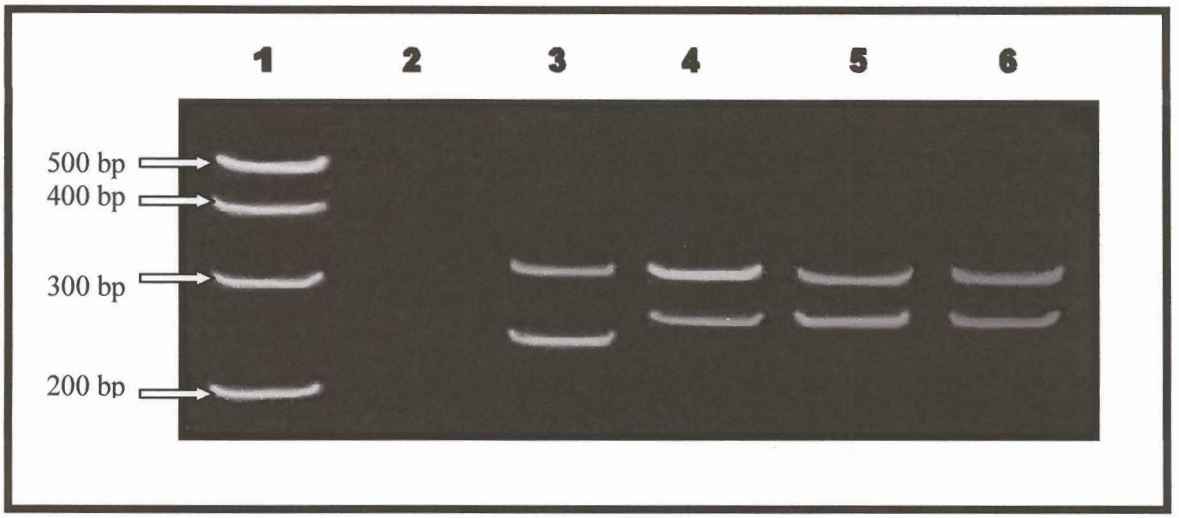


Figure 2.1. Microsatellite fingerprints of the *P. elliottii* (E 503) x *P. caribaea* (Pch 23) hybrid population. The fingerprints were obtained by PCR amplification with primer RPTest 9. Lane 1 contains 100 bp DNA ladder, while lanes 2 to 6 contain the fingerprints obtained using E 503 megagametophyte DNA, E 503 needle DNA, E 503 x Pch 23 embryo DNA, Pch 23 needle DNA and Pch 23 pollen DNA, respectively.

In Figure 2.2, in which the microsatellite fingerprints of the E 503 x Ach 271 hybrid population are recorded, there is no definitive evidence as to the gene flow that occurred during fertilization. If the controlled pollination was successful, then the hybrid must have inherited the polymorphic allele (i.e. the 275 bp fragment in lane 4, Figure 2.2) from *P. elliottii* and not from *P. caribaea.* Another possibility is that the controlled pollination was not successful and that the embryo produced was actually the result of *P. elliottii* self fertilization.

For specific hybrid populations, such as the E 503 x Ach 49 hybrid population presented in Figure 2.3, null alleles were detected using polymorphic marker RPTest 9. The absence of an amplification product of between 250 – 280 bp was verified by altering the stringency of the PCR conditions to ensure that the result was not merely due to poor amplification at that locus.

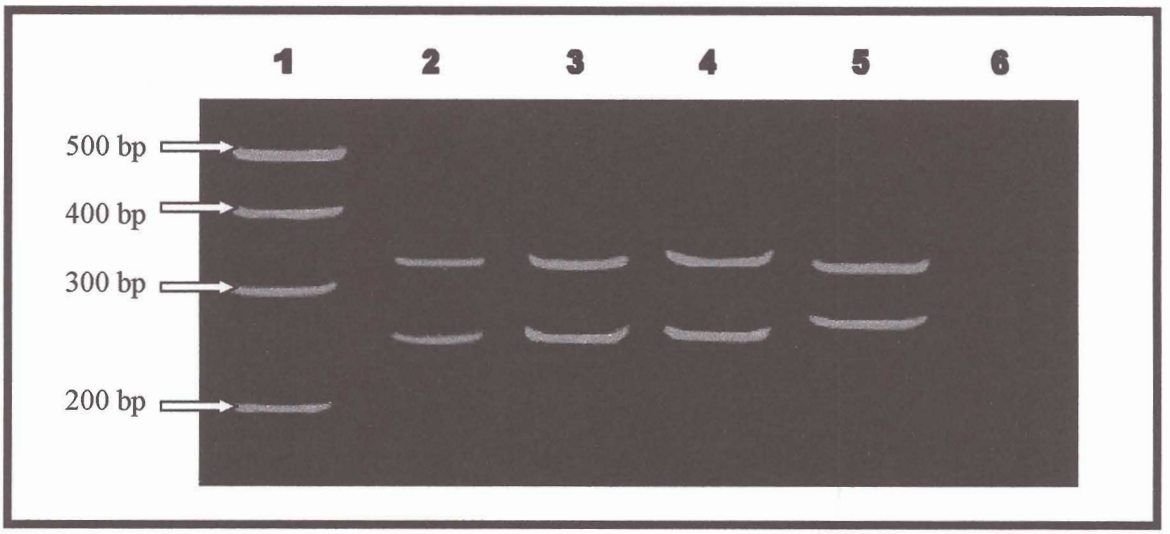


Figure 2.2. Microsatellite fingerprints of the *P. elliottii* (E 503) x *P. caribaea* (Ach 271) hybrid population. The fingerprints were obtained by PCR amplification with primer RPTest 9. Lane 1 contains 100 bp DNA ladder, while lanes 2 to 6 contain the fingerprints obtained using E 503 megagametophyte DNA, E 503 needle DNA, E 503 x Pch 23 embryo DNA, Ach 271 needle DNA and Ach 271 pollen DNA, respectively.

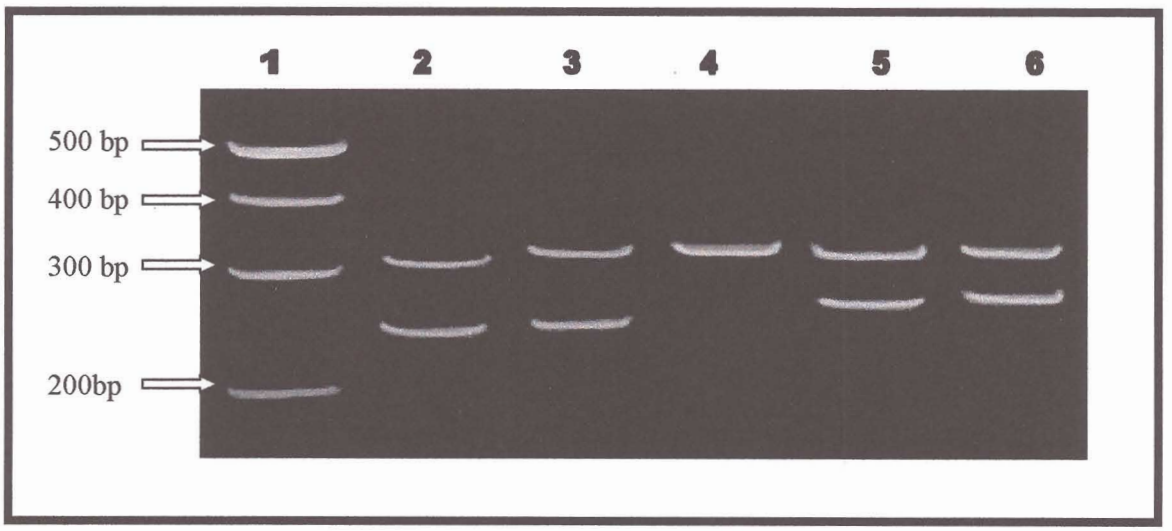


Figure 2.3. Microsatellite fingerprints of the *P. elliottii* (E 503) x *P. caribaea* (Ach 49) hybrid population. The fingerprints were obtained by PCR amplification with RPTest 9. Lane 1 contains 100 bp DNA ladder, while lanes 2 to 6 contain the fingerprints obtained using E 503 megagametophyte DNA, E 503 needle DNA, E 503 x Pch 23 embryo DNA, Ach 49 needle DNA and Ach 49 pollen DNA, respectively.

2.1.4 Discussion

The evolutionary conservation of DNA sequences flanking microsatellite regions allows the cross-species amplification of microsatellite markers in various related species (Echt *et al.*, 1999). According to Primmer *et al.* (1996) the probability of successful cross-species amplification of microsatellites seems to correlate with the taxonomic distance between the species. This would imply that cross-species amplification, while not uncommon, does not always occur and the greater the taxonomic distance between the species the smaller the chance that it will occur.

Pinus elliottii and *Pinus caribaea* both belong to the subgenus *Pinus*, the hard pines (Wright, 1976). The eleven microsatellite markers screened were developed for both the taxonomic subgenera *Pinus* (*P. radiata* and *P. taeda* - the hard pines) and *Strobus* (*Pinus strobus* – the soft pines). Of the eleven microsatellite markers screened, seven (63.6%) produced DNA fingerprints (Table 2.3). Of these seven markers that produced DNA fingerprints, only two (28.6%) produced polymorphic fingerprints, while the remaining five (71.4%) produced monomorphic DNA fingerprints. Both of the polymorphic microsatellite markers were developed using species in the subgenus *Pinus*. The monomorphic microsatellite markers were derived from both the *Pinus* and *Strobus* subgenera. These results are consistent with other cross-species amplification results, where suitable informative markers were obtained from only 20 – 24% of primer pairs screened (Echt *et al.*, 1996; Pfeiffer *et al.*, 1997). Relatedness, therefore, seems to play an

important role in the type of polymorphism (i.e. polymorphic vs. monomorphic) observed, as well as the success rate encountered during cross-species amplification.

The important role which microsatellite markers can play in population studies is demonstrated by their two-fold ability to detect polymorphisms between the amplification products of both *P. elliottii* and *P. caribaea* parents, and to determine gene flow and parental contribution in *P. elliottii x P. caribaea* hybrid progeny. According to Queller *et al.* (1993) if the genotypes of the mother and its offspring are known, then it is straightforward to exclude incompatible paternal genotypes, but all other males must be excluded before the father can be assigned. The results tend to indicate that microsatellite fingerprints, of the hybrid populations, obtained using single polymorphic primer pairs are not always conclusive (Figure 2.2). However, in studies using more than one polymorphic microsatellite marker it is possible to clearly distinguish between species and hybrid material and to eliminate some of the uncertainty surrounding gene flow.

Not all polymorphic markers produce DNA fingerprints that are equally informative. The usefulness of a marker is determined by its degree of polymorphism in the species that is being tested and the extent to which gene flow and parental contribution can be determined from the fingerprint produced. For example, the polymorphic fingerprint produced by primer RPTest 6 on the E 503 x Pch 23 hybrid population (Figure 2.1) could be considered useful, as it is possible to distinguish the E 503 maternal parent from the Pch 23 pollen donor using this marker. In the same way this marker can also be used to determine gene flow and parental contribution, as the E 503 x Pch 23 hybrid fingerprint

mirrors the fingerprint obtained for the Pch 23 pollen donor. This means that based upon the DNA fingerprints produced by primer RPTest 6 it is possible to accept the null hypothesis that the embryos were the result of the controlled pollination between the *P. elliottii* and *P. caribaea* parents.

The polymorphic fingerprint produced by primer RPTest 6 on the E 503 x Ach 271 hybrid population (Figure 2.2) is less informative as the E 503 x Ach 271 hybrid fingerprint mirrors that of the E 503 maternal parent and it is therefore impossible to determine gene flow or parental contribution. In this scenario two possible options exist. Firstly, that the controlled pollination was unsuccessful and that the E 503 x Ach 271 "hybrid" was actually the progeny of E 503 self-fertilization. The second option is that it is assumed that the controlled fertilization between E 503 maternal parent and the Ach 271 pollen parent was successful, in which case the E 503 x Ach 271 hybrid must have inherited the polymorphic allele (i.e. the 275 bp fragment in lane 4, Figure 2.2) from the E 503 maternal parent. This means that based upon the DNA fingerprints produced by primer RPTest 6 it is not possible to reject or accept the null hypothesis that the embryos were the result of the controlled pollination between the *P. elliottii* and *P. caribaea* parents.

Monomorphic microsatellite markers are not useful as it is impossible to distinguish between species or to determine gene flow or parental contribution. However, the fact that cross-species amplification does occur indicates that sequence conservation does exist within the genus *Pinus* and that those specific loci were more conserved than others.

A second type of uninformative microsatellite marker is a marker that produces fingerprints that contain null alleles. Null alleles are the manifestations of mutations in one or both of the microsatellite primer binding regions (Callen *et al.*, 1993). These mutations inhibit the primers from binding and may result in the loss of the PCR product. An example of the uninformative nature of null alleles, in the current study, is evident in the microsatellite fingerprints produced by primer RPTest 6 in the E 503 x Ach 49 hybrid population (Figure 2.3). From these fingerprints it is impossible to distinguish the E 503 maternal parent from the Ach 49 pollen donor as the polymorphic allele (i.e. a fragment of 250 – 280 bp) is absent. This means that based upon the DNA fingerprints produced by primer RPTest 6 it is impossible to accept the null hypothesis that the embryos were the result of the controlled pollination between the *P. elliottii* and *P. caribaea* parents as the polymorphic allele is absent.

The ability to determine gene flow and parental contribution via cross-species amplification of microsatellite markers, holds important implications for the application of this technique on a large scale in the forestry industry. This technique not only reduces the time and costs involved in marker development, but also the time and costs involved during the nursery phase and the selection of superior clones for future plantations. For example, once superior *P. elliottii x P. caribaea* hybrids are identified, based on field trial performance, it will be possible to screen the seedlings produced in order to verify that they really are the progeny of the controlled pollination between the superior parents. This procedure will allow seedlings that do not conform to the microsatellite fingerprints

expected for the hybrid to be discarded early on in the screening phase and ensure that only the correct genotypes enter into the bulking-up phase.

2.1.5 Conclusion

The fact that microsatellite markers developed in other species can be used successfully in the DNA profiling of related species, makes this an extremely powerful and accessible technique. This cross-species amplification also greatly reduces the cost and time requirements involved in DNA profiling studies, by eliminating the need for sequence information and marker development (Echt *et al.,* 1999; Weising and Gardner, 1999).

Not all microsatellite markers produce DNA fingerprints that are equally informative and useful. The usefulness of a marker is determined by its degree of polymorphism in the species that are being tested and the extent to which gene flow and parental contribution can be determined from the fingerprints produced. Polymorphic microsatellite markers tend to be highly informative and are therefore far more useful than monomorphic markers, which tend to be relatively uninformative.

The polymorphism displayed by microsatellites makes them useful as markers in linkage studies (Moore *et al.,* 1991). However, parental determination should generally be approached via a process of elimination in order to accurately accept or reject the null hypothesis that the embryo was the result of successful controlled pollination. It is therefore important that information from several loci be pooled and analyzed together,

as it is unlikely that a single locus will provide enough information upon which the null hypothesis may be rejected or accepted (Strassmann, 1998).

2.2 DEVELOPMENT OF SSR MARKERS IN *PINUS ELLIOTTII X PINUS CARIBAEA* HYBRIDS

2.2.1 Introduction

Microsatellites are repeated sequences that consist of 1 – 5 bp which are scattered throughout the eukaryotic genome (Litt and Luty, 1989). Variations in the number of these tandem repeats result in the generation of extensive polymorphisms (Hearne *et al.*, 1992). These polymorphisms make microsatellite loci extremely useful markers for genetic mapping, DNA typing and population genetic studies (Dayanandan *et al.*, 1998; Dib *et al.*, 1996; Hokanson *et al.*, 1998; Schmidt *et al.*, 2000).

In early studies microsatellites were either cloned and sequenced (Litt and Luty, 1989; Tautz, 1989), or were identified from sequence databases (Weber and May, 1989). Based on this information primers were designed to recognize sequences flanking the microsatellite region. The polymorphic amplification products obtained after PCR with these specifically designed primers were then separated on polyacrylamide gels (Wu *et al.*, 1994).

In 1994 Zietkiewicz *et al.* demonstrated a novel approach to measuring genetic diversity in plants and animals by using primers based on microsatellites. The Zietkiewicz technique combined the benefits of microsatellite and RAPD analysis (Hantula *et al.*, 1996; Hantula and Müller, 1997). Two types of polymorphism can be identified using this random amplified microsatellite (RAMs) technique. Firstly the on / off polymorphisms, similar to those found using RAPD markers, as variation is due to existence or absence of a marker. Secondly, polymorphism due to length differences in the sizes of the amplification products (Hantula *et al.*, 1996). The RAMs technique involved the amplification of DNA between the distal ends of two closely located microsatellite regions, by using random microsatellite primers (Zietkiewicz *et al.*, 1994; Hantula *et al.*, 1996, 1998).

The aim of this study was to develop microsatellite markers in the *P. elliottii x P. caribaea* hybrid population, using the RAMs approach, and to determine how informative these microsatellite markers could be with regard to DNA profiling.

2.2.2 Materials and Methods

2.2.2.1 Plant material and DNA isolation

Needle tissue of the female parent tree, *P. elliottii* E 503, supplied by SAFCOL, South Africa, was used as the main source of plant material in this study. DNA was isolated from this needle tissue using a modified CTAB method (Murray and Thompson, 1980) as described in section 2.1.2.2.

2.2.2.2 Primers and PCR conditions

Three different microsatellite primers were screened in this study, namely CA(8) (Appendix A), CT(8) and GTG(5). These primers were selected for their ability to produce amplification profiles linked to random microsatellites throughout the genome of *P. elliottii.*

All amplification reactions were carried out in 25 µl reaction volumes and DNA template concentrations ranging from 12.5 ng/µl to 75 ng/µl were tested. The reaction mixture contained template DNA, 1% (v/v) Promega thermophilic DNA polymerase 10X buffer, magnesium free (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% (v/v) Triton®X-100), 300 µM dNTPs, 3 mM $MgCl_2$, 0.5 µM primer, and 1 U Promega *Taq* DNA polymerase in storage buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA 1 mM DTT, 50% (v/v) glycerol, 0.5% (v/v) Tween®20, 1% (v/v) Triton®X-100). Temperature cycling was done using a, Perkin Elmer GeneAmp® PCR system 9700 (Applied Biosystems), thermal cycler programmed for an initial step of 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 47°C and 2 min at 72°C. The amplification cycles were followed by a final elongation step of 7 min at 72°C. The amplification products were analyzed by electrophoresis on 3% agarose gels (Whitehead Scientific) in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 5.3 V/cm for 30 min. The amplification products were detected by staining with ethidium bromide (0.5 µg/ml) and visualized by exposure to UV illumination. Molecular size marker III, prepared by

digesting lambda DNA with restriction enzymes *Eco*RI and *Hin*dIII, was included in all electrophoresis profiles.

2.2.2.3 Cloning and transformation

In order to ensure the cloning of a variety of amplification products, the profile displaying the greatest number and variety of bands was selected. Impurities, which may have influenced the effectiveness of the cloning and transformation reactions, were removed from the amplification products using the QIAquick™ PCR purification kit (QIAGEN). The amplification products were then randomly cloned into the T7 / SP6 cloning site of the pGEM®-T Easy plasmid vector, before being transformed into *Escherichia coli* JM 109 competent cells using the Promega pGEM®-T EASY vector system. The transformed *E. coli* cells were then plated out, at various concentrations, on LB plates (1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl, 1.5% (w/v) Agar, pH 7.4) containing Ampicillin (50 mg/ml), IPTG (100 mM) and XGAL (2% w/v) and incubated at 37°C for 16 to 24 h. Transformed colonies, identified using the blue / white selection criteria of the IPTG / XGAL system, were used to inoculate liquid LB, containing Ampicillin (50 mg/ml), and were incubated for 24 h at 37°C.

2.2.2.4 Colony screening

In order to identify transformed colonies containing inserts of interest screening was done directly on each colony using 25 µl PCR reactions with primers homologous to the flanking regions of the recombinant plasmids. DNA was added to each reaction by lightly pressing a sterile pipette tip to the selected colony, inserting the tip into the eppendorf tube containing the reaction mixture and pipetting up and down. The reaction mixtures contained 1% (v/v) Promega thermophilic DNA polymerase 10X buffer, magnesium free, 100 µM dNTPs, 1 mM $MgCl_2$, 0.5 µM of each microsatellite primer, 0.5 µM primer SP6, 0.5 µM primer T7 and 1 U Promega *Taq* DNA polymerase in storage buffer A. Temperature cycling was done using a Perkin Elmer GeneAmp® PCR system 9700 (Applied Biosystems), thermal cycler programmed for 25 cycles of 30 s at 94°C, 45 s at 50°C and 2 min at 72°C, followed by an elongation step of 5 min at 72°C. The amplification products were analyzed by electrophoresis on 2% agarose gels in 1 X TAE buffer at 5.3 V/cm for 20 min. The amplification products were detected by staining with ethidium bromide (0.5 µg/ml) and visualized by exposure to UV illumination. Molecular size marker III was included in all electrophoresis profiles.

2.2.2.5 Plasmid isolation and PEG precipitation

Based on the amplification profiles obtained during the screening procedure, transformed colonies were selected and their plasmids isolated using the Nucleospin® Plasmid kit (Macherey-Nagel) The isolated plasmid DNA was then purified further by polyethylene

glycol (PEG) precipitation. The precipitation involved the addition of 625 mM NaCl and 9.75% (w/v) PEG_{6000} to the isolated plasmid DNA. The mixture was mixed gently by pipetting and left on ice for 30 min. The mixture was centrifuged at 12 000 x *g*, at 4°C, for 30 min. The aqueous phase was discarded and the pellet was washed twice in 70% ethanol. The pellet was air-dried, at 40°C on a heating block, until all traces of ethanol evaporated. The pellet was resuspended in 20 µl sterile water and the DNA concentration was determined spectrophotometrically.

2.2.2.6 Sequencing

Sequencing of the insert was done on a Perkin Elmer GeneAmp® 96000 thermal cycler, using the ABI Prism™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to manufacturers recommendations. The cycle sequencing consisted of 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The sequencing reaction was followed by 0.7 X (v/v) 95% ethanol and 42 mM sodium acetate, pH 4.6, precipitation. The mixture was then left on ice for 10 min before being centrifuged at 12 000 x *g* for 30 min. The supernatant was discarded and the pellet was rinsed twice in 70% ethanol prior to air-drying on a heating block, at 40°C, until all traces of ethanol had evaporated. The DNA sequence was subsequently obtained using a Perkin Elmer ABI 377 automated sequencer and analyzed using the Sequence Navigator™ programme, version 1.01.

2.2.2.7 Primer development and testing

Once analyzed the sequence was searched for regions containing microsatellites. Primers, of between 17 and 21 nucleotides and of G + C percentages of ca. 50% - 70%, were designed in the regions flanking the microsatellites. The primer design utility on the Cybrgene AB website (http://www.cybergene.se) was used to ensure that the primer sequences selected did not contain hairpin loops or palindromic sequences, which would reduce the efficiency of the primer-template interaction.

All of the primers were subsequently tested in 25 µl PCR reactions. The reaction mixture contained 50 ng/µl *P. elliottii* E 503 needle DNA, 1% (v/v) Promega thermophilic DNA polymerase 10X buffer - magnesium free, 300 µM dNTPs, 3 mM $MgCl_2$, 0.25 µM of each primer, and 1 U Promega *Taq* DNA polymerase in storage buffer A. Temperature cycling was done using a Perkin Elmer GeneAmp® PCR system 9700 (Applied Biosystems). The reaction conditions were optimized for each primer by starting at an annealing temperature 5°C lower than the Tm of the primers and gradually increasing this temperature by 5°C with each subsequent PCR. The thermal cycler was programmed for an initial step of 4 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at various annealing temperatures and 45 s at 72°C. The amplification cycles were followed by a final elongation step of 5 min at 72°C. The PCR reactions were screened for amplification products on 2% agarose gels in 1 X TAE buffer, at 5.3 V/cm for 20 min, and were detected by staining with ethidium bromide (0.5 µg/ml). Molecular size marker III was included in all electrophoresis profiles.

PCR reactions which were found to contain amplification products were further analyzed by separation on 7.5% non-denaturing polyacrylamide gels (3.75 % (v/v) FMC® Long Ranger Gel Solution, 1% (v/v) TBE buffer, 0.1% (w/v) APS, 0.004% (v/v) TEMED) in 1 X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 26.6 V/cm for one and a half hours. After separation the amplification products, which were detected by staining with (1:10 000) Sybr®GreenI Nucleic Acid Gel stain (Boehringer Mannheim), were visualized by exposure to UV illumination. Boehringer Mannheim 100 bp DNA ladder was included as the molecular size marker in all electrophoresis profiles.

The amplification products were then cut out of the PAGE gels and extracted from the plugs by using the QIAquick™ PCR purification kit (QIAGEN) according to manufacturers recommendations. The purified amplification products were then cloned into the T7 / SP6 cloning site of the pGEM®-T Easy plasmid vector, before being transformed into *Escherichia coli* JM 109 competent cells using the Promega pGEM®-T EASY vector system. The transformed *E. coli* cells were then plated out, at various concentrations, on LB plates containing Ampicillin (50 mg/ml), IPTG (100 mM) and XGAL (2% w/v) and incubated at 37°C for 16 to 24 hours. Transformed colonies, identified using the blue / white selection criteria of the IPTG / XGAL system, were then selected and their plasmids isolated using the Nucleospin® Plasmid kit (Macherey-Nagel). The isolated plasmid DNA was then purified further by PEG precipitation, before sequencing of the insert was done on a Perkin Elmer GeneAmp® 96000 thermal cycler, using the ABI Prism™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to manufacturers recommendations.

2.2.3 Results

The careful screening of transformed colonies resulted in the selection of ten colonies that contained inserts potentially suitable for sequencing. The ten sequences obtained are given in Appendix A. The ten sequences were analyzed and subsequently screened for regions containing microsatellites. Primers, of between 17 and 21 nucleotides, were then designed in the regions flanking these microsatellite loci. A summary of the primers designed is presented in Table 2.4, while the position of the primers relative to the sequences from which they were designed can be seen in Appendix A.

Two of the ten microsatellite primer pairs that were designed were found to produce amplification products (primer JD7-AMO and JD9-AMO), while the remaining eight would not produce any amplification products (primers JD1-AMO, JD2-AMO, JD3-AMO, JD4-AMO, JD5-AMO, JD6-AMO, JD8-AMO and JD10-AMO).

The microsatellite markers, JD7-AMO and JD9-AMO, produced monomorphic amplification products. Figure 2.4 shows the microsatellite fingerprints, of the E 503 x Pch 23 hybrid population, which were obtained using microsatellite marker JD9-AMO, while figure 2.5 shows the microsatellite fingerprints of the same population which were obtained using microsatellite marker JD7-AMO.

Microsatellite marker JD9-AMO was designed to produce an amplification product of approximately 300 base pairs. This corresponds with the amplification product shown in

figure 2.4 as well as with the sequencing results obtained, which overlap substantially with the original RAMs sequence 3 (Appendix A, Figure A-3).

Table 2.4. Summary of RAM primers developed.

Primer	Forward sequence (5' – 3')	Reverse Sequence (5' – 3')
JD1-AMO	F1: ATAATCAAGCATACAAG	R1: GGTTGTAGTGGTGTCTC
JD2-AMO	F2: GCCCACATCTACCCAAG	R2: CTGATGTGGAAGGTCTG
JD3-AMO	F3: GCAGGACAACGCCAA	R3: GCAAAACAAGATAAT
JD4-AMO	F4: GCATCTCATTTTCTATT	R4: GCGTGTTTAGATACTTTA
JD5-AMO	F5: CCCAACACTCCTGGGCT	R5: GAGCAGGACAACGCCAA
JD6-AMO	F6: TGGACAAACGCCTAAC	R6: AGGTCAAGGGAGACAA
JD7-AMO	F7.1: TGGATCCCCCAAGTCT F7.2: TAGTCCAGCCTTCAGTG	R7: CAAAGAGGAAACGAACAG
JD8-AMO	F8: CACACACACATGGACGCA TA	R8.1: CGAACTGAATGGGTTGTGTG R8.2: GTTTACCGCAGTTGGTTG
JD9-AMO	F9.1:GCACACACACACACACAAAAAAAACT F9.2: CACCAGGGGAAACCGAG	R9: TTGCTCAAGCCAGCCAG
JD10-AMO	F10: CCCGGACGATTTCCTTG	R10: GTGATTGACCCGCCTCC

Microsatellite marker JD7-AMO was designed to produce an amplification product of approximately 250 base pairs. However, as is shown in figure 2.4, an amplification product of approximately 350 base pairs was obtained. When the annealing temperature

was raised, thereby increasing the stringency of the PCR reaction, the same amplification product was obtained.

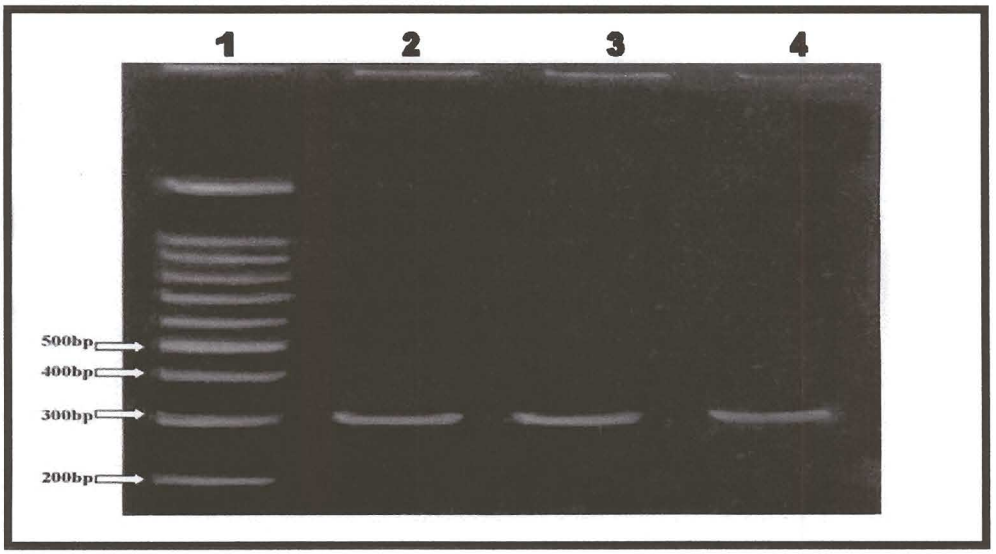


Figure 2.4. Microsatellite fingerprints of the *P. elliottii* (E 503) *x P. caribaea* (Pch 23) hybrid population. The fingerprints were obtained by PCR amplification with primer JD9-AMO. Lane 1 contains 100 bp DNA ladder, while lanes 2 to 4 contain the fingerprints obtained using E 503 needle DNA, E 503 x Pch 23 embryo DNA and PCH 23 needle DNA, respectively.

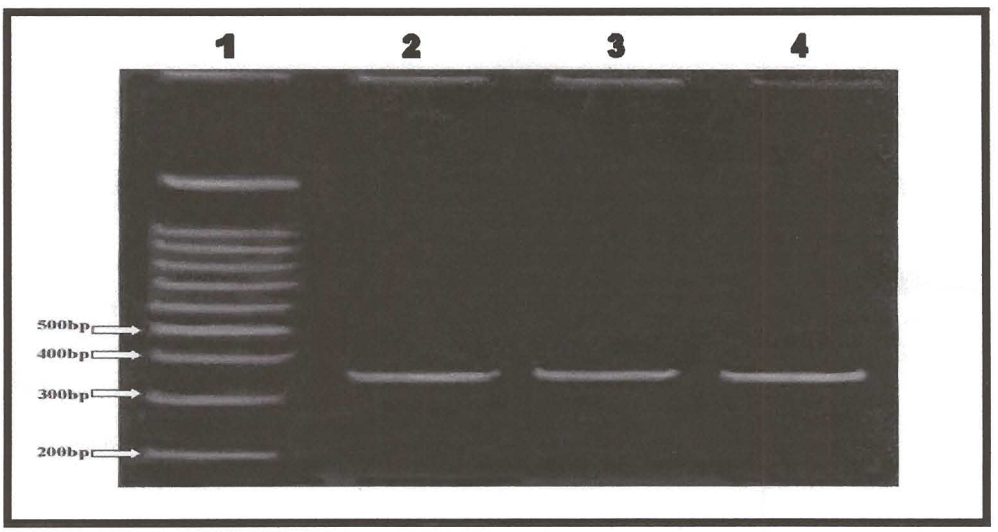


Figure 2.5. Microsatellite fingerprints of the *P. elliottii* (E 503) *x P. caribaea* (Pch 23) hybrid population. The fingerprints were obtained by PCR amplification with primer JD7-AMO. Lane 1 contains 100 bp DNA ladder, while lanes 2 to 4 contain the fingerprints obtained using E 503 needle DNA, E 503 x Pch 23 embryo DNA and PCH 23 needle DNA, respectively.

These results seem to indicate that the JD7-AMO reverse primer bound further down-stream than it was designed to, thereby amplifying an additional, untargeted section of the *Pinus* genome. The sequencing results verify these results as substantial sequence homology was found to exist with that of the original RAMs sequence 6 (Appendix A, Figure A-6).

2.2.4 Discussion

In 1994 Zietkiewicz *et al.* found that RAMs could be used to generate species specific DNA fingerprints. These species-specific DNA fingerprints can provide useful information pertaining to linkage, gene flow and parental contribution within and between populations (Hantula *et al.,* 1996).

Due to the costs involved in sequencing and primer design only ten inserts were sequenced. The sequences were analyzed and ten primer pairs were designed. Upon testing it was found that only two of the ten primer pairs (20%) resulted in the production of amplification products. In both cases the amplification products produced were monomorphic. As discussed in section 2.1.4 monomorphic microsatellite markers are not as useful as polymorphic markers, as it is impossible to distinguish between species or to determine gene flow or parental contribution. Although the amplification products produced were monomorphic the results indicate that this method has potential for microsatellite marker development in pines.

The fact that marker JD-7AMO produced an amplification product which was approximately 100 bp larger than it should have been indicates the need for marker screening to ensure that the amplification product obtained actually corresponds with the expected microsatellite region being targeted. In this scenario the marker does not need to be discarded as it amplified a conserved region in *P. elliottii*, *P. caribaea* and in the *P. elliottii x P. caribaea* hybrid.

In section 2.1.4 the important role which polymorphic microsatellite markers can play in population studies was discussed. Similarly, polymorphic microsatellite markers developed using RAMs would be just as useful for linkage, gene flow and genotype determination.

2.2.5 Conclusion

The RAMs approach to microsatellite marker development, although not as cost effective as simply using markers developed in related species, is a relatively fast way of developing species specific microsatellites. Although the source of variability in the RAMs fragments is still unknown, Hantula *et al.* (1996) proposed that the most likely polymorphisms would be length polymorphisms as variability in these elements is mostly due to differences in the number of repeats. The species specific DNA fingerprints obtained using RAMs are highly reproducible and can be used to identify both inter- and intra-species specific polymorphisms.

The results of this study indicated that the RAMs technique is potentially an effective way of developing microsatellite markers specific to the *P. elliottii x P. caribaea* hybrids. The results are promising enough to justify further investigation into this approach for marker development and perhaps even investigation into the potential of these markers for cross-species amplification.

CHAPTER 3

THE INFLUENCE OF STORAGE AND MORPHOLOGY ON THE VIABILITY OF *PINUS CARIBAEA* POLLEN

3.1 Introduction

The hybrid between *P. elliottii* and *P. caribaea* displays many promising and desirable characteristics, such as rapid growth combined with excellent wood quality (Denison and Kietzka, 1993; Stanger *et al.,* 1999). These characteristics are mainly attributed to the hybrid vigor obtained during the cross and is the main reason why *P. elliottii x P. caribaea* hybrids are favored above improved parent species for commercial planting (Bester, 2000). However, a number of problems are associated with the *P. elliottii x P. caribaea* hybrid. Firstly, the *P. caribaea* pollen ripens approximately three months before the *P. elliottii* ovules are receptive (Mather, 2000); there is therefore a need to optimize the long-term storage conditions of the *P. caribaea* pollen. The second major problem facing the hybrid is one of incompatibility between *P. elliottii* and *P. caribaea,* which results in low seed set and a low number of viable hybrid seeds (Mather, 1996).

The odds of successful cross-pollination are increased by random wind dispersal and the millions of pollen grains that are produced (Baker and Baker, 1979). This ensures that at least a small percentage will reach its destination and result in pollination thereby ensuring the proliferation of the species. Of these millions of pollen grains a percentage will be sterile, others will be malformed and the vast majority will end up being dispersed by the wind and never even come near the pollination droplet (Singh, 1978; Wright, 1976). Other factors that have been found to influence pollination are male fertility (Schoen and Cheliak, 1987), pollen viability and pollen tube growth rates (Ottavariano *et al.,* 1980) and non-random embryo abortion (Sorensen, 1982).

Pollen viability is perhaps the most important factor known to influence reproduction in plants. Pollination and fertilization are directly dependent on pollen viability (Singh, 1978). According to Pacini (1996) cytoplasmic carbohydrates and sucrose are involved in protecting the pollen during exposure and dispersal, while Van Bilsen *et al.* (1994) found that a direct correlation exists between pollen viability and lipid degeneration during storage.

The entire reproductive cycle is dependent on the ability of the pollen grain to germinate once it is captured in the pollination droplet. Germination requires energy as well as the elaboration of cellular and structural materials within the pollen tube as it is formed (Baker and Baker, 1979). Pollen tube growth consists of two phases *in vivo*, namely an initial autotrophic phase followed by a heterotrophic phase (Bellani *et al.*, 1985). During the autotrophic phase energy and building blocks must be provided by the reserves, such as oils, sugars and starches in the pollen grain itself, as only limited additional material may be absorbed from the surrounding stylar tissue (Baker and Baker, 1979; Bellani *et al.*, 1985; Delph *et al.*, 1997; Willemse, 1968). During the heterotrophic phase polysaccharide reserves in the pistil are mobilized and enzymes for carbohydrate metabolism are induced in the vicinity of the growing pollen tube (Roggen, 1967).

Two broad aims were established for this investigation. The first aim was to optimize the storage conditions for the *P. caribaea* pollen during the three month storage period. The second aim was to investigate the relationship that exists between the morphology and viability of *P. caribaea* pollen.

3.2 Materials and Methods

3.2.1 Plant Material

Eighteen *P. caribaea* pollen types, which were provided by SAFCOL, South Africa, were used in this study. These eighteen types included pollen from: Ach 22, Ach 24, Ach 29, Ach 31, Ach 33, Ach 49, Ach 57, Ach 65, Ach 91, Ach 93, Ach 114, Ach 271, Acb 60, Acb 62, Pch 23, Pch 69, Pch 88 and Pch 105.

3.2.2 Influence of environmental factors on *P. caribaea* pollen viability

Ten of the eighteen *Pinus caribaea* pollen types (Ach 24, Ach 29, Ach 31, Ach 65, Ach 93, Ach 114, Pch 23, Pch 69, Pch 88 and Pch 105), which were provided by SAFCOL, Dukuduku, South Africa, were used in the germination trials to determine the effects of environmental conditions on viability. The factors investigated included the effects of pollen age, the temperature and humidity at which the pollen was stored and the developmental stage of the pollen cone at the time of harvesting on the viability of the pollen (Table 3.1). In order to facilitate germination, the pollen was placed on 3% (w/v) agar containing 10% (w/v) sucrose and left at room temperature for 72 hours, as described by Wright (1976). After 72 hours microscope slides were prepared and the samples were investigated using an Axiovert 35 28436 inverted light microscope. Germination percentage was determined by counting the number of germinated pollen grains per microscope field, at 20 X magnification, and by repeating the procedure over ten random microscope fields per pollen type.

Table 3.1. Environmental conditions investigated.

	Pollen age	Pollen cone developmental stage	Time of harvesting	Environmental conditions
***P. caribaea* Pollen**	• Fresh (1 – 2 weeks) • 3 months old • old (ca. one year)	• Young cones (ca. 10mm) • Medium cones (ca. 20 mm) • Old cones (ca. 35 mm)	• 08:30 • 12:00 • 16:30	• 4°C • 20°C • 20°C Low Humidity (LH) • 20°C High Humidity (HH) • -20°C • -80°C

3.2.3 The influence of environmental factors on the ultrastructure of *P. caribaea* pollen

P. caribaea pollen, Pch 23, Pch 29 and Pch 53, which were collected at various times of the day and which were exposed to various environmental conditions during storage, as described in section 3.2.2, was prepared for transmission electron microscopy. The pollen was fixed for two weeks in 3% (v/v) glutaraldehyde in 75 mM sodium phosphate buffer (pH 7.5). After the initial fixation the pollen was rinsed three times in 75 mM sodium phosphate buffer (pH 7.5) and then fixed further in 2% (w/v) aqueous osmium tetroxide for 2 hours, before being rinsed three times with distilled water. The pollen was subsequently stained overnight with 0.5% (w/v) uranylacetate-dihydrate, then rinsed

twice with distilled water and heated for a few seconds in a water bath at 65°C. Heated agar was subsequently added and the tubes covered with parafilm. Once the agar had cooled and solidified it was cut into small cubes. These pollen containing agar squares were then dehydrated in 30%, 50%, 75%, 95%, and twice in 100% ethanol for 30 minutes per dehydration step. The ethanol was subsequently replaced with 1.2 X propylene oxide and the samples were stored while the plastic embedding solution (10% (w/v) VCD, 26% (w/v) NSA, 6% (w/v) DER 736, 0.4% (w/v) DMAE) was prepared according to Spurr (1969). The pollen containing agar squares were then embedded in the plastic solution, which was cast in molds and left at 60°C for eight hours to allow polymerization. Excess plastic was trimmed from around the embedded pollen material and the material was subsequently sectioned, in Amstrong diameter, using the LKB ultrotome III ultramicrotome. The sections were caught up in a loop and transferred to 200-grid mesh. The section containing grids were then stained in 5% (w/v) uranylacetate for 30 minutes followed by staining with lead citrate as described by Reynolds (1963). The sections were examined using a Phillips CM 100 transmission electron microscope at 60 kV.

3.2.4 *P. caribaea* pollen morphology

3.2.4.1 Scanning electron microscopy (SEM)

The eighteen *P. caribaea* pollen types were prepared for Scanning Electron Microscopy according to the procedures outlined by Coetzee and Van der Merwe (1999). The procedure involved fixation of the pollen in 2.5% (v/v) glutaraldehyde in 75 mM sodium phosphate buffer (pH 7.5) for at least one hour. After fixation the pollen was rinsed three times in sodium phosphate buffer (pH 7.5) and fixed further in 0.25% (w/v) aqueous osmium tetroxide for approximately half an hour. The pollen was rinsed three times in distilled water and dehydrated for 15 minutes in 70% ethanol, followed by three times 15 minutes in 100% ethanol. The dehydrated pollen was then critical point dried in liquid carbon dioxide. Once dry, the pollen was mounted on a stub and sputtered with a colloidal gold solution (Duff *et al.*, 1993) using the Biorad SEM coating system. The prepared pollen stubs were examined using a Jeol Winsem JSM 6400 scanning microscope at 5 kV and 1 000 X magnification. Twenty images, in a side on view and twenty in a distal view of each of the eighteen pollen types were scanned in. As it was impossible to obtain a "size" estimate, such as total area or volume of individual pollen grains, five dimensions (representative of the size and shape of the pollen grains and their airbags) were measured using the UTHSCSA *Image Tool* programme to obtain a data set applicable for statistical analysis (Figure 3.1).

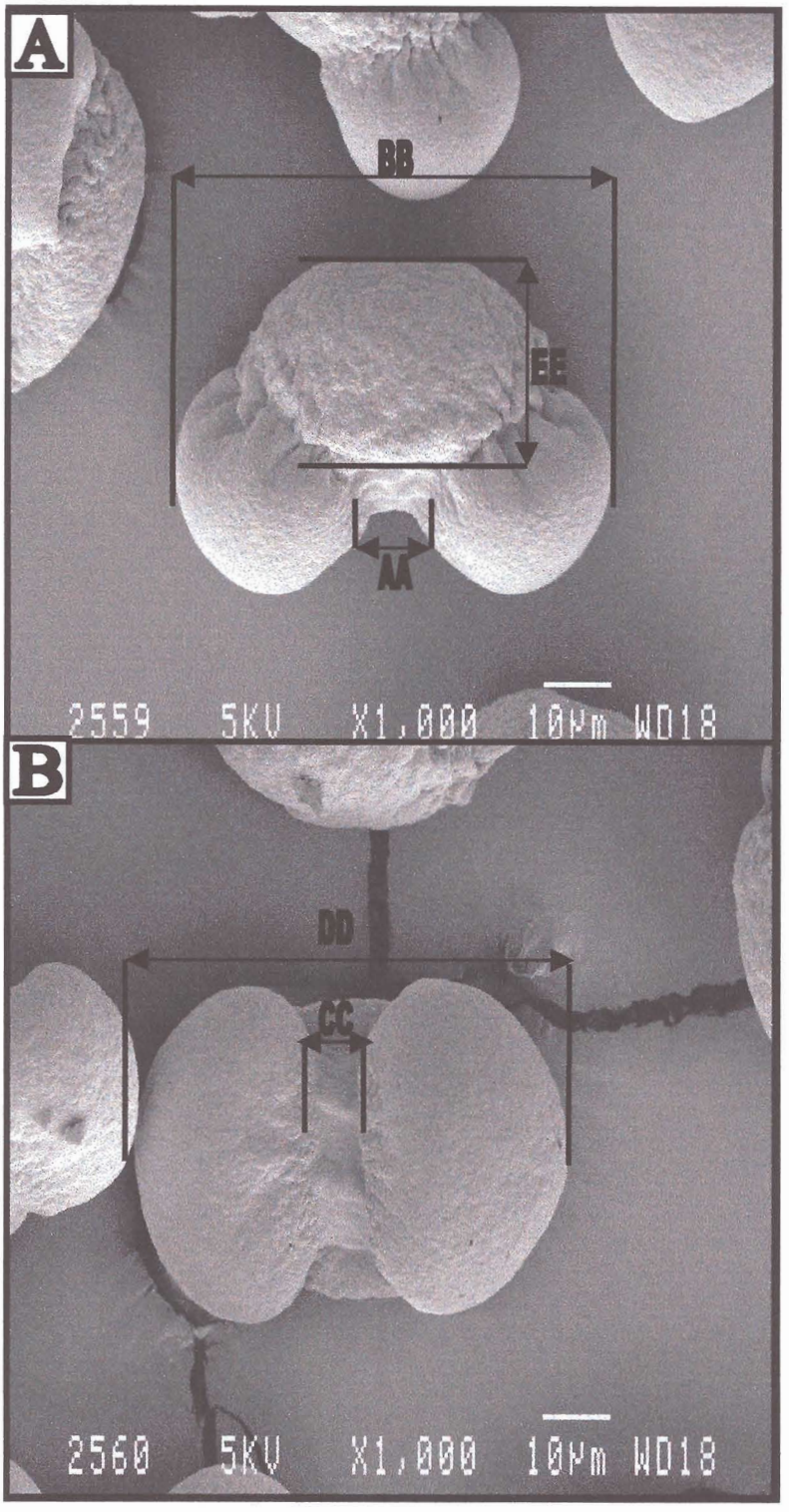


Figure 3.1. Five dimensions measured for each of the eighteen *P. caribaea* pollen types. (A). Side on view facilitating the measurement of dimensions AA, BB and EE. (B). Distal orientation facilitating measurement of dimensions CC and DD.

3.2.4.2 Transmission electron microscopy (TEM)

Seven *P. caribaea* pollen types (AP 30, AP 128, AP 168, AP 294, Pch 23, Pch 29 and Pch 53) were randomly selected and prepared for transmission electron microscopy as described in section 3.2.3.

3.2.5 Statistical analysis of *P. caribaea* germination vs. morphology

A number of questions arose from the cursory examination of the dimensional and germination data that had been obtained for the eighteen *P. caribaea* pollen types. In Table 3.2 these questions and the statistical analytic techniques that were used to investigate them are summarized.

Table 3.2. Statistical analytic techniques used to investigate the questions relating to the eighteen *P. caribaea* pollen types (SAS / STAT Users guide, 1989)

Question	Statistical technique
Are the eighteen types significantly different?	• Analysis of variance (ANOVA)
Is there an association between the dimensions of the types and their germination rate?	• Regression: Germination = dependent variable
Would the sum of the dimensions provide a reliable size variable?	• Cronbach coefficient alpha
If the sum of the dimensions is not reliable, could another aggregation technique help?	• Principle component analysis
Do the dimensions allow a clustering of the eighteen types into separate groups?	• Median Hierarchical cluster analysis • Ward's minimum variance cluster analysis

3.3 Results

3.3.1 Influence of environmental factors on *P. caribaea* pollen viability

The germination data displayed a direct correlation between pollen viability and the age of the *P. caribaea* pollen, the temperature and humidity at which the pollen was stored and the developmental stage of the pollen cone at harvesting. Highest germination percentages were observed the shorter the duration of storage (i.e. the fresher the pollen, Figure 3.2) and the lower the temperature at which the pollen was stored (Figure 3.3). High germination percentages were also observed for pollen that had been stored at room temperature and at low relative humidity (Figure 3.3).

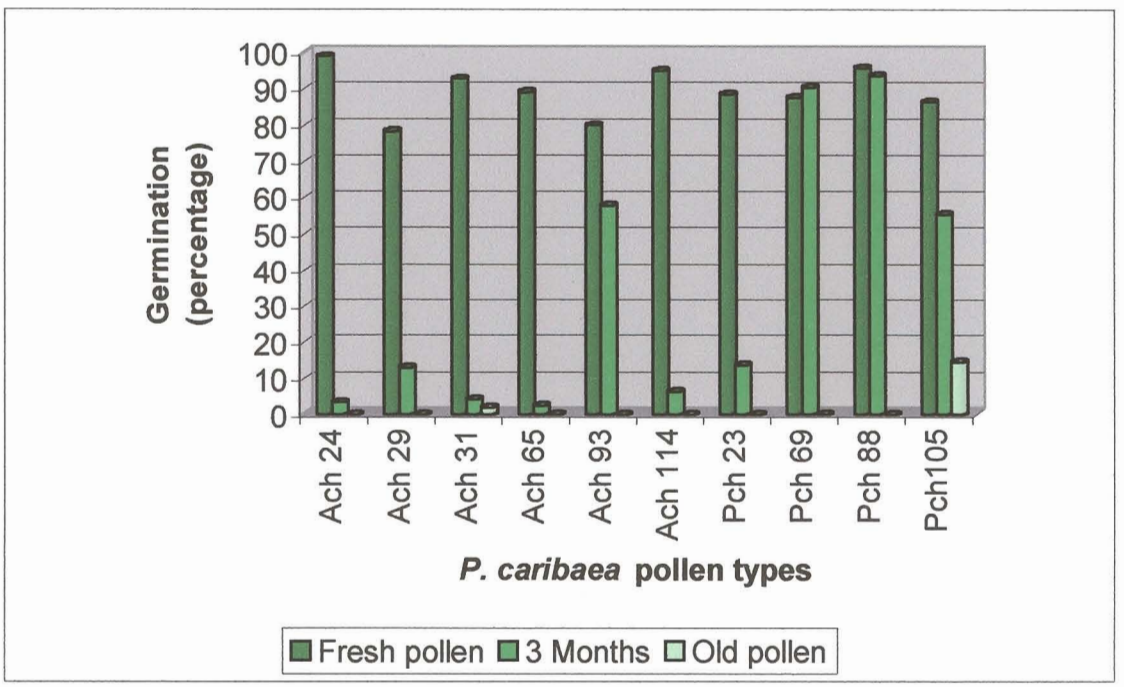


Figure 3.2. Germination percentages of *P. caribaea* pollen stored for various periods of time.

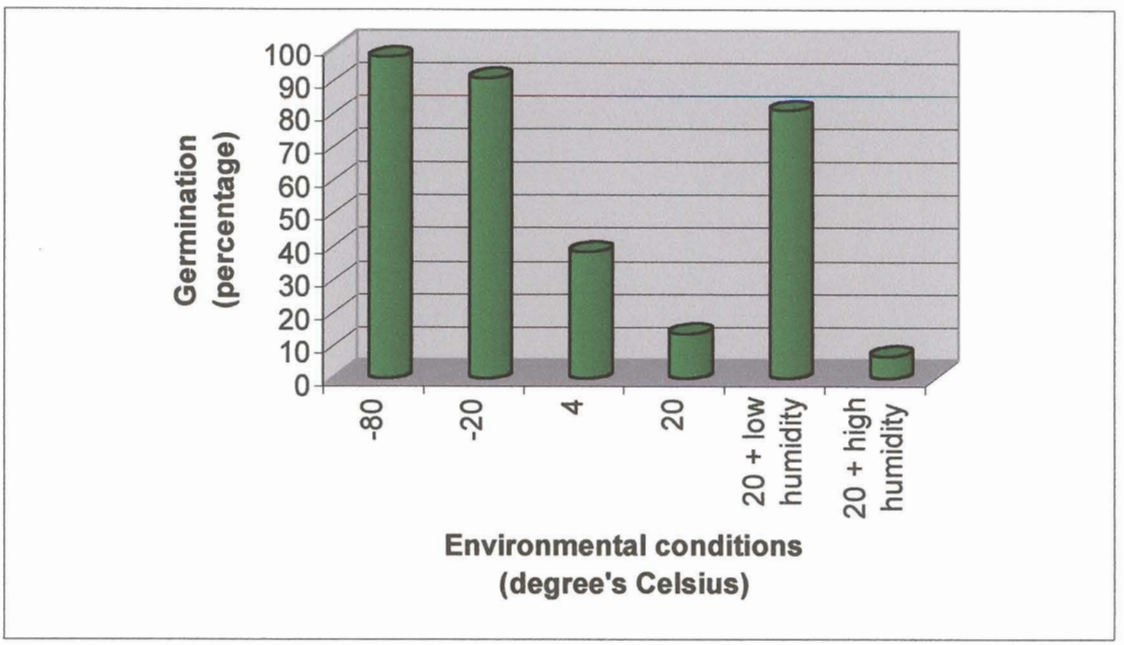


Figure 3.3. Germination percentages of *P. caribaea* pollen stored at various temperatures and humidity.

A correlation between the developmental stage of the pollen cone at the time of harvesting and pollen viability was also found to exist. High germination percentages were observed for the mature (medium and old) pollen cones and lowest germination percentages for young, immature pollen cones (Figure 3.4). The variable results obtained for the study on the effects of collection time on pollen viability indicated that this factor had little effect on the germination of the *P. caribaea* pollen. The results of this study were subjected to statistical analysis and were found to be significant.

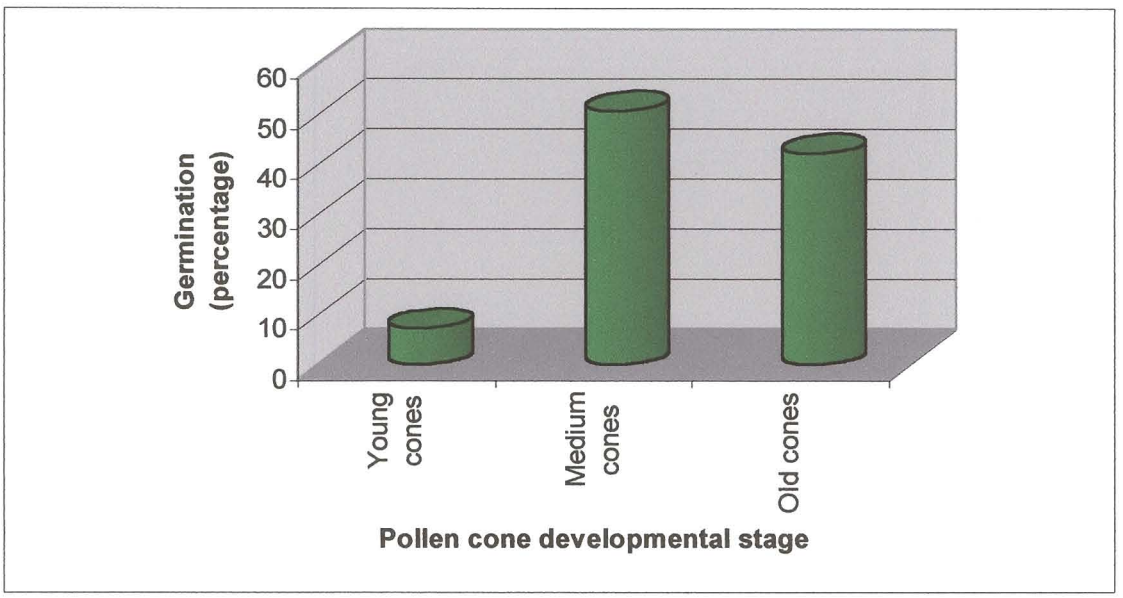


Figure 3.4. Influence of pollen cone developmental stage on germination rate of *P. caribaea* pollen.

3.3.2 The influence of environmental factors on the ultrastructure of *P. caribaea* pollen.

The results of the TEM investigation on the influence that environmental conditions exert on the ultrastructure of *P. caribaea* pollen were found to be variable and generally uninformative. No clear correlation was found which could link poor pollen viability with the time of harvest, the pollen cone developmental stage or even the temperature at which the pollen was stored.

3.3.3 *P. caribaea* pollen morphology

3.3.3.1 Scanning electron microscopy (SEM)

Based upon the SEM pictures it is possible to describe the general structure of *Pinus caribaea* pollen as being large, winged grains, which are strongly polarized, with proximal and distal regions. The wings are hemispheric, situated in the distal region and border on the germ furrow (Figure 3.5). The surface of the cap is sculptured, while the surface of the wings tends to be porous or pitted (Figure 3.6). *Pinus* pollen was similarly described by both Tomlinson (1994) and Pardi *et al.* (1996). The average size of the *P. caribaea* pollen based on the five dimensions, as described in Figure 3.1, is given in Table 3.3.

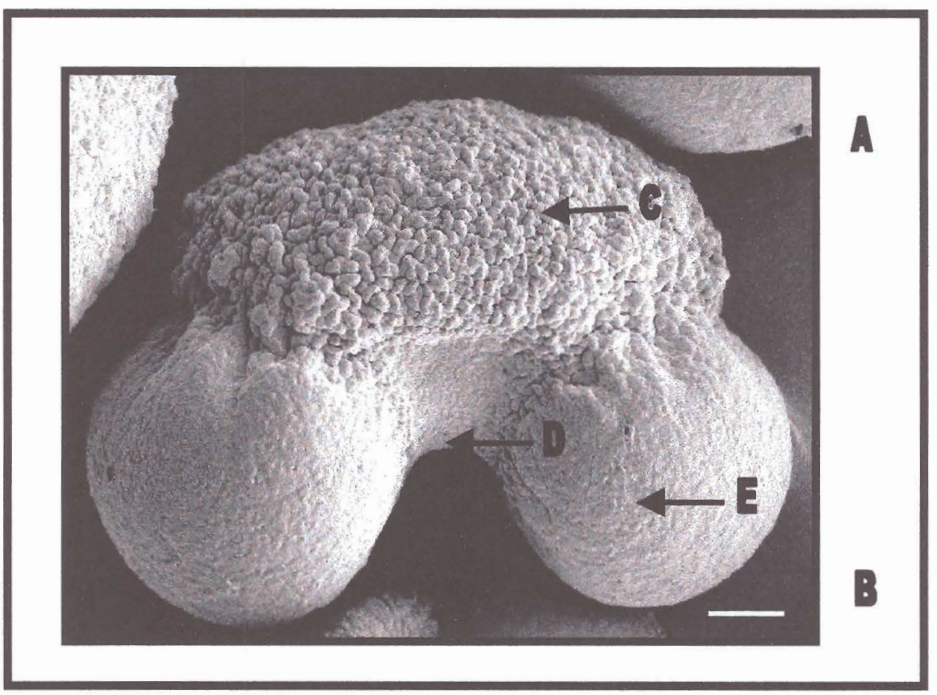


Figure 3.5. SEM picture of the general structure of *P. caribaea* pollen. (A) Proximal region. (B) Distal region. (C) Cap. (D) Germ furrow. (E) Hemispheric wing. 1 000 X Magnification, 10 μm Scale bar.

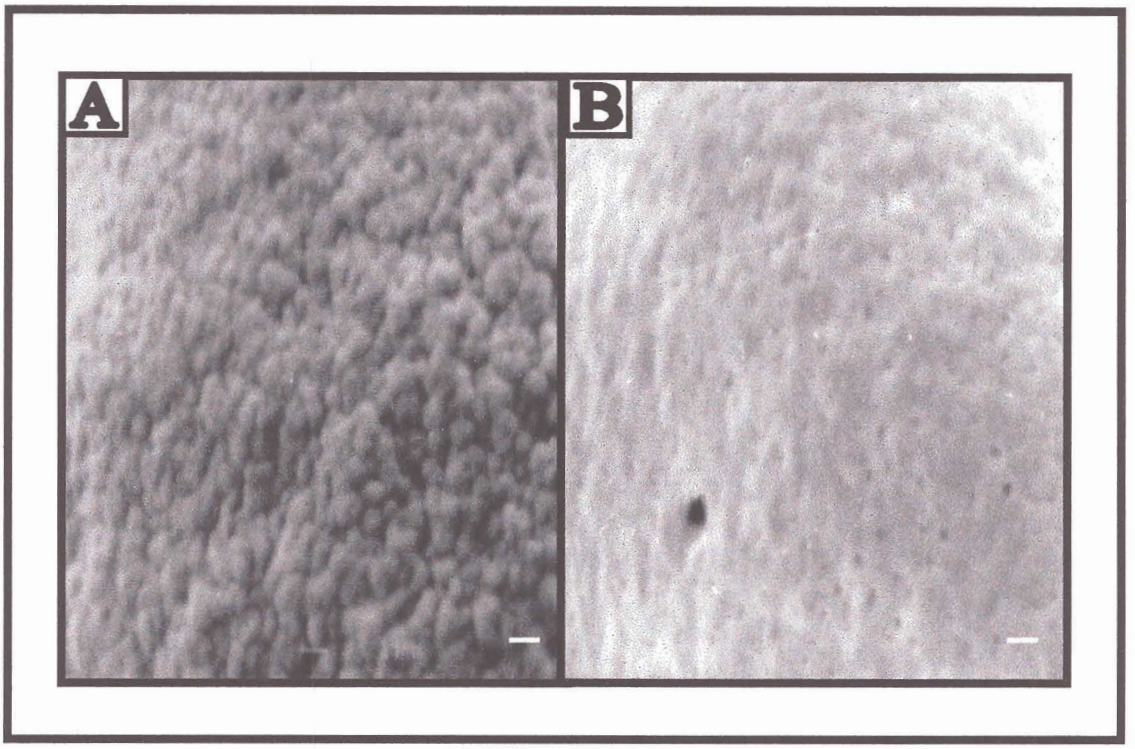


Figure 3.6. SEM pictures of the surface of *P. caribaea* pollen. (A) Sculptured surface of the pollen cap. (B) Pitted surface of the pollen wings. 2 000 X Magnification, 1μm Scale bar.

Table 3.3. Average size of *P. caribaea* pollen

	Dimensions in micrometers (μm)				
	AA	**BB**	**CC**	**DD**	**EE**
Mean	7.82	66.81	8.24	60.46	19.7
Standard deviation	1.96	3.42	2.26	3.94	1.8

3.3.3.2 Transmission electron microscopy (TEM)

The results of the TEM investigation correlated with the general ultrastructure of *Pinaceae* pollen grains as described by Tomlinson (1994) and Pardi *et al.* (1996). Various features of a mature *Pinus* pollen grain can be distinguished in the TEM micrographs. In Figure 3.7 features such as the intine, exine and endexine of the cell wall can be distinguished. The gas space in the wings and the germ furrow can also be clearly distinguished. The cell wall of the wings consists of a thin exine layer with many fingerlike projections that project inwards (Figure 3.8). The continuous exine is thicker in the proximal region, becoming thinner in the distal region from which the pollen tube will arise. The exine is made up of two distinct layers namely the endexine and the ektexine. The ektexine in turn is made up of three parts namely the footlayer, tectum and infratectum. In Figure 3.9 the TEM micrograph of the cell wall ultrastructure is given.

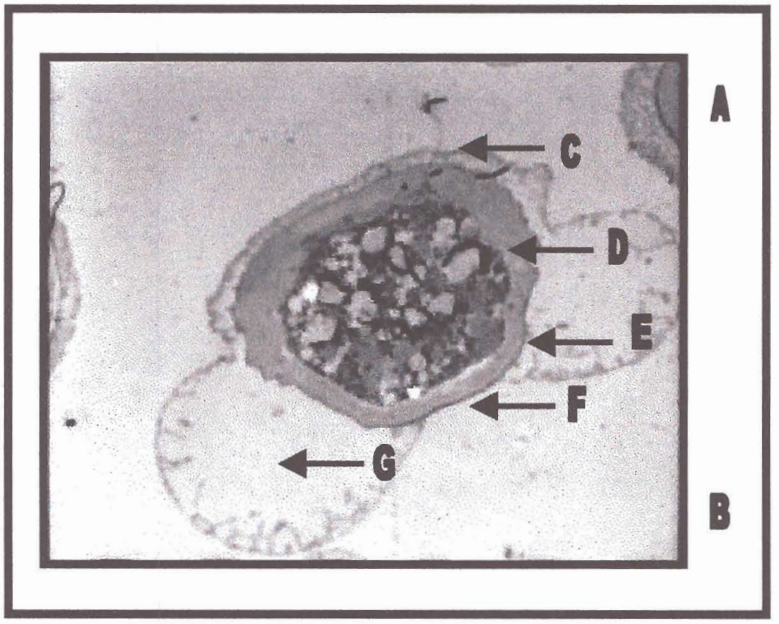


Figure 3.7. TEM micrograph of the general ultrastructure of *P. caribaea* pollen. (A) Proximal region. (B) Distal region. (C) Exine. (D) Intine. (E) Endexine. (F) Germ furrow. (G) Air space in wing. 360 X Magnification.

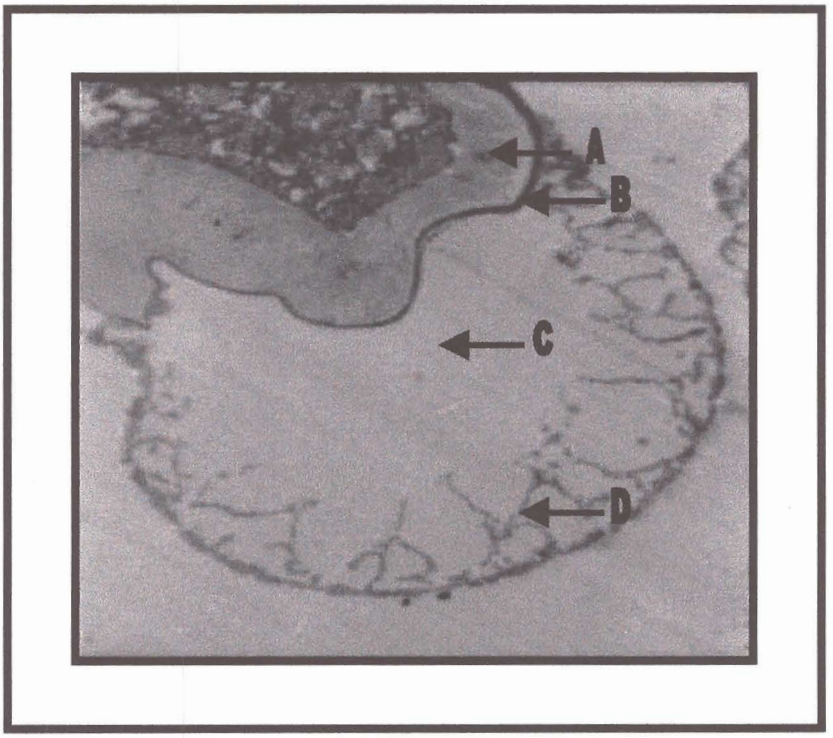


Figure 3.8. TEM micrograph of the ultrastructure of the *P. caribaea* pollen wing. (A) Intine. (B) Endexine. (C) Air space of wing. (D) Fingerlike projections of the exine. 870 X Magnification.

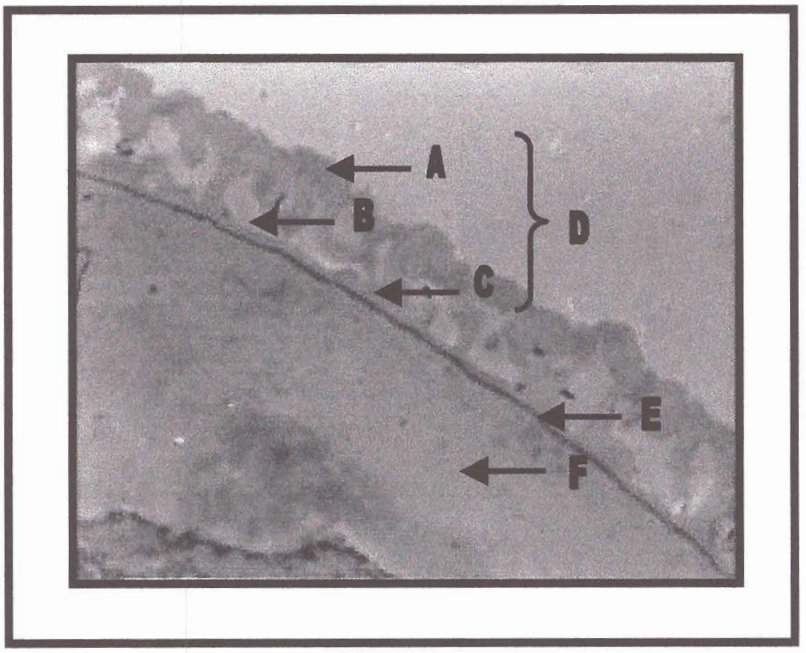


Figure 3.9. TEM micrograph of the ultrastructure of the *P. caribaea* pollen cell wall. (A) Tectum. (B) Infratectum. (C) Footlayer. (D) Ektexine. (E) Endexine. (F) Intine. 2 650 X Magnification.

3.3.4 Statistical analysis of *P. caribaea* germination vs. morphology

Using the UTHSCSA *Image Tool* programme the dimensional data pertaining to the eighteen *P. caribaea* pollen types was generated. This dimensional data in combination with the germination data (Figure 3.2) was then subjected to statistical analysis (SAS / STAT Users guide, 1989).

The first question investigated related to whether the eighteen pollen types could be shown to be significantly different, based upon the dimensional data. The results of the ANOVA (Appendix B) showed that the term for differences between the eighteen pollen types was highly significant (Pr = 0.0001).

The next question was to determine whether an association existed between the dimensional measurements and the germination rates of the eighteen *P. caribaea* pollen types. The following function was fit to the data:

$$Y_{Germination} = \beta_1 X_{AA} + \beta_2 X_{B\beta} + \beta_3 X_{CC} + \beta_4 X_{DD} + \beta_5 X_{EE}$$

where Y is the observed germination, β_i are the regression coefficients and X_{AA} to X_{EE} are the dimensional measurements, as described in Figure 3.1. This model was found to be significant at $Pr < 0.05$ and the correlation coefficient squared was 0.8668 (i.e. 86% of variation is determined by the model).

The third question involved an examination of the sum of the dimensional measurements for the eighteen pollen types. The analysis for consistency, which was done using the Cronbach coefficient (alpha), was found to be less than 0.7. Thereby indicating that the sum of the dimensional measurements was not representative of the size of a specific *P. caribaea* pollen type.

The forth point of interest was to determine the way in which the dimensional measurements aggregated. A principle component analysis (PCA) was therefore done. The results of the PCA showed that the first principle component (PC) is dominated by factor 1 (EE), the second by factor 2 (AA) and the rest by factors 3, 4 and 5 (DD, BB and CC, respectively) (Table 3.4).

Table 3.4. Results of PCA – Variance explained by each factor

Dimensional measurement	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
AA	0.07	**0.93**	0.16	0.22	0.24
BB	0.19	0.22	0.18	**0.93**	0.13
CC	0.08	0.24	0.27	0.13	**0.92**
DD	0.08	0.16	**0.93**	0.19	0.26
EE	**0.99**	0.06	0.07	0.17	0.07

The multiple regression of the scores from the PC's representing the dimensions AA, BB, CC, DD and EE as predictors of germination were found to be highly significant ($Pr < 0.05$) and the regression coefficients (correlation coefficient squared = 0.8691) were also

highly significant. These variables therefore contribute jointly to the size of the pollen grain and they are good predictors of germination.

The next step was to look whether it would be possible to cluster the *P. caribaea* pollen types. Two different clustering techniques were investigated. These techniques were Median hierarchical cluster analysis and Ward's minimum variance cluster analysis.

Using the Median hierarchical cluster analysis it was determined that the eighteen *P. caribaea* pollen types could be divided into three clusters, although the majority of the pollen lines tended to fall into only two clusters (Table 3.5). Using Ward's minimum variance cluster analysis three clusters were chosen in order to keep the data analysis as simple as possible (Table 3.6).

Table 3.5. Results of Median hierarchical cluster analysis

	Cluster 1		**Cluster 2**	
***P. caribaea* pollen lines**	Ach 22	Ach 24	Ach 57	Ach 65
	Ach 29	Ach 31	Ach 93	Ach114
	Ach 33	Ach49	Ach 271	Pch 23
	Ach 91	Acb 60	Pch 69	Pch 88
	Acb 62	Pch 105		

Table 3.6. Results of Ward's minimum variance cluster analysis

	Cluster 1		**Cluster 2**		**Cluster 3**	
***P. caribaea* pollen lines**	Ach 33	Ach 29	Ach 22	Acb 62	Ach 24	Ach 114
	Ach 91	Ach 49	Pch 105		Ach 93	Ach 57
	Ach 31	Acb 60			Ach 65	Ach 271
					Pch 23	Pch 69
					Pch 88	

When the results of the Median hierarchical cluster and Ward's minimum variance cluster analysis were compared basic similarities were observed. Both methods cluster Ach 29, Ach 31, Ach33, Ach 49, Ach 91 and Acb 60 in one group (Group A, Figure 3.10) and Ach 57, Ach 65, Ach 93, Ach 114, Ach 271, Pch 23, Pch 69 and Pch 88 in a separate group (Group B, Figure 3.11). When these results were compared with the original SEM images it was found that all the pollen types clustering in group B displayed intermediate dimensions (Figure 3.12 A & B), while all the pollen types clustering in group A either displayed smaller than average dimensions (Figure 3.12 C & D) or displayed larger than average dimensions (Figure 3.12 E & F). When these results were compared with the germination results obtained it was found that the pollen types which clustered into group B were amongst those types which displayed high germination percentages and those which clustered into group A were amongst those types which displayed low to intermediate germination percentages (Figure 3.13).

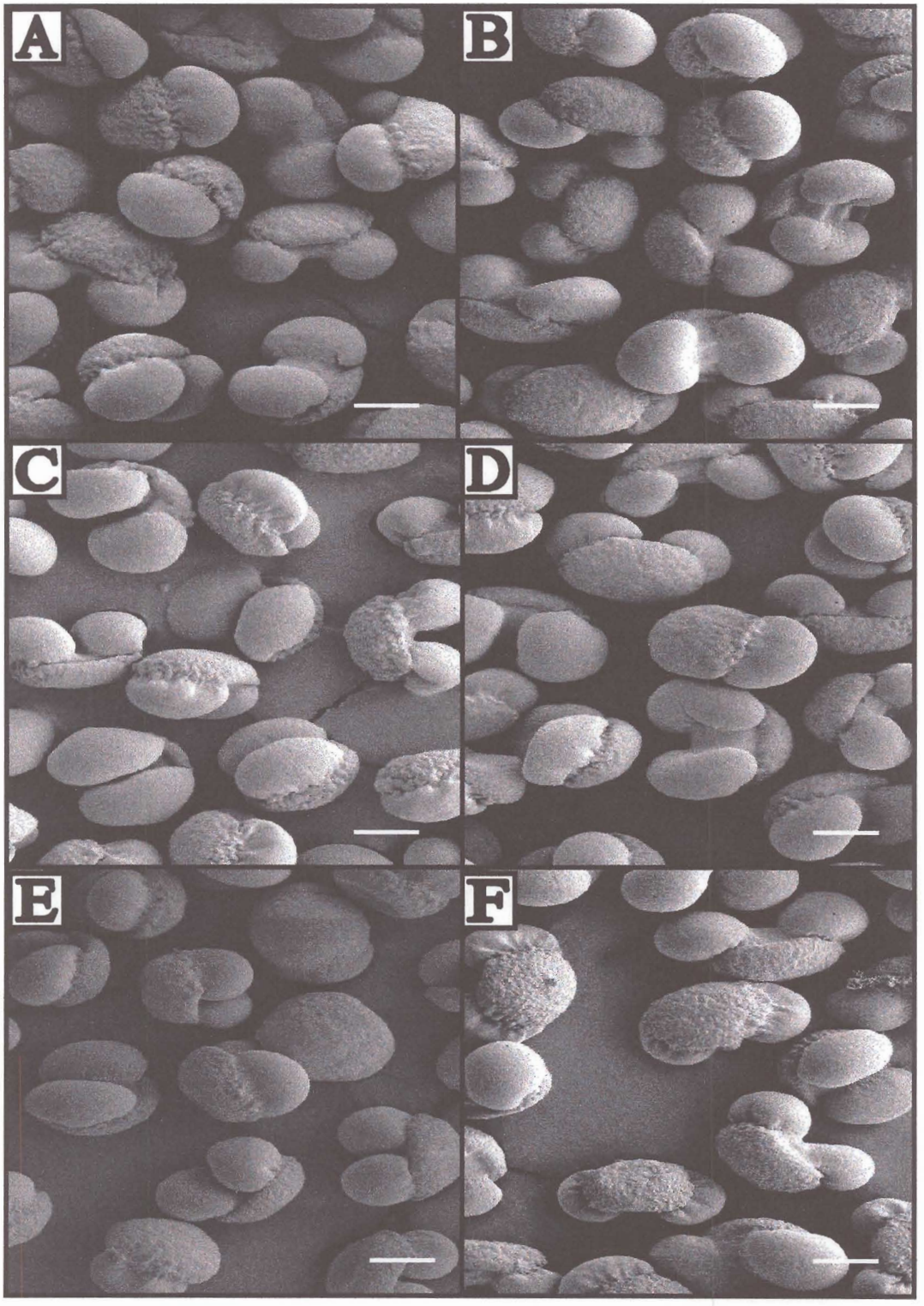


Figure 3.10. *P. caribaea* pollen types clustered into Group A. (A) Ach 29. (B) Ach 31. (C) Ach 33. (D) Ach 49. (E) Ach 91. (F) Acb 60. 250 X Magnification, 100 µm Scale bar.

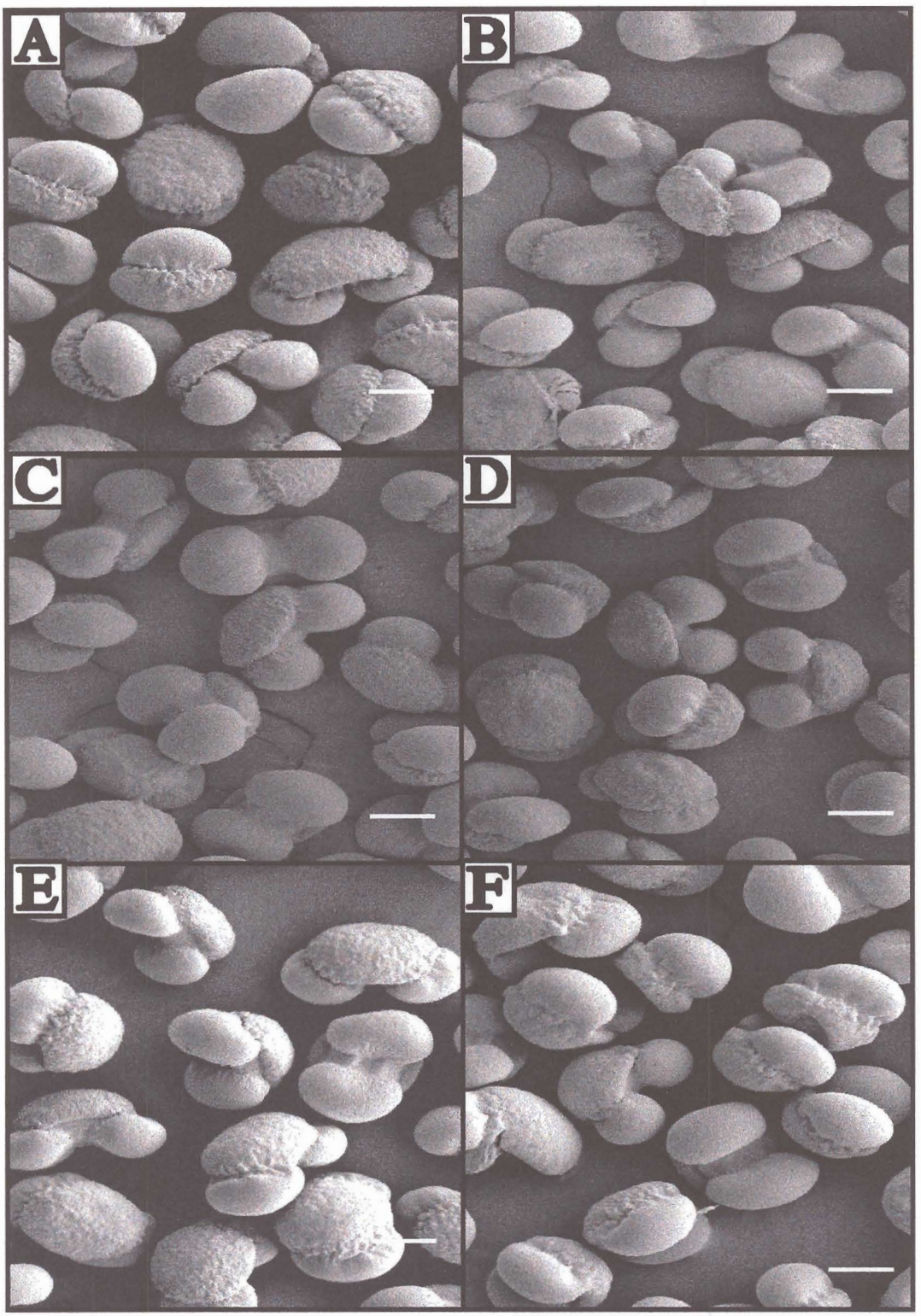


Figure 3.11. *P. caribaea* pollen types clustered into Group B. (A) Ach 57, (B) Ach 65, (C) Ach 93, (D) Ach 114, (E) Ach 271, (F) Pch 88. 250 X Magnification, 100 µm Scale bar.

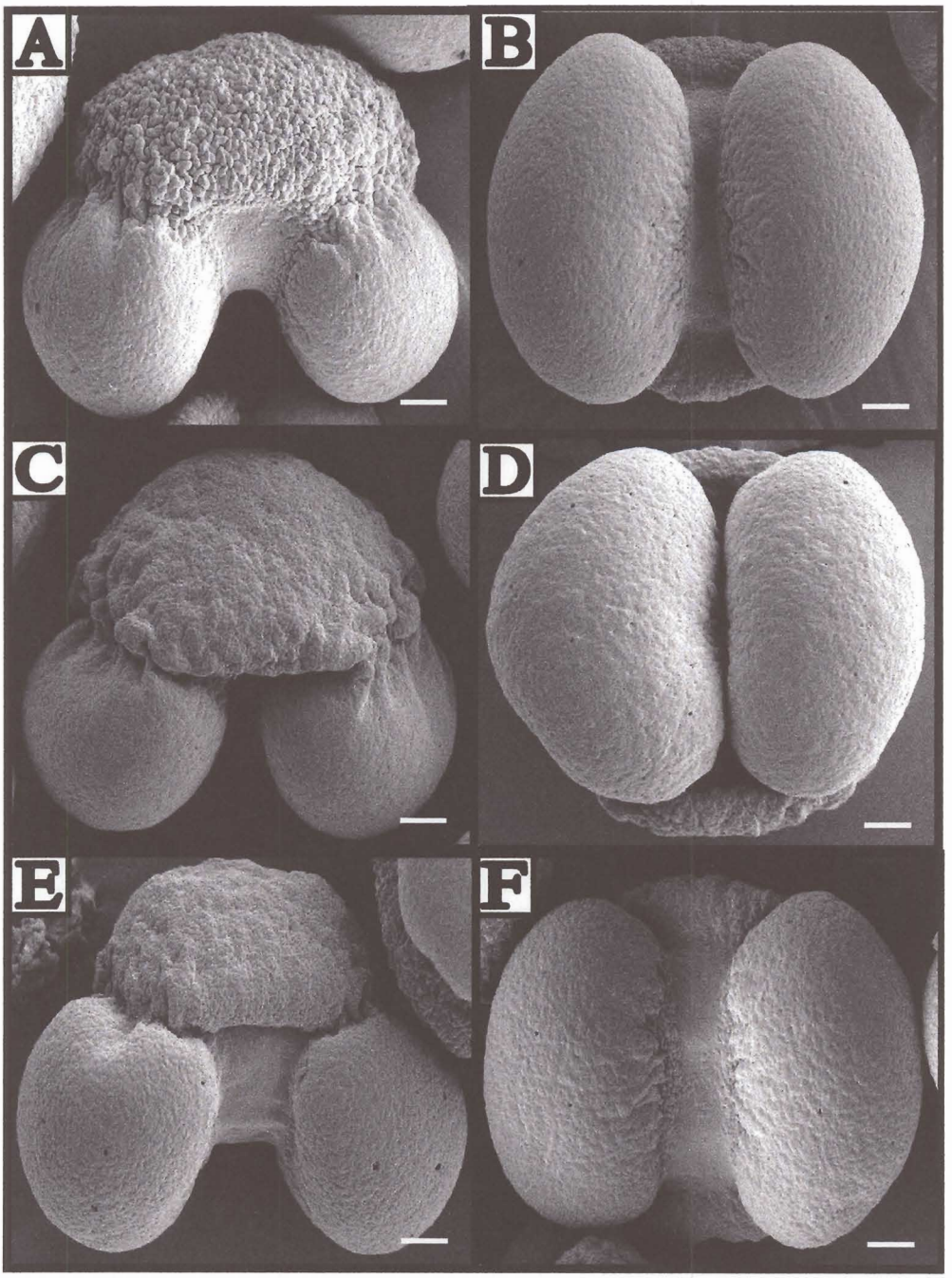


Figure. 3.12. SEM pictures of *P. caribaea* pollen clusters. (A). SEM picture of pollen with intermediate germ furrow in side view. (B). SEM picture of pollen with intermediate germ furrow in distal view. (C). SEM picture of pollen with narrow germ furrow in side view. (D). SEM picture of pollen with narrow germ furrow in distal view. (E). SEM of pollen with wide germ furrow in side view. (F). SEM of pollen with wide germ furrow in distal view. 1 000 X Magnification, 10 μm Scale bar

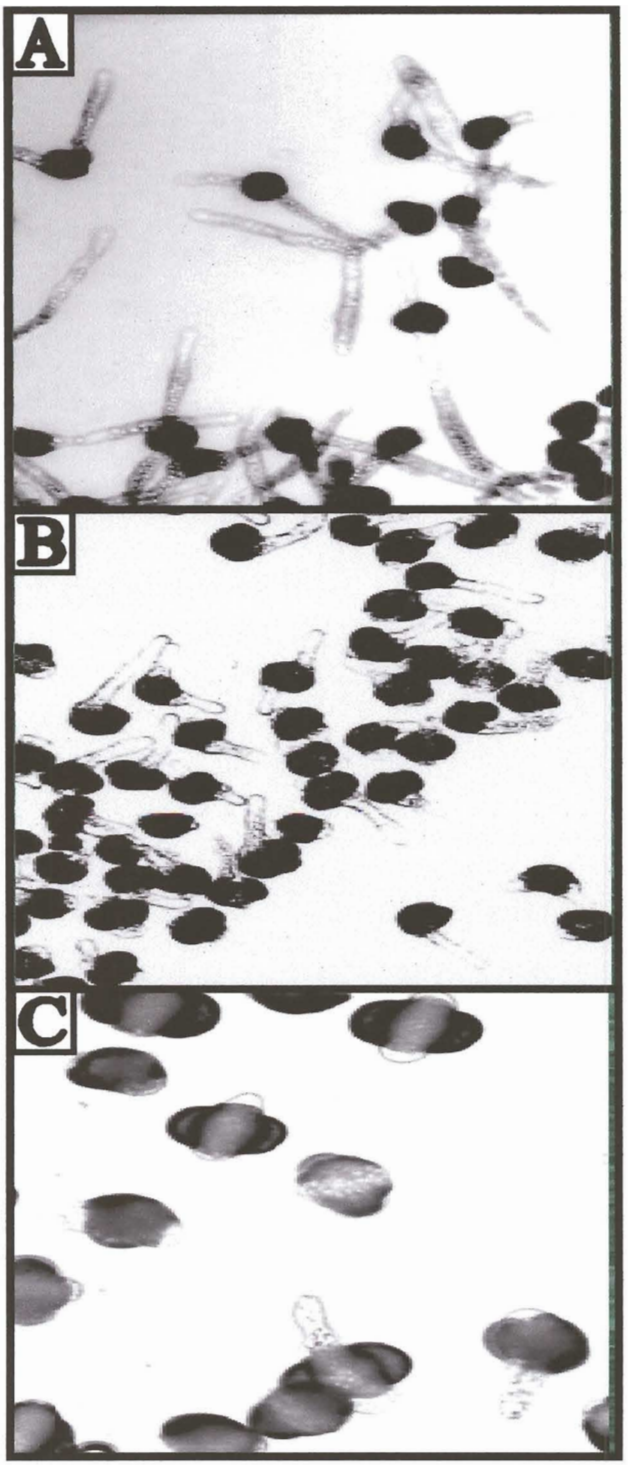


Figure 3.13. Light microscope pictures of *P. caribaea* pollen germination. (A) High germination (10 X Magnification). (B) Intermediate germination (10 X Magnification). (C) Low germination (20 X Magnification).

3.4 Discussion

Pollen viability is one of the most important limiting factors of reproductive success (Singh, 1978). The viability of gymnosperm pollen is very vulnerable due to its airborne dispersal and slow maturation (Wright, 1976), which facilitates the exposure of the pollen to various environmental pollutants (Pardi *et al.*, 1996). The viability of the pollen is, however, not only determined by atmospheric agents, but also by internal factors such as lipid (Van Bilsen *et al.*, 1994) and carbohydrate reserves (Pacini, 1996) and the duration and conditions under which it is stored (as found in this study).

The environmental conditions to which the pollen was exposed played a major role in germination and pollen viability. The results indicated that there was a direct decrease in viability with an increase in the age of the pollen, temperature at which the pollen was stored and exposure to high humidity during storage. These results correlate with those described by Van Bilsen *et al.* (1994) who found that the higher the relative humidity (i.e. 75% vs. 40%) during pollen storage the lower the viability. Van Bilsen *et al.* (1994) linked this decrease in viability to the deesterification of phospholipids that resulted in the degradation of membrane integrity. The pollen cone developmental stage at the time of harvesting was also found to influence viability as the highest germination percentages were observed for medium pollen cones. This would suggest that medium cones are more likely to contain mature, but fresh pollen. It is therefore proposed that the decrease in viability observed with the increase in pollen cone age is due to the pollen having passed it's optimal condition and entering into the declining viability phase. Based on these

results recommendations were made to SAFCOL, suggesting ways in which the viability of *P. caribaea* pollen could be prolonged. These recommendations are summarized in Table 3.7.

Table 3.7. Summary of recommendations pertaining to optimization of *P. caribaea* pollen storage conditions

Recommendations
The *P. caribaea* pollen should be:
• Dried prior to storage;
• Stored in small quantities;
• Stored at the lowest possible temperature;
• Stored in a dry place;
• Stored in sealed containers;
• Once opened, all left over pollen should be discarded.

The results of the TEM investigation on the influence that environmental conditions exert on the ultrastructure of *P. caribaea* pollen were found to be variable and generally uninformative. These results would seem to correlate with those obtained for the study on the effect of environmental factors on viability as those results were also found to be variable and uninformative. Of the environmental factors tested only temperature, humidity and the duration of storage were found to influence pollen viability. These differences in germination are, however, not clearly reflected in the ultrastructure of the

P. caribaea pollen. It is therefore proposed that a study needs to be done in which the effects of temperature, humidity and the duration of storage on the ultrastructure of *P. caribaea* pollen are the main focus. Such a study would help determine whether these results are a true reflection of the effects of environmental conditions on the ultrastructure of *P. caribaea* pollen or whether they are just a selectively skewed version of what is really happening.

According to Snow and Spira (1991) and Stephenson *et al.* (1988) only the most vigorous pollen tubes achieve fertilization under conditions of intense pollen competition. Pollen competition can occur when more pollen is deposited on the stigma than is required for fertilization of all the flower's ovules (Havens, 1994). The deposition of excess pollen occurs regularly during the controlled pollination of the *P. elliottii* ovules with the *P. caribaea* pollen. It would therefore be advantageous to only use the most vigorous *P. caribaea* pollen, which displays the fastest germination rate and pollen tube growth rate, for the controlled crosses.

As a cautionary note it must be included that pollen performance rates can not only be contributed to genetic factors, but that non-genetic factors such as: (i) temperature during pollination (Elgersma *et al.*, 1989), (ii) the location of the pollen on the stigma (Thomson, 1989) and (iii) the competitive environment within the style (Cruzan, 1986 and 1990), may play an important role on a seasonal basis (Charlesworth and Charlesworth, 1992). According to Delph *et al.* (1997) if the plant is exposed to unfavorable environmental conditions it may lead to a reduction in the resources available for pollen production and

ultimately to differences in the quality and / or the quantity of the storage products that are produced within those pollen grains.

In 1994 Tomlison described the general structure of a pollen grain based on five contributing factors. These factors included the size, overall shape, surface sculpture, sporoderm ultrastructure and the presence or absence of obvious apertures. The combined SEM and TEM investigations provide all the necessary information in order to be able to describe all these factors relating to *P. caribaea* pollen. The SEM investigation provided valuable information pertaining to four of the five factors, namely size, overall shape, surface sculpture and the presence of an obvious aperture in the form of the germ furrow. The SEM pictures were, however, limited to an external view of these factors using the scanning electron microscope. The TEM sections on the other hand provided the means necessary in order to investigate the sporoderm ultrastructure, as well as the other four factors on an internal level using the transmission electron microscope. In 1996 Pardi *et al.* similarly described the general structure of *Pinus pinea* and *Pinus pinaster* pollen.

Generally the results of the statistical analysis indicated that a highly significant relationship existed between germination and morphology of the *P. caribaea* pollen. To this end factors such as whether: (i) the eighteen pollen types were significantly different; (ii) an association between the dimensions of the pollen types and their germination existed; (iii) the sum of the dimensions would provide a reliable size variable; and (iv) the dimensions would allow the eighteen pollen types to be clustered into groups were investigated.

The first factor was investigated by doing an ANOVA of the dimensional data available. The results of the ANOVA indicated that the term for differences between the eighteen pollen types was highly significant, which suggests that the eighteen pollen types are significantly different from one another when their dimensions are taken into account.

Using regression a good association was found to exist between the dimensional measurements and germination of the pollen types, as it was possible to determine up to 86% of the variation. The association was therefore found to be highly significant.

The Cronbach coefficient (alpha) was used to determine whether the sum of the dimensions would provide a reliable size variable. The sum can only be accepted if the measures of the five dimensions add consistently to the total (i.e. their individual measurement error must be very small) (Steyn *et al.*, 1994). The analysis for consistency (i.e. the Cronbach coefficient) indicated that this was not the case and the sum of the dimensional measurements was therefore not representative of the size of a specific *P. caribaea* pollen type.

A principle component analysis (PCA) was carried out in order to determine the way in which the dimensional measurements aggregated. During a PCA the five dimensions are combined in a linear function (five linear functions are formed in this case), called a principle component (Steyn *et al.*, 1994). Differences in the size of the coefficients of the functions (PC's) indicate the relative importance of the dimensions. The results of the

PCA showed that the first PC was dominated by factor 1 (EE) and the second by factor 2 (AA) this means that of the five factors, factor 1 and factor 2 carry the most weight when the dimensions of the pollen grains are determined (Table 3.4).

The PC's represent independent aggregations of the dimensions and allow aggregate values, called scores, to be calculated for each entry (Steyn *et al.*,1994). These scores were used to regress on germination. The multiple regression of the scores from the PC's representing the dimensions AA, BB, CC, DD and EE as predictors of germination were found to be highly significant and up to 86.91% of the variation could be determined. It can therefore be concluded that these variables contribute jointly to the size of the pollen grain and they are good predictors of germination. These results clearly confirm that there is an association between morphology and germination, but also emphasize that the association is not a simple one.

Having determined that the eighteen *P. caribaea* pollen types were significantly different from one another (with reference to all five dimensions) and that there was a highly significant association between the dimension of the pollen and its germination rate, the next step was to look whether it would be possible to cluster the pollen types. Two different clustering techniques were investigated. These techniques were Median hierarchical cluster analysis and Ward's minimum variance cluster analysis. Using the Median hierarchical cluster analysis it was determined that the eighteen *P. caribaea* pollen types could be divided into three clusters, although the majority of the pollen types fall into only two clusters (Table 3.5). Using Ward's minimum variance cluster analysis

three clusters were chosen in order to keep the data analysis as simple as possible (Table 3.6). When the results of the Median hierarchical cluster analysis and Ward's minimum variance cluster analysis were compared it was found that many of the clusters overlapped, thereby confirming the clustering of the *P. caribaea* pollen types into two distinct groups (Group A, Figure 3.10 and Group B, Figure 3.11).

When the results of the cluster analysis were compared with the original SEM images and the germination results obtained it was found that all the pollen types clustering into group B displayed intermediate dimensions (Figure 3.12 A & B) and high germination percentages (Figure 3.13). Conversely the pollen types clustering into group A displayed either smaller than average dimensions (Figure 3.12 C & D) or larger than average dimensions (Figure 3.12 E & F) and low to intermediate germination percentages (Figure 3.13). This would suggest that a highly significant association seems to exist between *P. caribaea* pollen morphology and viability.

3.5 Conclusion

The results of the investigation on the effect of environmental conditions indicated that long term pollen viability can be maintained if the pollen is stored under specific conditions. The results indicated that only mature pollen from intermediate cones should be harvested and that if temperature and humidity are controlled during storage, then it should be possible to maintain *P. caribaea* pollen viability.

The highly significant association found to exist between *P. caribaea* pollen morphology and viability strongly suggests that a dimensional screening step would be beneficial during the selection of the pollen parent. This screening step would reduce the chances of inferior *P. caribaea* pollen parents from being used in crosses during hybrid production and therefore from entering into the hybrid performance trials in general. The correct storage of the *P. caribaea* pollen would ensure the viability of the pollen used in the cross and therefore result in increased pollination which should in turn result in increased fertilization.

CONCLUSION

Identification of pine hybrids using SSR loci.

The fact that microsatellite markers developed in other species can be used successfully in the DNA profiling in related species, and specifically for the profiling of the *P. elliottii x P. caribaea* hybrids, makes this an extremely powerful and accessible technique. By eliminating the need for sequence information and marker development cross-species amplification of microsatellite markers greatly reduces the cost and time requirements involved in DNA profiling studies.

The results also indicated that not all microsatellite markers produced DNA fingerprints that were equally informative and / or useful. Generally polymorphic microsatellite markers were found to be highly informative and therefore far more useful than monomorphic markers, which tend to be relatively uninformative. Parental determination should therefore be approached via a process of elimination in order to accurately accept or reject the null hypothesis, that the embryo was the result of successful controlled pollination. As it is unlikely that a single locus will provide enough information upon which the null hypothesis may be rejected or accepted, the information from several loci should be pooled and analyzed together.

The results of the preliminary study indicated that the RAMs technique, although not as cost effective as simply using markers developed in related species, is potentially an effective way of developing microsatellite markers specific to the *P. elliottii x P.*

caribaea hybrids. The species-specific DNA fingerprints obtained using RAMs are highly reproducible and can be used to identify both inter- and intra-specific polymorphisms. The results were therefore promising enough to justify further investigation into this approach for marker development and perhaps even investigation into the potential of these markers for cross-species amplification.

The influence of storage and morphology on the viability of *Pinus caribaea* pollen

The results of the investigation on the effect of environmental conditions indicate that long term pollen viability can be maintained if the pollen is stored under specific conditions. The results indicated that only mature pollen from intermediate cones should be harvested and that if temperature and humidity are controlled during storage, then it should be possible to maintain *P. caribaea* pollen viability.

The highly significant association found to exist between *P. caribaea* pollen morphology and viability strongly suggests that a dimensional screening step would be beneficial during the selection of the paternal parent. This screening step would reduce the chances of inferior *P. caribaea* pollen parents from being used in crosses during hybrid production and therefore from entering into the hybrid performance trials in general. The correct storage of the *P. caribaea* pollen would ensure the viability of the pollen used in the cross and therefore result in increased pollination which should in turn result in increased fertilization.

SUMMARY

The study found that cross-species amplification of microsatellite markers developed in other species can be used successfully for the DNA profiling of related species, and more specifically for the DNA profiling of the *P. elliottii x P. caribaea* hybrids. This study also found that not all microsatellite markers produce DNA fingerprints that are equally useful and informative, and that the information from several markers should be pooled and analyzed together in order to make valid predictions relating to parental contribution and gene flow. Furthermore, it was found that the preliminary study on the usefulness of the RAMs technique for marker development indicated that the RAMs technique displayed great potential as an effective way of developing microsatellite markers specific to the *P. elliottii x P. caribaea* hybrids.

The study also found that environmental conditions to which the *P. caribaea* pollen is exposed may influence its long-term viability and that the viability may be maintained if the pollen is stored under optimal conditions. Furthermore, a statistically highly significant association was found to exist between *P. caribaea* pollen morphology and viability, thereby strongly suggesting that a dimensional screening step would be beneficial for the forestry industry before the selection of the pollen donors are made for pollination studies.

OPSOMMING

In die studie is bevind dat kruis-spesie amplifisering van mikrosattelietmerkers wat in ander species ontwikkel is, ook suksesvol vir DNA-profiele van naverwante spesies, en meer spesifiek vir die profiele van die *P. elliottii x P. caribaea* hibried, gebruik kan word. In die studie is verder bevind dat nie alle mikrosattelietmerkers ewe informatief of bruikbaar is nie. Daar is verder bevind dat die inligting wat met verskillende merkers verkry is, verskieslik saam geanaliseer moet word om sinvolle afleidings met betrekking tot geenvloei en ouerlike bydrae, te verkry. Daar is ook verder in 'n voorlopige studie oor die bruikbaarheid van die RAMs-tegniek bevind dat die tegniek groot potensiaal inhou om *P. elliottii x P. caribaea* hibried-spesifieke mikrosatteliete op 'n effektiewe manier te ontwikkel.

Die studie het verder getoon dat die omgewingstoestande waaraan stuifmeel blootgestel word, die langtermyn kiemkragtigheid van stuifmeel beïnvloed. Kiemkragtigheid kan behou word indien die kondisies waarby die stuifmeel geberg word, optimaal is. Die studie het verder aangetoon dat daar 'n hoogs betroubare verwantskap tussen *P. caribaea* stuifmeelmorfologie en kiemkragtigheid bestaan. Dit wil dus voorkom asof dit voordelig vir die bosboubedryf sou wees om die dimensies van stuifmeel eers te bepaal, alvorens finale keuses van vaderlike donors vir bestuiwingstudies gemaak word.

LITERATURE CITED

Akkaya, M.S., Bhagwat, A.A. and Cregan, P.B. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics* **132**: 1131 – 1139.

Aronen, T.S., Nikkanen, T.O. and Häggman, H.M. 1998. Compatibility of different pollination techniques with microprojectile bombardment of Norway spruce and Scots pine. *Canadian Journal of Forest Research* **28**: 79 – 86.

Baker, H.G. and Baker, I. 1979. Starch in angiosperm pollen grains and its evolutionary significance. *American Journal of Botany* **66**: 591 – 600.

Bellani, L.M., Pacini, E. and Franchi, G.G. 1985. *In vitro* pollen grain germination and starch content in species with different reproductive cycle. I. *Lycopersicum peruvianum* Mill. *Acta Botanica Neerlandica* **34**: 59 – 64.

Bester, C. 2000. Breeding programme. *In:* Kassier, H. (ed.) *SAFCOl internal symposium on the P. elliottii x P. caribaea hybrid.* SAFCOL, South Africa.

Binelli, G. and Bucci, G. 1994. A genetic linkage map of *Picea abies* Karst., based on RAPD markers, as a tool in population genetics. *Theoretical and Applied Genetics* **88**: 283 – 288.

Brondani, R.P.V., Brondani, C., Tarchini, R. and Grattapaglia, D. 1998. Development, characterization and mapping of microsatellite markers in *Eucalyptus grandis* and *Eucalyptus urophylla*. *Theoretical and Applied Genetics* **97:** 816 – 827.

Bruford, M.W. 1999. Introduction. *In:* Bloomer, P. (convener) *Microsatellites: Their Application and analysis.* Department of Genetics, University of Pretoria, South Africa.

Butcher, P.A., Glaubitz, J.C. and Moran, G.F. 1999. Applications for microsatellite markers in the domestication and conservation of forest trees. http://www.fao.org/forestry/FOR/FORM/FOGENRES/genresbu/web27-en/micr-e.stm

Callen, D.F., Thompson, A.D., Shen, Y., Phillips, H.A., Richards, R.I., Mulley, J.C. and Sutherland, G.R. 1993. Incidence and origin of "Null" alleles in the (AC)n microsatellite markers. *American Journal of Human Genetics* **52**: 922 – 927.

Charlesworth, D. and Charlesworth B. 1992. The effects of selection in the gametophyte stage on mutational load. *Evolution* **46**: 703 – 720.

Charlesworth, B., Sniegowski, P. and Stephan, W. 1994. The evolutionary dynamics of repetitive DNA in eukaryotes. *Nature* **371**: 215 – 220.

Coetzee, J. and van der Merwe, C.F. 1999. Preparation of biological material for electron microscopy – a practical guide. Laboratory for microscopy and microanalysis, University of Pretoria, South Africa.

Colson, I. and Goldstein, D.B. 1999. Evidence for complex mutations at microsatellite loci in drosophila. *Genetics* **152**: 617 – 627.

Cruzan, M.B. 1986. Pollen tube distributions in *Nicotiana glauca*: evidence for density dependent growth. *American Journal of Botany* **73**: 902 – 907.

Cruzan, M.B. 1990. Pollen – pollen and pollen – style interactions during pollen tube growth in *Erythronium grandiflorum*. *American Journal of Botany* **77**: 116 – 122.

Dayanandan, S., Rajora, O.P. and Bawa, K.S. 1998. Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). *Theoretical and Applied Genetics* **96**: 950 – 956.

De Win, A.H.N., Knuiman, B., Pierson, E.S., Geurts, H., Kengen, H.M.P. and Derksen, J. 1996. Development and cellular organization of *Pinus sylvestris* pollen tubes. *Sexual Plant Reproduction* **9**: 93 – 101.

Delph, L.F., Johannsson, M.H. and Stephenson, A.G. 1997. How environmental factors affect pollen performance: ecological and evolutionary perspectives. *Ecology* **78**: 1632 – 1639.

Den Ouden, P. and Boom, B.K. 1978. Pinus. *In: Manual of cultivated conifers hardy in the cold- and warm-temperate zone*. Martinus Nijhoff publishers, The Hague, Boston, London.

Denison, N.P. and Kietzka, J.E. 1993. The use and importance of hybrid intensive forestry in South Africa. *South African Forestry Journal* **165**: 55 – 60.

Depeiges, A., Goubely, C., Lenoir, A., Cocherel, S., Picard, G., Raynal, M., Grellet, F. and Delseny, M. 1995. Identification of the most represented repeated motifs in *Arabidopsis thaliana* microsatellite loci. *Theoretical and Applied Genetics* **91**: 160 – 168.

Devey, M.E., Bell, J.C., Smith, D.N. and Neale, D.B. 1996. A genetic linkage map for *Pinus radiata* based on RFLP, RAPD, and microsatellite markers. *Theoretical and Applied Genetics* **92**: 673 – 679.

Devey, M.E., Sewell, M.M., Uren, T.L. and Neale, D.B. 1999. Comparative mapping in loblolly and radiata pine using RFLP and microsatellite markers. *Theoretical and Applied Genetics* **99**: 656 – 662.

Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N. and Vignal, A. 1996. A comprehensive genetic map of the human genome based on 5 264 microsatellites. *Nature* **380**: 152 – 154.

Dietrich, W.F., Miller, J., Steen, R., Merchnat, M.A., Damron-Boles, D., Husain, Z., Dredge, R., Daly, M.J., Ingalls, K.A., O'Connor, T.J., Evans, C.A., DeAngelis, M.M., Levinson, D.M., Kruglyak, L., Goodman, N., Copeland, N.G., Jenkins, N.A., Hawkins, T.L., Stein, L., Page, D.C. and Lander, E.S. 1996. A comprehensive genetic map of the mouse genome. *Nature* **380**: 149 – 152.

Dow, B.D., Ashley, M.V. and Howe, H.F. 1995. Characterization of highly variable (GA/CT)n microsatellites in the bur oak, *Quercus macrocarpa*. *Theoretical and Applied Genetics* **91**: 137 – 141.

Duff, D.G., Baiker, A. and Edwards, P.G. 1993. A new hydrosol of gold clusters. 1. Formation and particle size variation. *Langmuir* **9**: 2301 – 2309.

Echt, C. and Burns, R. 1999. PCR primer pair sequences and SSRs for various *P. taeda* ESTs. http://dendrome.ucdavis.edu/Data/ssr-est.html

Echt, C.S. and May-Marquardt, P. 1997. Survey of microsatellite DNA in pine. *Genome* 40: 9 – 17.

Echt, C.S., May-Marquardt, P., Hseih, M. and Zahorchak, R. 1996. Characterization of microsatellite markers in eastern white pine. *Genome* 39: 1102 – 1108.

Echt, C.S. and Nelson, C.D. 1997. Linkage mapping and genome length in eastern white pine (*Pinus strobus* L.). *Theoretical and Applied Genetics* **94**: 1031 – 1037.

Echt, C.S., Vendramin, G.G., Nelson, C.D. and Marquardt, P. 1999. Microsatellites DNA as shared genetic markers among conifer species. *Canadian Journal of Forest Research* **29**: 365 – 371.

Elgersma, A., Stephenson, A.G. and Den Nijs, A.P.M. 1989. Effects of genotype and temperature on pollen tube growth in perennial ryegrass (*Lolium perenne* L.). *Sexual Plant Reproduction* **2**: 225 – 230.

Ellegren, H. 1991. DNA typing of museum birds. *Nature* **354**: 113.

Erdtman, G. 1952. Pollen and spore morphology. *In: Pollen morphology and plant taxonomy – Angiosperms.* Almqvist and Wiksell, Stockholm.

Estoup, A., Solignac, M., Harry, M. and Cornuet, J-M. 1993. Characterization of (GT)n and (CT)n microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. *Nucleic Acids Research* **21**: 1427 – 1431.

Fisher, P.J., Richardson, T.E. and Gardner, R.C. 1998. Characteristics of single- and multi-copy microsatellites from *Pinus radiata. Theoretical and Applied Genetics* **96**: 969 – 979.

Groenen, M.A.M, Crooijmans, R.P.M.A, Veenendaal, A., Cheng, H.H., Siwek, M. and Van der Poel, J.J. 1998. A comprehensive microsatellite linkage map of the chicken genome. *Genomics* **49**: 265 – 274.

Hagelberg, E., Gray, I.C. and Jeffreys, A.J. 1991. Identification of the skeletal remains of a murder victim by DNA analysis. *Nature* **352**: 427 – 429.

Hantula, J., Dusabenyagasaniy, M. and Hamelin, R.C. 1996. Random amplified microsatellites (RAMs) – a novel method for characterizing genetic variation within fungi. *European Journal of Forest Pathology* **26**: 159 – 166.

Hantula, J., Maarit Niemi, E., Kaitera, J., Jalkanen, R. and Kurkela, T. 1998. Genetic variation of the resin top fungus in Finland as determined by random amplified microsatellites (RAMs). *European Journal of Forest Pathology* **28**: 361 – 372.

Hantula, J., and Müller, M.M. 1997. Variation within *Gremmeniella abietina* in Finland and other countries as determined by Random Amplified Microsatellites (RAMS). *Mycological Research* **101**: 169 – 175.

Havens, K. 1994. Clonal repeatability of *in vitro* pollen tube growth rates in *Oenothera organensis* (Onagraceae). *American Journal of Botany* **81**: 161 – 165.

Hearne, C.M., Ghosh, S. and Todd, J.A. 1992. Microsatellites for linkage analysis of genetic traits. *Trends In Genetics* **8**: 288 – 294.

Helentjaris, T., King, G., Slocum, M., Siedenstrang, C. and Wegman, S. 1985. Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. *Plant Molecular Biology* **5**: 109 – 118.

Hicks, M., Adams, D., O'Keefe, S., MacDonald, E. and Hodgetts, R. 1998. The development of RAPD and microsatellite markers in lodgepole pine (*Pinus contorta* var. *latifolia*). *Genome* **41**: 797 – 805.

Hinze, W. 2000. History and development of the hybrid. *In:* Kassier, H. (ed.) *SAFCOl internal symposium on the P. elliottii x P. caribaea hybrid.* SAFCOL, South Africa.

Hokanson, S.C., Szewc-McFadden, A.K., Lamboy, W.F. and McFerson, J.R. 1998. Microsatellite (SSR) markers reveal genetic identities, genetic diversity and relationships in a *Malus x domestica* Borkh. core subset collection. *Theoretical and Applied Genetics* **97**: 671 – 683.

Hufford, T.L. 1978. The vascular plants – A survey. *In: Botany – Basic concepts in plant biology.* Harper & Row publishers, New York.

Jarne, P. and Lagoda, P.J.L. 1996. Microsatellites, from molecules to populations and back again. *TREE* **11**: 424 – 429.

Johnson, H. 1978. The pines of North America. *In: The international book of trees.* Mitchell Beazely Limited, London.

Karhu, A., Dieterich, J.-H. and Savolainen, O. 2000. Rapid expansion of microsatellite sequences in Pines. *Molecular Biology and Evolution* **17**: 259 – 265.

Kashi, Y., King, D. and Soller, M. 1997. Simple sequence repeats as a source of quantitative genetic variation. *Trends In Genetics* **13**: 74 – 78.

Kostia, S., Varvio, S-L., Vakkari, P. and Pulkkinen, P. 1995. Microsatellite sequences in a conifer, *Pinus sylvestris. Genome* **38**: 1244 – 1248.

Kurmann, M.H. 1991. Exine stratification in extant gymnosperms: a review of published transmission electron micrographs. *Kew Bulletin* **47**: 25 – 39.

Lagerkrantz, U., Ellegren, H. and Andersson, L. 1993. The abundance of various polymorphic microsatellite motifs differ between plants and vertebrates. *Nucleic Acids Research* **21**: 1111 – 1115.

Lathrop, G.M., Lalouel, J.M., Julier, C. and Ott, J. 1985. Multilocus linkage analysis in humans: detection of linkage and estimation of recombination. *American Journal of Human Genetics* **37**: 482 – 498.

Lavi, U., Akkaya, M., Bhagwat, A., Lahav, E. and Cregan, P. 1994. Methodology of generation and characteristics of simple sequence repeat DNA markers in avocado. *Euphytica* **80**: 171 – 177.

Lee, S.B. and Taylor, J.W. 1990. Isolation of DNA from fungal mycelia and single spores. *In*: Innis, M.A., Gelfand, D.H., Sninsky, JJ. and White, T.J. (eds) *PCR protocols: a guide to methods and applications.* Academic Press, San Diego.

Lehn, C.A. and Davis, S.K. 1999. Construction of libraries for the isolation of species-specific microsatellites. *In:* Bloomer, P. (convener) *Microsatellites:Their Application and analysis.* Department of Genetics, University of Pretoria, South Africa.

Litt, M. and Luty, J.A. 1989. A hypervariable microsatellite revealed by *in vitro* amplification of dinucleotide repeats within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**: 397 – 401.

Mader, S.S. 1998. Diversity of Life: The plants. *In: Biology.* McGraw-Hill publishers.

Malan, F. 2000. Wood quality. *In:* Kassier, H. (ed.) *SAFCOL internal symposium on the P. elliottii x P. caribaea hybrid.* SAFCOL, South Africa.

Malan, F.S. 1995. The basic wood properties and sawtimber quality of South African grown *Pinus elliottii x Pinus caribaea. South African Forestry Journal* **173**: 35 – 41.

Mather, J. 1996. Research report: 1 April 1993 – 30 June 1996. SAFCOL, South Africa.

Mather, J. 2000. Personal communication on the routine annual operations in the Kwazulu / Natal Region.

Maurizio, R., Sheperd, M., Harriss, F., Henry, R., Lee, S. and Cordeiro, G. 1999. Cross species amplification of microsatellite loci – a valuable tool for genetic studies in plants. *In: Plant and animal genome conference VII. Town & country hotel, San Diego, CA, January 17-21, 1999.*

Mellersh, C.S., Langston, A.A., Acland, G.M., Fleming, M.A., Ray, K., Wiegand, N.A., Francisco, L.V., Gibbs, M., Aguirre, G.D. and Ostrander, E.A. 1997. A linkage map of the canine genome. *Genomics* **46**: 326 – 336.

Moore, R., Clark, W.D., Stern, K.R. and Vodopich, D. 1995. Gymnosperms. *In: Botany.* Wm. C. Browm Publishers, Dubuque, IA.

Moore, S.S., Sargeant, L.L., King, T.J., Mattick, J.S., Georges, M. and Hetzel. J.S. 1991. The conservation of dinucleotide microsatellites among mammalian genomes allows the use of heterologous PCR primer pairs in closely related species. *Genomics* **10**: 654 – 660.

Moran, C. 1993. Microsatellite repeats in pig (*Sus domestica*) and chicken (*Gallus domesticus*) genomes. *The Journal of Heredity* **84**: 274 – 280.

Murray, M.G. and Thompson, W.F. 1980. Isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**: 4321 – 4325.

Nakamura, R.R. and Wheeler, N.C. 1992. Pollen competition and paternal success in Douglas-Fir. *Evolution* **46**: 846 – 851.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. and White, R. 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* **235**: 1616 – 1622.

Nikkanen, T., Aronen, T., Häggman, H. and Venäläinen, M. 2000. Variation in pollen viability among *Picea abies* genotypes – potential for unequal paternal success. *Theoretical and Applied Genetics* **101**: 511 – 518.

Nkongolo, K.K. 1999. RAPD variations among pure and hybrid populations of *Picea mariana, P. rubens,* and *P. glauca* (Pinaceae), and cytogenetic stability of *Picea* hybrids: identification of species-specific RAPD markers. *Plant Systematics and Evolution* **215**: 229 – 239.

Ostrander, E.A., Jong, P.M., Rine, J. and Duyk, G. 1992. Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. *Proceedings of the National Academy of Science U.S.A.* **89**: 3419 – 3423.

Ottaviano, E., Sari-Gola, M. and Mulcahy, D.L. 1980. Pollen tube growth rates in *Zea mays*: Implications for genetic improvement of crops. *Science* **210**: 437 – 438.

Owens, J.N., Takaso, T. and Runions, C.J. 1998. Pollination in conifers. *Trends in Plant Science* **3**: 479 – 485.

Pacini, E. 1996. Types and meaning of pollen carbohydrate reserves. *Sexual Plant Reproduction* **9**: 362 – 366.

Pacini, E., Franchi, G.G. and Ripaccioli, M. 1999. Ripe pollen structure and histochemistry of some gymnosperms. *Plant Systematics and Evolution* **217**: 81 – 99.

Pardi, M.L., Viegi, L., Renzoni, G.C., Franchi, G.G. and Pacini, E. 1996. Effects of acidity on the insoluble polysaccharide content of *Pinus pinea* L. and *Pinus pinaster* Aiton. *Grana* **35**: 240 – 247.

Parker, P.G., Snow, A.A., Schug, M.D., Booton, G.C. and Fuerst, P.A. 1998. What molecules can tell us about populations: Choosing and using a molecular marker. *Ecology* **79**: 361 – 382.

Pemberton, J.M., Slate, J., Bancroft, D.R. and Barrett, J.A. 1995. Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology* **4**: 249 – 252.

Petersen, G., Johansen, B. and Seberg, O. 1996. PCR and sequencing from a single pollen grain. *Plant Molecular Biology* **31**: 189 – 191.

Pfeiffer, A., Oliveri, A.M. and Morgante, M. 1997. Identification and characterization of microsatellites in Norway spruce (*Picea abies* K.). *Genome* **40**: 411 – 419.

Piffanelli, P., Ross, J.H.E. and Murphy, D.J. 1998. Biogenesis and function of the lipidic structures of pollen grains. *Sexual Plant Reproduction* **11**: 65 – 80.

Poynton, R.J. 1979. The pines. *In: Report to the Southern African Regional Commission for the Conservation and Utilization of the Soil (SARCCUS) on tree planting in Southern Africa*. Vol. 1. S.A. Forestry Research Institute, Department of Forestry, Republic of South Africa.

Primmer, C.R., Moller, A.P. and Ellegren, H. 1996. A wide-range survey of cross-species microsatellite amplification in birds. *Molecular Ecology* **5**: 365 – 378.

Queller, D.C., Strassmann, J.E. and Hughes, C.R. 1993. Microsatellites and kinship. *TREE* **8**: 285 – 288.

Rafalski, J.A. and Tingey, S.V. 1993. Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. *Trends in Genetics* **9**: 275 – 280.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**: 208 – 213.

Rico, C., Zadworny, D., Kuhnlein, U. and Fitzgerald, G.J. 1993. Characterization of hypervariable microsatellite loci in the threespine stickleback (*Gasterosteus aculeatus*). *Molecular Ecology* **2**: 271 – 272.

Robbins, W.W., Weier, T.E. and Stocking, C.R. 1964. The vascular plants: Phylum XV. Pterophyta. *In: Botany – An introduction to plant science (3rd Ed.).* John Wiley and Sons, Inc. New York.

Robinson, J.P. and Harris, S.A. 1999. Amplified fragment length polymorphisms and microsatellites: A phylogenetic perspective. *Chpter 12 In:* Gillet, E.M. (ed.) *Which DNA marker for which purpose? Final compendium of the research project: Development, optimization and validation of molecular tools for assessment of biodiversity in forest trees in the European Union DGXII Biotechnology FW IV.* http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.html

Roggen, H.P.J.R. 1967. Changes in enzyme activities during the progame phase in *Petunia hybrida. Acta Botanica Neerlandica* **16**: 1 - 31.

Rossetto, M., Mclauchlan, A., Harriss, F.C., Henry, R.J., Baverstock, P.R., Lee, L.S., Maguire, T.L. and Edwards, K.J. 1999. Abundance and polymorphism of microsatellite markers in the tea tree (*Melaleuca alternifolia, Myrtaceae). Theoretical and Applied Genetics* **98**: 1091 – 1098.

Rowley, J.R., Skvarla, J.J. and Walles, B. 1999. Microsporogenesis in *Pinus sylvestris* – VII. Exine expansion and tapetal development. *Taiwania* **44**: 325 – 344.

SAS / STAT Users Guide. 1989. Version 6, 4th edition, vol. 1 and 2. SAS Institute, Cary, USA.

Schmidt, A., Doudrick, R.L., Heslop-Harrison, J.S. and Schmidt, T. 2000. The contribution of short repeats of low sequence complexity to large conifer genomes. *Theoretical and Applied Genetics* **101**: 7 – 14.

Schoen, D.J. and Cheliak, W.M. 1987. Genetics of the polycross. 2. Male fertility variation in Norway spruce, *Picea abies* (L.) Karst. *Theoretical and Applied Genetics* **74**: 554 – 559.

Singh, H. 1978. Embryology of gymnosperms. *In:* Zimmerman,W. *et al.* (Ed's) *Encycolpedia of plant anatomy 10/2*. Gebrüder Borntraeger, Berlin.

Sinnott, E.W. and Wilson, K.S. 1957. The Gymnosperms. *In: Botany – Principles and problems (6th Ed)*. McGraw-Hill Book Company, inc. New York.

Slee, M.U. and Abbott, D.C. 1990. Pollination investigations for production of the hybrid between slash and caribbean pines. *South African Forestry Journal* **152**: 7 – 16.

Smith, D.N. and Devey, M.E. 1994. Occurrence and inheritance of microsatellites in *Pinus radiata*. *Genome* **37**: 977 – 983.

Snow, A.A. and Spira, T.P. 1991. Pollen vigor and the potential for sexual selection in plants. *Nature* **352**: 796 – 797.

Soranzo, N., Provan, J. and Powell, W. 1999. An example of microsatellite length variation in the mitochondrial genome of conifers. *Genome* **42**: 158 – 161.

Sorensen, F.C. 1982. The roles of polyembryony and embryo viability in the genetic system of conifers. *Evolution* **36**: 725 – 733.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**: 31 – 43.

Stanger, T.K., Shaw, M.J.P., Braunstein, R. and Nikles, D.G. 1999. A comparison of the kraft pulp properties of *P. elliottii* and the *P. elliottii x P. caribaea* var. *hondurensis* hybrid grown in Queensland, Australia. *South African Forestry Journal* **186**: 9 – 14.

Stephenson, A.G., Winsor, J.A., Schlichting, C.D. and Davis, L.E. 1988. Pollen competition, nonrandom fertilization, and progeny fitness: a reply to Charlesworth. *The American Naturalist* **132**: 303 – 308.

Steyn, A.G.W., Smith, C.F., Du Toit, S.H.C. and Strasheim, C. 1994. *In: Modern statistics in practice (1st Ed.).*

Strassmann, J.E. 1998. DNA microsatellites. http://es.rice.edu/projects/Bios321/DNA.html

Strauss, S.H., Lande, R. and Namkoong, G. 1992. Limitations of molecular-marker-aided selection in forest tree breeding. *Canadian Journal of Forest Research* **22**: 1050 – 1061.

Tanaka, K., Tsumura, Y. and Nakamura, T. 1999. Development and polymorphism of microsatellite markers for *Fagus crenata* and the closely related *Fagus japonica*. *Theoretical and Applied Genetics* **99**: 11 – 15.

Tautz, D. 1989. Hypervariability of simple sequences as a general source of polymorphic DNA markers. *Nucl. Acids Res.* **17**: 6463 – 6471.

Thomas, B.R., Macdonald, S.E., Hicks, M., Adams, D.L. and Hodgetts, R.B. 1999. Effects of reforestation methods on genetic diversity of lodgepole pine: an assessment using microsatellite and randomly amplified polymorphic DNA markers. *Theoretical and Applied Genetics* **98**: 793 – 801.

Thomas, M.R. and Scott, N.S. 1993. Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STS's). *Theoretical and Applied Genetics* **86**: 985 – 990.

Thomson, J.D. 1989. Germination schedules of pollen grains: Implications for pollen selection. *Evolution* **43**: 220 – 223.

Ting, W.S. and Tseng, C.C. 1965. Electron-microscopic studies of the pollen wall of *Pinus balfouriana* Grev. Et Balf. *Pollen Spores* **7**: 9 – 12.

Tomlinson, P.B. 1994. Functional morphology of saccate pollen in conifers with special reference to *Podocarpaceae. Int. J. Plant Sci.* **155**: 699 – 715.

Truter, N. 2000. Economics. *In:* Kassier, H. (ed.) *SAFCOl internal symposium on the P. elliottii x P. caribaea hybrid.* SAFCOL, South Africa.

Ujino, T., Kawahara, T., Tsumura, Y., Nagamitsu, T., Yoshimaru, H. and Ratnam, W. 1998. Development and polymorphism of simple sequence repeat DNA markers for *Shorea curtisii* and other *Dipterocarpaceae* species. *Heredity* **81**: 422 – 428.

Van Bilsen, D.G.J.L., Van Roekel, T. and Hoekstra, F.A. 1994. Declining viability and lipid degeneration during pollen storage. *Sexual Plant Reproduction* **7**: 303- 310.

Van de Ven, W.T.G. and McNicol, R.J. 1996. Microsatellites as DNA markers in Sitka spruce. *Theoretical and Applied Genetics* **93**: 613 – 617.

Van der Merwe, L. and Wentzel, C. 2000. Seed production and hedge material. *In:* Kassier, H. (ed.) *SAFCOl internal symposium on the P. elliottii x P. caribaea hybrid.* SAFCOL, South Africa.

Van der Nest, M.A., Steenkamp, E.T., Wingfield, B.D. and Wingfield, M.J. 2000. Development of simple sequence repeat (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). *Plant Breeding* **119**: 433 – 436.

Van Gelderen, D.M. 1989. Pinus / Pinaceae. *In: Conifers.* (2nd Ed.) Timber Press publishers, London.

Vendramin, G.G. and Ziegenhagen, B. 1997. Characterization and inheritance of polymorphic plastid microsatellite in *Abies*. *Genome* **40**: 857 – 864.

Vendramin, G.G., Anzidei, M., Madaghiele, A. and Bucci, G. 1998. Distribution of genetic diversity in *Pinus pinaster* Ait. As revealed by chloroplast microsatellites. *Theoretical and Applied Genetics* **97**: 456 – 463.

Vidaković, M. 1991. Pinus. *In: Conifers – morphology and variation.* Grafićki Zavod Hrvatske publishers.

Wakamiya, I., Newton, R.J., Johnston, J.S. and Price, H.J. 1993. Genome size and environmental factors in the genus *Pinus*. *American Journal of Botany* **80**: 1235 – 1241.

Wang, Z., Weber, J.L., Zhong, G. and Tanksley, S.D. 1994. Survey of plant short tandem DNA repeats. *Theoretical and Applied Genetics* **88**: 1 – 6.

Weber, J.L. and May, P.E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics* **44**: 388 – 396.

Weising, K. and Gardner, C. 1999. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome* **42**: 9 – 19.

Wentzel, C. and Oliveir, N. 2000. Nursery practice. *In:* Kassier, H. (ed.) *SAFCOl internal symposium on the P. elliottii x P. caribaea hybrid* .SAFCOL, South Africa.

White, G. and Powell, W. 1997. Cross-species amplification of SSR loci in the Meliaceae family. *Molecular Ecology* **6**: 1195 – 1197.

Willemse, M.T.M. 1968. Development of the micro- and macrogametophyte of *Pinus silvestris* L. *Acta Botanica Neerlandica* **17**: 330 – 331.

Willemse, M.T.M. 1971 a. Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. I. Morphological changes from zygotene until prometaphase I. *Acta Botanica Neerlandica* **20**: 261 – 274.

Willemse, M.T.M. 1971 b. Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. II. Morphological changes from prometaphase I until the tetrad stage. *Acta Botanica Neerlandica* **20**: 411 – 427.

Willemse, M.T.M. 1971 c. Morphological and quantitative changes in the population of cell organelles during microsporogenesis of *Pinus sylvestris* L. III. Morphological changes during the tetrad stage and in the young microspore. A quantitative approach to the changes in the population of cell organelles. *Acta Botanica Neerlandica* **20**: 498 – 523.

Williams, C.G., Elsik, C.G. and Barnes, R.D. 2000. Microsatellite analysis of *Pinus taeda* L. in Zimbabwe. *Heredity* **84**: 261 – 268.

Winter, P. and Kahl, G. 1995. Molecular marker technologies for plant improvement. *World Journal of Microbiology and Biotechnology* **11**: 438 – 448

Wright, J.W. 1976. *Introduction to forest genetics*. Academic Press, Inc. New York.

Wu, K-S., Jones, R., Danneberger, L. and Scolnik, P.A. 1994. Detection of microsatellite polymorphisms without cloning. *Nucleic Acids Research* **22**: 3257 – 3258.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genomic fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification. *Genomics* **20**: 176 – 183.

APPENDIX A

SEQUENCE DATA

0 5' CACACACACACACACACTGGACGCATATAATCAAGCATACAAGAATAT

50 GATCAAGCATACAAAATGATACATGTAAGTGACATATAACAACATGTA

100 ACCAATCATACAATGATCATGATGCATCCCACAACAACAACCGCAACT

150 TCCACATGGAGACACCACTACAACCATGATCAACACTAGACATTGTAA

200 TAGGCAGATGGAATGCAAGCGTAAGAGGCATACATGAAACCAACATGC

250 ATTCATTAACAATGCACACAACCCATTCAGTTCGAGGGGTGAACCATG

300 CTTCGATATGAATCTGTAACACCCTCCGCCCACATCTACCCAAGTAAC

350 CAACCAACTGCGGTAAACATGCGACGAGAGGATACCATGATGAGATTC

400 AACCTCCTAGGTGACCTTAAGGAAATGGAATGAAAGGAAAAACAACAT

450 ACAGACCTTCCACATCAGCTATAGCACCCTTGACCTCGTCC 3'

Figure A-1. RAMs Sequence 1. Development of primers JD1-AMO and JD2-AMO. Where: represents the CA_8 RAMs primer sequence; represents the microsatellite sequences; represents the JD1-AMO forward primer sequence; represents the JD1-AMO reverse primer sequence; represents the JD2-AMO forward primer sequence; and represents the JD2-AMO reverse primer sequence.

0 5' CACACACACACACACATAAATTTAATATTTTTGGGGTTATAATGTAAT

50 GTTATGTTGACATGACAACATCTCGGCCTCATATTTTTGTTTTAATGT

100 AACATTATGTTTTTTTTTTCTCTATAAAAGCTATTTCGTATTGAACTA

150 GTAAACATGATAATATTACATGAGGTAGGCGGCTGCAGAAGCCCGCAC

200 TTGCTAATACTTTACAAAATTTCCCCATTACAGAAGAGATGGGGAATC

250 TCAACAAAACTAACAGCCAAATCAATTGACGTTACAAACATACTGAGA

300 GAGGAAGAACTGAAAATAAGCATATGACCGGAGCTGAAGAACCCTTGA

350 CCTCGTACCCGCTGGTT 3'

Figure A-2. RAMs Sequence 2. Development of primers JD3-AMO. Where: represents the CA_8 RAMs primer sequence; represents the microsatellite sequences; represents the JD3-AMO forward primer sequence; and represents the JD3-AMO reverse primer sequence.

0 5' CACACACACACACACAAAAAAAACTATAAATTTTAATAAAACAAATCT

50 TTTTTTTTTTGTAGGCAAAGGTAGTGCATCTCATTTTCTATTAACATA

100 TCAGAAAATTACATCAAAATTAGTGGACCAGCTACCCGCCCACCAGGG

150 GAAACCGAGTAGGGTATCCACCTAAAGTTTTTAACACCATACCAAAAA

200 AAGAAGTCACGTAGTCTCTTTTTCCTGGCTAAATAGACATTACAGTCC

250 TTCCCTGGTCTAATTCTAGTGATATGCATATATAAACTGTCCATTACA

300 AAAAGAGGGAAGCTGCCTAAAGTATCTAAACACCGCAAAATCATCAGA

350 AGGGAGACAAGAACTAAAACTATTCAACAGCATTGGCAAAGATTGGAG

400 AGTCCTCCTTGGAGCTCCTCTGCTGGCTGGCTTGAGCAATTTCTGAAT

450 CTCTTCCAGCTGCTT 3'

Figure A-3. RAMs Sequence 3. Development of primers JD4-AMO and JD9-AMO. Where: ▭ represents the CA_8 RAMs primer sequence; ▭ represents the microsatellite sequences; ▭ represents the JD4-AMO forward primer sequence; ▭ represents the JD4-AMO reverse primer sequence; ▭ represents the JD9.1-AMO forward primer sequence; ▭ represents the JD9.2-AMO forward primer sequence; and ▭ represents the JD9-AMO reverse primer sequence.

0 5' CACACACACACACACATAGCAAAACAAGATAATCAAGGAGTTCACCCA

50 ACACTCCTGGGCTACATCTCCTGGTCCAACCTTCAGTAGTGACCTCTC

100 TATTATGTCTTTAAGAATATAATTTTACAAAAGAGAGCTCAATTTGAG

150 TCTCACACAACAGATACAAAACAACAAGGCTTTACTTGGATACCCCAA

200 GTCTTTCTGAGTACACAAACTCTCCTCACTGACTCTCTGTCTCCGTTT

250 TCTCTCTGTCTCAACAGTGCTTGCCTCTGTCTCTATCATTCAGTGGCT

300 TTTAAGCCAATTGGCGTTGTCCTGCTCGTTTCCTCTTCTGGGCTTTCT

350 GGCCCTTGACCTCGTCCCGCTGGTT 3'

Figure A-4. RAMs Sequence 4. Development of primers JD5-AMO. Where: ▭ represents the CA_8 RAMs primer sequence; ▭ represents the microsatellite sequences; ▭ represents the JD5-AMO forward primer sequence; and ▭ represents the JD5-AMO reverse primer sequence.

0 5' CACACACACACACACACACCATATAAAATCTGTATGAATGATTAAA

50 TGTGAaAATACATTGATTTTGTAAATGATTGACACATCTTCCAAATTG

100 GAAATATGGGTATGTTTTAGGAATATTTAGTGTTCTTGGACAAACGCC

150 TAACCCTCTAGATCTAATAGCAGGTCTAGAAACAACAAACAAAGTAAT

200 CTCAACCTTTTCTATAATCAATAGATAAAACAAGAGAGAAGAGAAACC

250 TACTTGTAGTTTTCGCCAGCGCCTTGTCTCCCTTGACCTCGTCCCGCT

300 GGTT 3'

Figure A-5. RAMs Sequence 5. Development of primers JD6-AMO. Where: represents the CA_8 RAMs primer sequence; represents the microsatellite sequences; represents the JD4-AMO forward primer sequence; and represents the JD4-AMO reverse primer sequence.

0 5'CACACACACACACACACATAACACAGCAAGATAATCAAGGAGTTCACC

50 CAAGACTCCTGGGCTACATCTCCTAGTCCAGCCTTCAGTGGTGACCTC

100 TCTATTATGTCTTCAAGAGCGGAGTTTTACAAAAGTGAGCTCAATTTG

150 AGTCTCACACAATAGATACAAAATAACAAGGCTTTACTTGGATCCCCC

200 AAGTCTTTCTGAGTACACAGATTCTCCTCACTAACTCTCTATCTCAGC

250 ACTTCTTGTCCCTGTCTCTGTCATTCTACGGCTTTTAAGCCAATGGGT

300 GTTGTCTGTTCGTTTCCTCTTTGGGTTCTCTGGCCCTTGACCTCGTCC

350 CGCTGGTT 3'

Figure A-6. RAMs Sequence 6. Development of primers JD7-AMO. Where: represents the CA_8 RAMs primer sequence; represents the microsatellite sequences; represents the JD7.1-AMO forward primer sequence; represents the JD7.2-AMO forward primer sequence; and represents the JD7-AMO reverse primer sequence.

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0    CACACACACACACACATGGACGCATATAATCAAGCATACAAGAATATG
50   ATCAAGCATACAAAATGATACATGTAAGTGACATATAACAACATGTAA
100  CCAATCATACAATGATCATGATGCATCCCACAACAACAACCGCAACTT
150  CCACATGGAGACACCACTACAACCATGATCAACACTAGACATTGTAAT
200  AGGCAGATGGAATGCAAGCGTAAGAGGCATACATGAAACCAACATGCAT
250  TCATTAACAATGCACACAACCCATTCAGTTCGAGGGGTGAACCATGCT
300  TCGATATGAATCTGTAACACCCTCCGCCCACATCTACCCAAGTAACCA
350  ACCAACTGCGGTAAACATGCGACGAGAGGATACCATGATGAGATTCAA
400  CCTCCTAGGTGACCTTAAGGNAATGGAATGAAAGGAAAAACAACATAC
450  AGACCTTCCACATCAGCTATAGCACCCTTGACCTCGTCCCGCTGGTT
```

Figure A-7. RAMs Sequence 7. Development of primers JD8-AMO. Where: ▭ represents the CA_8 RAMs primer sequence; ▭ represents the microsatellite sequences; ▭ represents the JD8-AMO forward primer sequence; ▭ represents the JD8.1-AMO reverse primer sequence; and ▭ represents the JD8.2-AMO reverse primer sequence.

```
0    5'CACACACACACACACAGAGAATTGCAACTAGTTGCAGCAGCGCGAAAG
50   GCGTCATTGGTTGTCTGAGGGCCTCCTGCATTTCTTTCAATCGACATT
100  CGGTGAACGGAATGATGGAAACAAGAAAGAAGGCTCGGCTTGCAGAGG
150  AAATGGAAGCTGTGCGGACCAAGGTGGCTGATAGGAAAGGTAAAGGGA
200  TTGCGAAAGAGGAGGTCCCGGACGATTTCCTTGAGGTCCCAGACACTT
250  CAGACGAGGGAGGGAGCAGCGACCCGAGTATGAATGCTGAAGAGTGGG
300  AACGGTATGCCATGGGTGTCAGTATGAGGAGCCGGCGTGGTCGAAGGG
350  TTCCTACGCGCGGGCGACGGAGGCGGGTCAATCACAATGGCTGCGAGA
400  CTGCTCCCAACCGTGGTCAGAACAACGCGGCCAGT 3'
```

Figure A-8. RAMs Sequence 8. Development of primers JD10-AMO. Where: ▭ represents the CA_8 RAMs primer sequence; ▭ represents the microsatellite sequences; ▭ represents the JD10-AMO forward primer sequence; ▭ represents the JD10-AMO reverse primer sequence.

0 CACACACACACACACACATAACACAGCAAGATAATCAAGGAGTTCACC

50 CAAGACTCCTGGGCTACATCTCCTAGTCCAGCCTTCAGTGGTGACCTC

100 TCTATTATGTCTTCAAGAGCGGAGTTTTACAAAAGTGAGCTCAATTT

150 GAGTCTCACACAATAGATACAAAATAACAAGGCTTTACTTGGATCCCC

200 CAAGTCTTTCTGAGTACACAGATTCTCCTCACTAACTCTCTATCTCAG

250 CACTTCTTGTCCCTGTCTCTGTC

Figure A-9. RAMs Sequence 9. No Primers developed for this sequence. Where: ▭ represents the CA_8 RAMs primer sequence; ▭ represents the microsatellite sequences.

0 5' CACACACACACACACATAACCATGTAACTTGCCTCCAGGAAAACACCT

50 TCGTGCAGTGACCCGACCTCCCTACCCTTGGTTCTTCACACCTTTGTG

100 TTGCCACCTGACTCTAGTGAACATTGTTCTACCACAGGTCTGAACATG

150 GCCAATGTTGTACAACCTAATTGTTGAACTGTTTACATGTCGATTATC

200 CTTCCATAGAAACCCCAA 3'

Figure A-10. RAMs Sequence 10. No primers developed for this sequence. Where: ▭ represents the CA_8 RAMs primer sequence; ▭ represents the microsatellite sequences.

APPENDIX B

ANOVA TABLES

Table B-1. Results of ANOVA where the eighteen pollen types were compared based on the dimensional measurement AA.

	A22	**A24**	**A29**	**A31**	**A33**	**A49**	**A57**	**A65**	**A91**	**A93**	**A114**	**A271**	**Ac60**	**Ac62**	**P23**	**P69**	**P88**	**P105**
A22	NA	/	/	/	/	/	*	/	/	*	/	*	/	*	*	*	/	/
A24	/	NA	/	/	*	/	*	*	*	*	*	*	/	*	*	*	*	/
A29	/	/	NA	/	/	/	*	/	/	*	/	/	/	/	*	/	/	/
A31	/	/	/	NA	/	/	*	/	/	*	*	*	/	*	*	*	/	/
A33	/	*	/	/	NA	/	*	/	/	*	/	/	*	/	*	/	/	/
A49	/	/	/	/	/	NA	*	/	/	*	/	*	/	*	*	*	/	/
A57	*	*	*	*	*	*	NA	*	*	*	*	*	*	*	*	*	*	*
A65	/	*	/	/	/	/	*	NA	/	*	/	/	/	/	*	/	/	/
A91	/	*	/	/	/	/	*	/	NA	*	/	/	/	/	*	/	/	/
A93	*	*	*	*	*	*	*	*	*	NA	*	*	*	*	/	/	*	*
A114	/	*	/	*	/	/	*	/	/	*	NA	/	*	/	*	/	/	*
A271	*	*	/	*	/	*	*	/	/	*	/	NA	*	/	*	/	/	*
Ac60	/	/	/	/	*	/	*	/	/	*	*	*	NA	*	*	*	/	/
Ac62	*	*	/	*	/	*	*	/	/	*	/	/	*	NA	*	/	/	*
P23	*	*	*	*	*	*	*	*	*	/	*	*	*	*	NA	*	*	*
P69	*	*	/	*	/	*	*	/	/	/	/	/	*	/	*	NA	/	*
P88	/	*	/	/	/	/	*	/	/	*	/	/	/	/	*	/	NA	/
P105	/	/	/	/	/	/	*	/	/	*	*	*	/	*	*	*	/	NA

* = Significantly different

Table B-2. Results of ANOVA where the eighteen pollen types are compared based on the dimensional measurement BB.

	A22	**A24**	**A29**	**A31**	**A33**	**A49**	**A57**	**A65**	**A91**	**A93**	**A114**	**A271**	**Ac60**	**Ac62**	**P23**	**P69**	**P88**	**P105**
A22	NA	/	*	*	*	*	/	/	/	/	/	/	*	/	/	/	/	/
A24	/	NA	/	/	/	/	*	/	/	/	/	/	/	/	/	*	*	/
A29	*	/	NA	/	/	/	*	*	/	*	*	*	/	/	*	*	*	/
A31	*	/	/	NA	/	/	*	*	/	*	*	*	/	*	*	*	*	/
A33	*	/	/	/	NA	/	*	*	/	*	*	*	/	*	*	*	*	/
A49	*	/	/	/	/	NA	*	*	/	*	*	*	/	*	*	*	*	/
A57	/	*	*	*	*	*	NA	/	*	/	/	/	*	*	/	/	/	*
A65	/	/	*	*	*	*	/	NA	*	/	/	/	*	/	/	/	/	/
A91	/	/	/	/	/	/	*	*	NA	*	/	/	/	/	*	*	*	/
A93	/	/	*	*	*	*	/	/	*	NA	/	/	*	/	/	/	/	/
A114	/	/	*	*	*	*	/	/	/	/	NA	/	*	/	/	/	/	/
A271	/	/	*	*	*	*	/	/	/	/	/	NA	*	/	/	/	*	/
Ac60	*	/	/	/	/	/	*	*	/	*	*	*	NA	*	*	*	*	/
Ac62	/	/	/	*	*	*	*	/	/	/	/	/	*	NA	/	/	*	/
P23	/	/	*	*	*	*	/	/	*	/	/	/	*	/	NA	/	/	*
P69	/	*	*	*	*	*	/	/	*	/	/	/	*	/	/	NA	/	*
P88	/	*	*	*	*	*	/	/	*	/	/	*	*	*	/	/	NA	*
P105	/	/	/	/	/	/	*	/	/	/	/	/	/	/	*	*	*	NA

* = Significantly different

Table B-3. Results of ANOVA where the eighteen pollen types are compared based on the dimensional measurement CC.

	A22	**A24**	**A29**	**A31**	**A33**	**A49**	**A57**	**A65**	**A91**	**A93**	**A114**	**A271**	**Ac60**	**Ac62**	**P23**	**P69**	**P88**	**P105**
A22	NA	/	/	*	/	*	*	/	/	*	*	/	/	*	*	*	/	*
A24	/	NA	/	/	/	/	*	*	/	*	*	*	/	*	*	*	/	/
A29	/	/	NA	*	/	*	*	/	/	*	*	/	/	*	*	*	/	/
A31	*	/	*	NA	*	/	*	*	*	*	*	*	*	*	*	/	*	/
A33	/	/	/	*	NA	*	*	/	/	*	/	/	/	/	*	*	/	*
A49	*	/	/	/	*	NA	*	*	*	*	*	*	*	*	*	/	*	/
A57	*	*	*	*	*	*	NA	*	*	*	*	*	*	*	/	*	*	*
A65	/	*	/	*	/	*	*	NA	/	/	/	/	/	/	*	/	/	*
A91	/	/	/	*	/	*	*	/	NA	*	*	/	/	*	*	*	/	/
A93	*	*	*	*	*	*	*	/	*	NA	/	/	*	/	/	/	*	*
A114	*	*	*	*	/	*	*	/	*	/	NA	/	*	/	/	/	*	*
A271	/	*	/	*	/	*	*	/	/	/	/	NA	/	/	*	/	/	*
Ac60	/	/	/	*	/	*	*	/	/	*	*	/	NA	*	*	*	/	/
Ac62	*	*	*	*	/	*	*	/	*	/	/	/	*	NA	/	/	*	*
P23	*	*	*	*	*	*	/	*	*	/	/	*	*	/	NA	/	*	*
P69	*	*	*	*	*	*	*	/	*	/	/	/	*	/	/	NA	*	*
P88	/	/	/	*	/	*	*	/	/	*	*	/	/	*	*	*	NA	/
P105	*	/	/	/	*	/	*	*	/	*	*	*	/	*	*	*	/	NA

* = Significantly different

Table B-4. Results of ANOVA where the eighteen pollen types are compared based on the dimensional measurement DD.

	A22	**A24**	**A29**	**A31**	**A33**	**A49**	**A57**	**A65**	**A91**	**A93**	**A114**	**A271**	**Ac60**	**Ac62**	**P23**	**P69**	**P88**	**P105**
A22	NA	/	/	/	*	*	*	/	/	/	/	/	*	/	/	/	/	/
A24	/	NA	/	*	*	*	*	/	/	/	/	/	*	/	/	/	/	/
A29	/	/	NA	/	/	/	*	*	/	*	*	*	/	/	*	*	*	/
A31	/	*	/	NA	/	/	*	*	/	*	*	*	/	*	*	*	*	/
A33	*	*	/	/	NA	/	*	*	/	*	*	*	/	*	*	*	*	/
A49	*	*	/	/	/	NA	*	*	/	*	*	*	/	*	*	*	*	/
A57	*	*	*	*	*	*	NA	/	*	/	*	*	*	*	/	*	/	*
A65	/	/	*	*	*	*	*	NA	*	/	/	/	*	/	/	/	/	*
A91	/	/	/	/	/	/	*	*	NA	*	/	/	/	/	*	/	*	/
A93	/	/	*	*	*	*	/	/	*	NA	/	/	*	/	/	/	/	*
A114	/	/	*	*	*	*	*	/	/	/	NA	/	*	/	/	/	/	*
A271	/	/	*	*	*	*	*	/	/	/	/	NA	*	/	/	/	/	*
Ac60	*	*	/	/	/	/	*	*	/	*	*	*	NA	*	*	*	*	/
Ac62	/	/	/	*	*	*	*	/	/	/	/	/	*	NA	/	/	/	/
P23	/	/	*	*	*	*	/	/	*	/	/	/	*	/	NA	/	/	*
P69	/	/	*	*	*	*	*	/	/	/	/	/	*	/	/	NA	/	/
P88	/	/	*	*	*	*	/	/	*	/	/	/	*	/	/	/	NA	*
P105	/	/	/	/	/	/	*	*	/	*	*	*	/	/	*	/	*	NA

* = Significantly different

Table B-5. Results of ANOVA where the eighteen pollen types are compared based on the dimensional measurement EE.

	A22	**A24**	**A29**	**A31**	**A33**	**A49**	**A57**	**A65**	**A91**	**A93**	**A114**	**A271**	**Ac60**	**Ac62**	**P23**	**P69**	**P88**	**P105**
A22	NA	/	*	/	*	/	/	/	/	/	/	/	/	/	/	/	/	/
A24	/	NA	/	/	/	/	/	/	/	/	/	/	/	/	*	/	/	*
A29	*	/	NA	/	/	/	*	*	/	/	/	*	*	*	*	*	*	*
A31	/	/	/	NA	/	/	*	*	/	/	/	/	/	*	*	/	/	*
A33	*	/	/	/	NA	/	/	*	/	/	/	*	*	*	*	*	*	*
A49	/	/	/	/	/	NA	*	*	/	/	/	/	/	/	*	/	/	*
A57	/	/	*	*	*	*	NA	/	*	/	/	/	/	/	/	/	/	/
A65	/	/	*	*	*	*	/	NA	*	/	/	/	/	/	/	/	/	/
A91	/	/	/	/	/	/	*	*	NA	/	/	/	/	/	*	/	/	*
A93	/	/	/	/	/	/	/	/	/	NA	/	/	/	/	*	/	/	/
A114	/	/	/	/	/	/	/	/	/	/	NA	/	/	/	/	/	/	/
A271	/	/	*	/	*	/	/	/	/	/	/	NA	/	/	/	/	/	/
Ac60	/	/	*	/	*	/	/	/	/	/	/	/	NA	/	/	/	/	/
Ac62	/	/	*	*	*	/	/	/	/	/	/	/	/	NA	/	/	/	/
P23	/	*	*	*	*	*	/	/	*	*	/	/	/		NA	/	/	/
P69	/	/	*	/	*	/	/	/	/	/	/	/	/	/	/	NA	/	/
P88	/	/	*	/	*	/	/	/	/	/	/	/	/	/	/	/	NA	/
P105	/	*	*	*	*	*	/	/	*	/	/	/	/	/	/	/	/	NA

* = Significantly different

APPENDIX C

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

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LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APS	Ammonium persulphate
bp	Base-pairs
ca.	Approximately
CTAB	Cetyltrimethylammonium bromide
D.E.R. 736	Diglycidyl ether of polypropylene glycerol
DMAE	Dimethylaminoethanol
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ha.	Hectare
HCl	Hydrochloric acid
IPTG	Isopropylthio-β-D-galactoside
KCl	Potassium chloride
LB	Luria-Bertani medium
NaCl	Sodium chloride
NaOAc	Sodium acetate
NSA	Nonenyl succinic anhydride
M	Molar
$MgCl_2$	Magnesium chloride

PC	Principle component
PCA	Principle component analysis
PCR	Polymerase chain reaction
PVP	Polyvinylpolypyrrolidone
RAMs	Ramdomly amplified microsatellites
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SAFCOL	South African Forestry Company Limited
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
SSR	Simple sequence repeats
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TEM	Transmission electron microscope
TEMED	N,N,N'N'-tetra-methylethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
U	One unit of *Taq* DNA polymerase. i.e. the amount of enzyme required to catalyze the incorporation of 10 nanomoles of dNTPs into acid insoluble material in 30 minutes at 74°C.
UV	Ultraviolet
V	Volts
VCD	Vinyl cyclohexene dioxide

v/v	Volume per volume
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside
w/v	Weight per volume