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.

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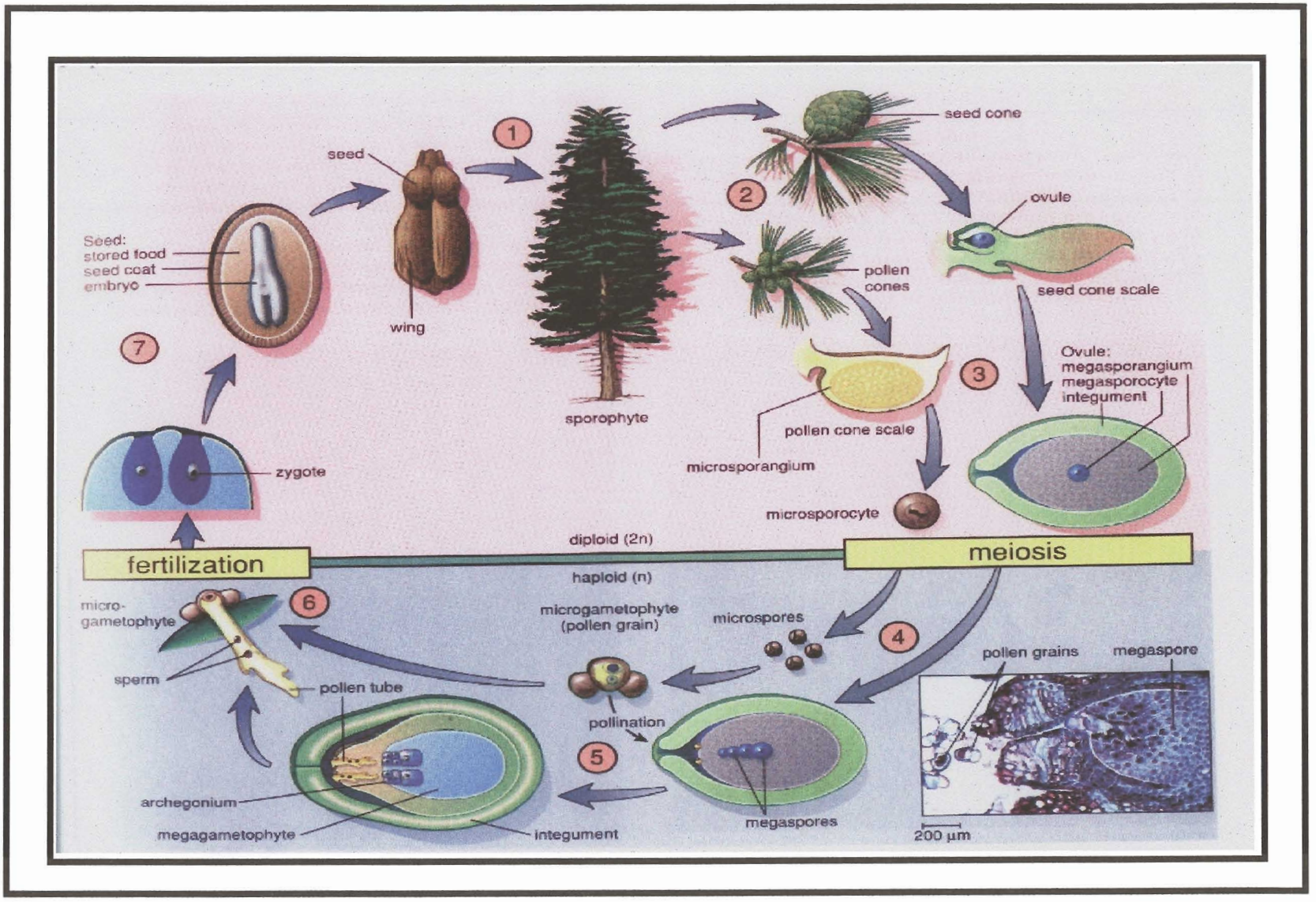

Figure 1.1. Life cycle of the pines (Mader, 1998).

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

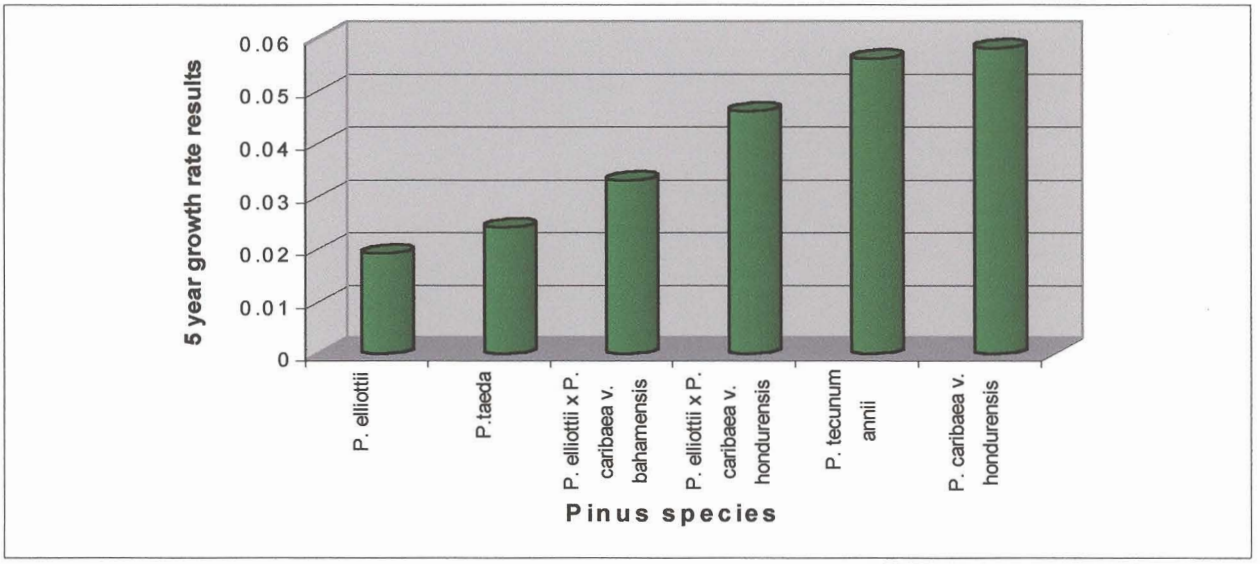

Figure 1.2. Comparison of *P. elliottii x P. caribaea* hybrid growth rate with the growth rates of the parent species and other pine species. (Bester, 2000)

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.