Genetic characterisation of *Colophospermum mopane* (s*ensu lato*) using RAPD analyses

By

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DECLARATION

I declare that this dissertation hereby submitted to the University of Limpopo for the degree Master of Science has not been previously submitted by me for a degree at or any other university, that is my own work in design and execution, and that all material contained therein has been duly acknowledged.

Signed: _____

Date: _____

DEDICATION

This Dissertation is dedicated to my late father, Freddy Legodi; my mother, Pauline Legodi; my son Matome; my nieces Refilwe, Khomotso, Matlou and Getse; my nephews Freddy and Tumi; my sister Julia Sebola, and my brothers Charles, Jonas, Managa and Lesley.

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ABSTRACT

Colophospermum mopane (*sensu lato*) is currently recognised on morphological and physiological characteristics. To add to the suite of taxonomic characters, the genetic variability of *C. mopane* (*sensu lato*) was investigated using the RAPD technique. DNA was extracted from young seedlings and mature leaves using the CTAB method. Initially, the DNA extraction was problematic due to the presence of polysaccharides, making PCR nearly impossible. An additional phenol precipitation step was introduced to purify the DNA used to perform RAPD analyses. Twenty random primers were tested for their suitability and reproducibility to reveal polymorphism in *C. mopane* (*sensu lato*). Nine of the primers tested amplified the genomic DNA. Subsequently, three primers (OPA 03, OPA 08 and OPA 09) were selected based on their reproducibility and demonstration of polymorphism. OPA 03 amplified most of the samples tested whereas OPA 08 and OPA 09 amplified 50% of the samples. RAPD bands ranged from 180 bp to 2000 bp. RAPD profiles of *C. mopane* (*sensu lato*) with three random primers showed few polymorphisms. Individual trees of different ecotypes show similar RAPD banding pattern, instances were found where trees of the same ecotype showed different bands. The total character difference based on presence and absence of bands revealed both variability and similarity of *C. mopane* (*sensu lato*). Phylogenetic trees from individual primers and combined primers were constructed using Neighbour Joining and Parsimony analysis. The phylogenetic tree from the combined primers of bootstrap parsimony generated three clades with low and high parsimony bootstrap values. The first clade receives weak support (61%) while the second and third clades receive support of 90% and 70%, respectively. The other remaining entities collapsed resulting in basal polytomy. The third clade shows some members of Alba (Alba 11 Phala, Alba 1 Phala and Alba 7 Musina) grouped together. The overall results of *C. mopane* (*sensu lato*) show high (84.1%) genetic similarity. No ecotypic marker was obtained. Most of the ecotypes have not diverged genetically far from one another or from the parental material (Mopane – *sensu stricto*). The genetic results partially support the perceived morphological differences. In this study the RAPD technique has established its value as an additional tool to express the genetic variability in *C. mopane* (s*ensu lato*).

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LIST OF ABBREVIATIONS

bp: base pairs

cm: centimetre

CTAB: Cetyltrimetylammonium bromide

DNA: Deoxyribose Nucleic Acid

dNTPs: deoxynucleotide 5' triphosphates

EDTA-Ethylene Diamine Tetra Acetic acid

kb: kilobases

m: metre

M: molar

$MgCl_2$: Magnesium Chloride

ml: millimetres

mM: millimolar

NaCl: Sodium Chloride

ng: nanogram

PAUP: Phylogenetic Analysis Using Parsimony

PCR: Polymerase Chain Reaction

PVP: Polyvinylpopyrrolidone

RNAse: Ribonuclease

TAE: Tris Acetic EDTA buffer

TE: Tris EDTA buffer

TRIS: Tris hydroxymethyl amino methane

µl: microlitre

µM: micromolar

EPONYMY

The following working names are chosen to distinguish the ecotypes from each other:
Alba was chosen as a name to emphasise the whitish seed pods.
Acuminata highlight the pointed shape of the leaflets.
Lesliei, named after Leslie Wessels for sharing responsibility in typification of the ecotypes.
Laevigatum was assigned to an ecotype with smooth bark and small seeds.
Parvispermum was assigned to an ecotype with rough bark and small seeds.

TERMS AND DESIGNATIONS

For the purpose of this dissertation, the following designations will be applied to distinguish between *C. mopane* and its ecotypes.
C. mopane (s*ensu lato*) refers to *C. mopane* (*sensu stricto*) and its ecotypes
Mopane (Mop) refers to *C. mopane* (*sensu stricto*)
Alba refers to an ecotype of *C. mopane* (s*ensu lato*)
Acuminata (Acum) refers to an ecotype of *C. mopane* (*sensu lato*)
Lesliei (Les) refers to an ecotype of *C. mopane* (*sensu lato*)
Parvispermum (Parvi) refers to an ecotype of *C. mopane* (*sensu lato*)
Laevigatum (Laevi) refers to an ecotype of *C. mopane* (*sensu lato*)

CHAPTER 1

INTRODUCTION

1.1 Rationale for the study

Colophospermum mopane (J. Kirk ex Benth.) J. Kirk ex J. Léonard is one of the most valuable tree species indigenous to southern Africa (Léonard 1999). The tree provides a valuable timber and is widely used as construction material (Madzibane and Potgieter 1999), for medicinal purposes (Mashabane et al., 2000) and by browsers as a source of food (Styles and Skinner 1997). Ecological variants exist within *C. mopane* (*sensu stricto*) indicating a degree of phenotypic plasticity (Madams 1990). Wessels et al. (1998) informally described three ecotypes of *C. mopane* (Acuminata, Alba and Lesliei) based on differences in their morphology, physiology (e.g. tolerance to cold and drought) and habitat. Later, two additional ecotypes, with the working names; Laevigatum and Parvispermum, were distinguished. The latter two ecotypes have not yet been informally described.

From the observed morphological characteristics of ecotypes it is not clear as to whether the morphological differences observed in the Zambian biotypes (Madams 1990) and different ecotypes of *C. mopane* (*sensu stricto*) are the result of environmental factors (e.g. water availability, irradiance, edaphic conditions) or the result of a genetic disposition (Wöhrmann 1990). However, during summer, Alba and Parvispermum are not distinguishable from *C. mopane* (*sensu stricto*). Further evidence from DNA analysis could possibly support the morphological classification.

Plant taxonomic studies have been conducted for several years based on morphological and cytological analysis. As the current identification methods rely on morphological characteristics whose expression may be affected by developmental and environmental factors (light, humidity etc.), a morphological character as a marker, therefore, can be ambiguous (Liu and Furnier 1993).

Biochemical analysis such as allozyme and protein analysis have also been used in plant identification studies. However, the tissue specific expression of proteins has limited the use of protein analyses in identification of many crops (Claros et al., 2000). Proteins are also limited by the small number of discriminating loci providing limited coverage of the genome (Guasmi et al., 2006). Protein expression is often dependant on the environmental conditions of plant tissue used for protein extraction (Winter and Kahl 1995).

Villoen (2003) investigated the allozyme variation in five populations of *Colophospermum mopane* (*sensu stricto*) using isozyme techniques. Villoen (2003) could not observe heterozygote deficiencies as all loci conformed to Hardy-Weinberg expectations. The genetic differentiation among populations of *C. mopane* (*sensu stricto*) did not reflect geographical separation by genetic distance.

DNA fingerprinting markers (Restriction Fragments Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Microsatellites) are appropriate methods for studying the

genetic variation in plants (Avise 1994). These techniques have a broad application in familial relationship analyses, (Kirby 1992; Claros et al., 2000), genetic differentiation and plant species identification (William et al., 1990; Rajaseger et al., 1997). The use of DNA fingerprinting markers in plant taxonomy provides an opportunity to determine relationships more precisely than morphological and biochemical markers, as they are potentially unlimited in number, are not affected by environmental conditions, and can be organized into linkage maps (Niehuis et al., 1995).

DNA-based markers have largely overcome the disadvantages of morphological and isozyme analyses and have been successfully applied to discriminate among genotypes in a wide range of agricultural crops (Weising et al., 1991; Jain et al., 1994; Nynbom 1994; Rath et al., 1998; Rajaseger et al., 1999; Brenna 2004). An example is the lines Azucena and PR 304 rice cultivars that have been classified as *indicas* using morphological characters, whereas they behave like *japonicas* in crossing. These two samples were, however, clearly revealed to be *japonicas* after being analysed by molecular markers (Virk et al., 1997). It is hypothesised that DNA fingerprinting can be applicable on *C. mopane* (*sensu lato*), thereby providing evidence of morphological differences.

DNA-based marker techniques, such as Random Amplified Polymorphic DNA (RAPD), have been widely adopted as a convenient and powerful technique in detecting differences among closely-related organisms (Welsh and McClelland 1990; Williams et al., 1990). RAPD markers are considered as a useful tool in species identification

(Demeke et al., 1992). RAPD has been found to aid in distinguishing between major taxonomic sections and varieties (Adams and Demeke 1993).

RAPD analyses can be used effectively for initial assessment of levels and partitioning of genetic variation within plant species, particularly in species with limited genetic diversity information (Lowe et al., 2000). RAPD analyses also categorise plants that cannot be classified by morphological characters (Melchinger et al., 1994). In addition, it reveals a large amount of genetic variation with good coverage of the entire genome (Rajaseger et al., 1997).

If RAPD analyses support morphological differences, the ecotypes could be published as new specific or infra-specific entities of *C. mopane* (*sensu stricto*). This typification is of importance to game farmers, as they could plant the ecotypes selectively in suitable habitats on their farms ensuring improved/prolonged availability of browse during the dry winter months.

Value also lies in investigating the ecotypes in terms of ecological and even ecophysiological studies. It is only once the identity of the ecotypes has been established that meaningful ecological/ ecophysiological research can be undertaken. Such studies will provide answers as to the complex interaction between the ecotypes and *C. mopane* (*sensu stricto*) as well as between the ecotypes and other woodland species.

The results can also be helpful in reforestation projects especially in rural communities, as *C. mopane* (*sensu stricto*) has become threatened through indiscriminate use around local villages of Lubango and Namibe (Neto 2000). Care must, however, be taken not to contaminate the gene pool in areas where the ecotypes do not occur. This study aims to provide answers as to the genetic distance between the commonly found *C. mopane* (*sensu stricto*) and its ecotypes, and hence it's interbreeding potential.

1.2 Aim and specific objectives

1.2.1 Aim

To elucidate the use of DNA fingerprinting techniques in understanding the genetic variation of *Colophospermum mopane (sensu lato)*.

1.2.2 Objectives

The objectives of this research were:

1.2.2.1 To ascertain by means of RAPD the genetic variation between and within:

- The different ecotypes.
- Different individuals of *C. mopane* (*sensu stricto*) trees.
- Different trees of the same ecotype.
- Two different populations of the same ecotype.

1.2.2.2. To investigation the genetic variation of various ecotypes and *C. mopane* (*sensu stricto*) trees

1.2.2.3. To construct a phylogenetic tree of *C. mopane* (*sensu lato*).

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of mopane

Colophospermum Kirk ex J. Léonard is a monospecific genus first described by Léonard in 1949, and is classified as belonging to the Tribe Detarieae (Lock 1989) of the subfamily Caesalpinioideae, family Fabaceae (Leguminosae). Dr John Kirk who collected the two type specimens (*s.n.*) (Chrimaba specimen = holotype and Lupata specimen = isotype as per designation) during April 1860 in Shiramba and Lupata in Mozambique, suggested the name *Colophospermum* (colloquially known as mopane).

Previously (1865), *C. mopane* was placed by Bentham in the genus *Copaifera* L. (*Copaifera mopane* Kirk ex Benth.), along with *Copaifera conjugata* (Bolle) Milne-Redh. (now *Guibortia conjugata* Bolle J. Léonard) and *Copaifera coleosperma* Benth. (now *Guibortia coleosperma* (Benth.) J. Léonard), to which it is closely related (Bentham 1865). *Colophospermum mopane* was also placed in the genus *Copaiba* (*Copaiba mopane* (Kirk ex Benth.) O. Kuntze around the turn of the 19th century (1891) (Kuntze 1891).

Breteler et al. suggested in 1997 that *Colophospermum* be sunk under the Indian genus *Hardwickia* Roxburgh (previously also monotypic with the species *H. binnata* Roxb.) on the grounds of comparative anatomy, pollen morphology and wood anatomy. Breteler et al. (1997) argue that Léonard (1949) described *Colophospermum* to accommodate the aberrant *Copaifera mopane*. These authors further state that most characters which are

mentioned by Léonard to distinguish *Colophospermum* from *Copaifera* are in fact characters that characterize *Hardwickia*. In their 1997 article, these authors, however, omitted mentioning the fruit characters, which differ substantially. *Colophospermum mopane* then changed to *Hardwickia mopane* (Kirk ex Benth.) Breteler.

In response, Smith et al. (1998) proposed the retention of the name *Colophospermum mopane* based on the major economic importance of the species in southern Africa and the confusion that the name change would cause to users.

However, in 1999 Léonard provided a convincing argument, based on taxonomic grounds, for not accepting the name change proposed by Breteler and co-workers. Léonard (1999) mentions, amongst other, that several characters cited as common to the two genera are not exclusive to them, but are also found in other genera of the tribe. Léonard (1999) also stated that it is noted that numerous distinctive characters between these two genera are not mentioned by Breteler et al. (1997). Léonard concluded that Breteler and his colleagues, unconsciously no doubt, worked in a subjective manner. Considering Léonard's conclusive article, it is clear that Smiths 1998 article is superfluous.

2.2 Description of *Colophospermum mopane (sensu stricto*)

Colophospermum mopane (sensu stricto) ranges from a small shrub of 1 to 2 m, to a tree of 4 to 18 m, in height. Cathedral forms are predominately very tall (up to 18 to 20 m high) and single-stemmed, while dwarf and shrub forms of *C. mopane (sensu stricto*) are

commonly short and multi-stemmed (Timberlake 1995). The tree has an erect narrow crown, a dark grey to brown, rough and longitudinally-fissured bark. Leaves are alternate, with a single pair of nearly triangular leaflets. *Colophospermum mopane* (*sensu stricto*) trees are deciduous with leaves sometimes remaining on the tree well into the dry season (Fanshawe 1962; Palmer and Pitman 1972). The secondary root system is generally shallow, around 30 to 120 cm in depth (Thompson 1960), although in deep soils these roots can reach a depth of 2 m (Timberlake and Calvert 1993). The flowers are small, greenish-white or greenish-yellow, and in Zimbabwe and South Africa appear irregularly from December to March. Fruit pods are indehiscent, compressed and yellowish–brown, light and papery, and are dispersed by wind and rainwash. The seeds are large and compressed, usually kidney-shaped and corrugated, with numerous small, sticky, reddish glands (Timberlake 1995).

2.3 Distribution of *Colophospermum mopane* (*sensu stricto*)

Colophospermum mopane (*sensu stricto*) occurs in Angola, Namibia, Zambia, Botswana, Malawi, Zimbabwe, Mozambique and South Africa. This xerophytic tree species occurs in a 571 500 km² area of southern Africa. In the northern parts of South Africa, *C. mopane* (*sensu stricto*) extends over an area of about 23 000 km² (Mapaure 1994), ranging from north of the Soutpansberg in the Limpopo Province, to just south of the Olifants River in Mpumalanga. The distribution of *C. mopane* (*sensu stricto*) is determined by various ecological factors such as minimum rainfall, frost incidences and minimum temperatures (Timberlake 1995). Generally, mopane occurs on clay-rich soils,

in areas with a mean annual rainfall of 100 to 800 mm, and at altitudes ranging from 200 to 1 200 m above sea level (Timberlake 1995).

2.4 Biotypes of *Colophospermum mopane* (*sensu stricto*)

Inhabitants of mopani veld in Zambia recognise two distinct types of *C. mopane* (*sensu stricto*), based on bark morphology. According to Madams (1990), these biotypes are known by their Kunda names of "Mmkwelambulu" (trees with longitudinally fissured bark) and "Kapalamamba" (trees with bark fissured longitudinally and horizontally).

2.5 Description of *Colophospermum mopane* (*sensu stricto*) ecotypes

Turresson (1992) coined the term 'ecotype' to define an ecological subunit that arises as a result of a genotypic response to a particular habitat. An ecotype can also be defined as a community of individuals of the same species, differentiating according to the substrate in which it grows, in other words phenotypic plasticity (Tootill 1984). Ecotypes adapt to tolerant conditions such as temperature or light intensities which the others members of the same species are not exposed to. These adaptations may result in morphological or physiological changes within the species. However, interbreeding with other ecotypes does occur and fertile progeny is produced. Ecotypes may sometimes be sufficiently distinct to be given infra-specific names and are then termed ecospecies (Tootill 1984). According to Williamson (1996) an ecotype is an invasive capacity of species that depends at least partly on the ability to adapt to new habitats.

The ecotypes to be investigated have been informally described by Wessels et al. (1998) as outlined in the section that follows. Distinguishing characters of each ecotype are summarised in Table 1.

2.5.1 Ecotype Lesliei

This ecotype (Fig. 1 C) occurs abundantly along the river banks and among stands of other ecotypes. Trees of ecotype Lesliei may reach heights up to 7 m. The leaves remain mostly green during the dry winter months, probably due to the presence of underground water and to a tolerance for winter temperatures. Leaflets of this ecotype are on average 64 mm long and 26 mm wide. An outstanding characteristic of this ecotype is the cluster of four to seven dead leaves, which forms in the vicinity of inflorescence axes. The amber-coloured dead leaves contrast sharply with the green leaves and the large, light brown seed pods. The leaflets of this ecotype are generally longer than those of *C. mopane* (*sensu stricto*), but not as wide. The fruits of ecotype Lesliei are longer and thinner than those of *C. mopane* (*sensu stricto*) and other ecotypes. These fruits are semi-kidney-shaped, differing clearly from those of *C. mopane* (*sensu stricto*) and other ecotypes. Fruits are light brown, have a rough mat texture and a flat wing. The fruit pod is on average 45 mm long, 25 mm wide and 2 mm in thickness. Seeds of ecotype Lesliei are intricately convoluted, with numerous resin glands scattered over their surfaces. On average, the seeds and pods have a mean mass of 0.5 g. The inside of the fruits gives off a sharp turpentine smell like when opened. The bark of this ecotype is light grey, deeply vertically fissured, and flakes off in narrow strips (Fig. 2 C).

2.5.2 Ecotype Alba

Ecotype Alba individuals are found in stands of *C. mopane* (*sensu stricto*) trees (Fig. 1 A). During winter individuals of ecotype Alba stand out amongst the brown- leaved or leafless *C. mopane* (*sensu stricto*) individuals and remain mostly green during the dry winter months. Individuals of this ecotype reach heights of up to 4.5 m. These dissimilarities as opposed to *C. mopane* (*sensu stricto*) are in all probability due to differences in cold tolerance and/or physiological, anatomical and morphological characteristics. This ecotype generally has a more compact growth form compared to *C. mopane* (*sensu stricto*) trees. The leaflets of ecotype Alba are wider than those of *C. mopane* (s*ensu stricto*) and other ecotypes. Leaflets are on average 60 mm long and 27 mm wide. The production of light brown (whitish) fruits is an outstanding characteristic of this ecotype (Fig. 3 A). The fruit of ecotype Alba is longer, wider and thicker than those of the other ecotypes and *C. mopane* (*sensu stricto*). The fruit pod are on average 39 mm long, 22 mm wide and 3 mm thick. On average, the seeds and pods have a mean mass 0.4 g. The fruits are also lighter in weight than the fruits of the other ecotypes and *C. mopane* (s*ensu stricto*). This may be due to the small-sized seed of ecotype Alba. The bark of this ecotype is light grey, with deep vertical and lateral fissures, and flakes as scales (Fig. 2 D).

2.5.3 Ecotype Acuminata

Individuals of ecotype Acuminata occur among *C. mopane* (*sensu stricto*) trees in the veld, but also along the courses of drainage systems. Ecotype Acuminata is less tolerant to cold than the other ecotypes. Ecotype Acuminata has longer and narrower leaflets than

those of *C. mopane* (*sensu stricto*) and the other ecotypes (Fig. 4). Leaflets are on average 74 mm long and 25 mm wide. The fruits are light brown and have a rough matt texture. They are often slightly sun-bleached on the side exposed to the sun. The fruits of ecotype Acuminata are deeply incised and kidney-shaped. The fruit pods are of average 38 mm long, 21 mm wide and 3 mm thick. The seeds and pods have a mean mass of 0.5 g. Ecotype Acuminata has the shortest fruits and are thinner but wider than the fruits of *C. mopane* (s*ensu stricto*). This ecotype has dark grey to blackish bark, deeply fissured vertically and horizontally (Fig. 2 B). This gives the bark a "blocked" appearance.

2.5.4 Ecotype Laevigatum

Ecotype Laevigatum is not abundant and only concentrated to the west of Musina, where they grow on sandy soil, which originated from Clarens sandstone (Fig. 1 B). Trees of this ecotype appear intermixed with the *C. mopane* (s*ensu stricto*) forms. The trees are medium-sized, 2-5 m high, but can also obtain heights of 15-20 m under wet conditions where regular water supplies exist such as on river banks. Bark of this ecotype is grey and smoother than the bark of *C. mopane* (s*ensu stricto*) and other ecotypes, even for mature trees (Fig. 2 A). Unlike other ecotypes, no reddish exudates appear (between the wood and periderm) when the bark is removed. Also, the periderm appears late during twig development, so that lenticels are visible over large parts of young stems of 3 to 4 growth periods.

Leaves of ecotype Laevigatum remain green during the dry winter months. Leaflets of this ecotype are on average 44 mm long and 21 mm wide. The petiole is generally 19 mm long. The fruits of Laevigatum are light brown with a rough matt texture and flat wing (Fig. 3 A). Fruit pods are on average 31 mm long and 17 mm wide. This ecotype has the smallest fruits of all the ecotypes and *C. mopane* (*sensu stricto*). Seeds are on average heavier than those of the other ecotypes, with a mean mass of 0.8 g.

It is assumed that the distribution of this ecotype continues into Botswana (Wessels and Potgieter, personal communication). The precise northern and western distribution range of this ecotype is yet to be established. Presumed hybrids have been observed that morphologically resemble both *C. mopane* (*sensu stricto*) and Laevigatum. These trees have the smooth bark of Laevigatum and the larger fruits of *C. mopane* (s*ensu stricto*).

2.5.5 Ecotype Parvispermum

Ecotype parvispermum grows on calcareous soils. Trees of this ecotype occur intermingled with *C. mopane* (*sensu stricto*) and attain heights of 5 - 7 m. Leaflets of this ecotype are on average 43 mm long and 16 mm wide. The petiole is generally 21 mm long. The fruits of ecotype Parvispermum are light brown with a rough matt texture. Fruit pods are on average 29 mm long, 12 mm wide and 2mm in thickness. On average, the fruit has a mean mass of 0.46 g and the seed has a mean mass of 0.25 g. The bark of ecotype Parvispermum is rough, dark grey.

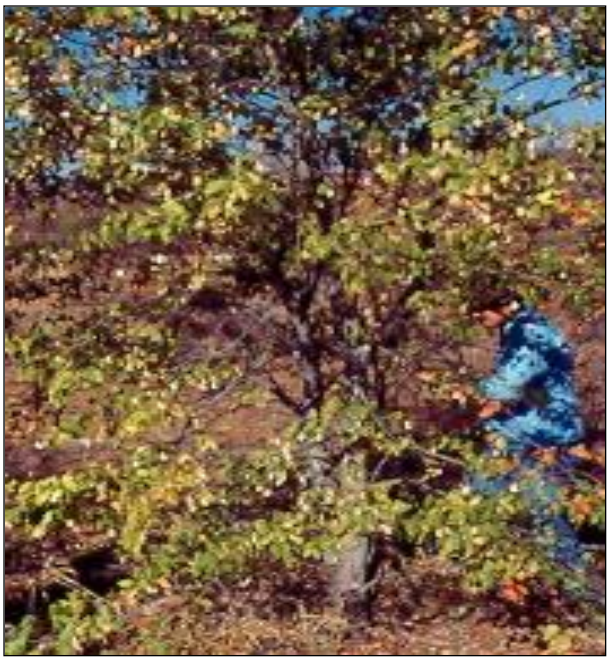
Table 1: Distinguishing morphological characters of the ecotypes, the most differentiating characters are underlined

	Alba	**Lesliei**	**Acuminata**	**Laevigatum**	**Parvispermum**
Bark colour and texture	Light grey Rough	Light grey, Rough	Dark grey–Black, Rough	Dark grey, Smooth	Dark grey, Rough
Leaflet shapes	Narrowest leaflet	Sharpest leaflet apex	Pulvinus on upper 1/3 of leaflets	No unique characters	No unique characters
Fruit colour and mean mass	Whitish brown, 0.4 g	Light brown, 0.5 g	Light brown 0.5 g	Brown, 0.8 g	Brown, 0.46 g

A

Alba

B



Laevigatum (Photo credit: D. Wessels)

C

Lesliei

Figure 1: Habit of the ecotypes.

A

Laevigatum

B

Acuminata

C

Lesliei

D

Alba

Figure 2 : Bark characteristics of the ecotypes.

A

Alba

B

C. mopane (*sensu stricto*) and other ecotypes

Figure 3: The fruits of *C. mopane* (*sensu lato*)

A

Acuminata

B

C. mopane (*sensu stricto*) and other ecotypes (Photos credit: D. Wessels)

Figure 4: The leaflets of *C. mopane* (*sensu lato*).

2.6 Previous studies on genetic diversity of *C. mopane* (*sensu stricto*)

Villoen et al. (2003) studied allozyme variation in five populations of *Colophospermum mopane* using isoenzyme techniques. The study was done to determine if ecological variants exist within *C. mopane* (*sensu stricto*) populations and to screen for genetic variation. The results show that no heterozygote deficiencies were observed as all loci conformed to Hardy-Weinberg expectations. Villoen et al. (2003) concluded that the genetic differentiation among populations of *C. mopane* (*sensu stricto*) did not reflect geographical separation by distance and the genetic variation within *C. mopane* (*sensu stricto*) was sufficient to ensure adaptability of the species. The study suggested that further DNA analysis should be done on ecotypes to prove their existence.

2.7 Random Amplified Polymorphic DNA (RAPD)

RAPD is a molecular tool for measuring genetic relationship among plants and plant cultivars described by Williams et al. (1990). RAPD is based on PCR procedure and a small amount of DNA is necessary in the reaction (Achenbach et al., 1996). The amplification protocol differs from standard PCR conditions in that only a single random oligonucleotide is employed. The PCR reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step. In the first cycle the DNA is made a single strand by raising the temperature to 94°C (denaturation step). In the second cycle, lowering of the temperature to between 40°C and 65°C results in annealing of the primer to their target sequences on the template DNA (annealing step). In the third cycle, a temperature is chosen where the activity of the thermostable *Taq* DNA polymerase is optimal, usually 72°C (elongation step).

Amplification products are separated by gel electrophoresis and visualised by ethidium bromide staining (Williams et al., 1990). The most crucial factors that need to be optimized in a RAPD reaction are the magnesium concentration, enzyme concentration, DNA concentration and annealing temperature of the primer. The Guanine and Cytosine (GC) content of the primers should be 40% to 60% and care should be taken to avoid sequences that produce internal secondary structures. The annealing temperature for a PCR cycle is generally 3°C to 5°C below the melting temperature of the primer (Williams et al., 1990).

The use of a single 10-mer oligonucleotide promotes generation of several discrete DNA products which are considered to originate from different genetic loci. The amplification products are resolved on agarose gel (Williams et al., 1990). This new technique received a high recognition as a powerful and efficient tool with application to ecology, systematic, evolution studies, and especially to population biology (Welsh and McClelland 1990). RAPD technology has quickly gained widespread acceptance and application because it has provided a tool for genetic analysis in biological systems that have not previously benefited from the use of molecular markers (Hadrys et al., 1992). RAPD markers have been used for the development of genetic maps, for targeting genetic markers and in population genetics (Hadrys et al., 1992). The area of research that has shown the most growth with respect to the use of RAPD technology is that of population genetics (Hadrys et al., 1992).

RAPD markers have been used to create fingerprints for the study of individual identity and taxonomic relationship in both eukaryotic and prokaryotic organisms. RAPD markers are being used effectively to assess the amount of genetic diversity in germplasm collections. RAPD markers show to discriminate between individuals in a germplasm collection and to distinguish between closely related individuals was simply a function of the number of RAPD bands that were observed. This technique can also be used to determine taxonomic identity, assess kinship relationship, detect interspecific gene flow, analyse hybrid speciation, and create specific probes (Hadrys et al., 1992).

The absence and presence of bands can be scored and the data converted into similarity matrices for calculating genetic distances (Ellsworth et al., 1993; Karp et al., 1996). The use of such techniques for germplasm characterisation facilitates conservation and utilization of plant genetic resources, permitting the identification of unique accessions or sources of genetically diverse germplasm. The ability of this method to distinguish between taxa also has useful implications in botanical quality analysis (Soltis et al., 1992; He et al., 1995).

RAPDs are suitable for studies on anonymous genomes (Hadrys et al., 1992). RAPD requires no DNA probes, and no prior knowledge of sequence information is required for the design of specific primers. The procedure involves no blotting or hybridisation. The technique is simple, quick and efficient. RAPD require only a small amount of DNA (Williams et al., 1990).

RAPD is a Yes or No test, either that a specific sequence of code matches other samples or it does not. If it does not, then that sequences is "polymorphic" (many forms) meaning the two plants are not of the same genotype. Sets of short primers (usually 10-mers) suitable for RAPD amplification are available commercially or can easily be synthesized and, apart from a thermocycler and an agarose gel assembly, no special equipment is required (Hadrys et al., 1992).

RAPD does not require special staining procedures to visualise polymorphisms. RAPD have been reported to detect higher levels of polymorphism compared to RFLP in cases where the two have been employed together (Williams et al., 1990; Lu et al., 1996). The data derived from RAPD have their strength in being able to distinguish individuals, cultivars and accessions (Karp et al., 1996).

Limitations of RAPD are that it is a dominant marker, hence it is impossible to distinguish homozygous individuals from heterozygous individuals in the absence of a pedigree analysis. In addition, the identity of individuals in the multi-profile is not known and there can be uncertainties in assigning markers to specific loci. Single bands on the gel can sometimes consist of several co-migrating amplification products (Karp et al., 1996). RAPD techniques lack reproducibility. This is because RAPD analysis provides only a medium level of polymorphism, being less suitable to distinguish very close genomes (i.e. different clones from the same cultivars or siblings) where similar banding patterns are expected (Oliveira et al., 1999).

2.8 RAPD as a tool for identification of plant species

2.8.1 African tree species

RAPDs have been applied to assess genetic variation and relationships in African tree species. These includes the following; *Prunus africana (*Dawson and Powell 1999), *Prunus mahaleb* (Jordano and Godoy 2000), *Irvingia* species (Lowe et al., 2000), *Musa* species (Pillay et al., 2001), *Vitellaria paradoxa* (Bouvet et al., 2004), *Vitex fischeri* (Lengkeek et al., 2006), and *Manihot esculenta* (Zacarias et al., 2006).

2.8.2 Non African trees

2.8.2.1 Cultivated fruit trees

Many cultivated fruit trees have been differentiated using RAPD due to difficulty in observing morphological traits after grafting, help in identifying accessions and also in avoiding duplications or mislabeling of genotypes at gene banks. Some of the cultivated fruit trees include, *Prunus persica* cultivars (Lu et al., 1996), *Ixora* species (Rajaseger et al., 1997), *Pyrus* species (Oliveira et al., 1999), *Citrus reticulata* accessions (Coletta Filho et al., 2000), *Feijoa sellowiana* (Dettori and Palombi 2000), *Lansium domesticum* accessions (Song et al., 2000), *Lithocerasus* species (Shimada et al., 2001), *Pistacia* species (Kafkas 2002), *Vitis* species (Kim et al., 2002), *Asimina triloba* (Huang et al., 2003), *Prunus dulcis* (MirAli and Nabulsi 2003), *Fragaria* species (Zebrowska and Tyrka 2003), *Diospyros taitoensis* (Yamagishi et al., 2005).

2.8.2.2 Wild woody trees

Most wild trees vary with age and sometimes with the habitat. Therefore, RAPD has been used to differentiate species and to ascertain the taxonomic status of a population.

Wild woody tree species include the following: *Gliricidia* species (Chalmers et al., 1992), *Theobroma cocoa* clones *(*Wilde et al., 1992), *Picea abies* (Bucci and Menozzi 1995), *Populus tremuloides* (Yeh et al., 1995*), Picea* species (Perron et al., 1995), *Pinus* species (Nkongolo et al., 2002), *Avicemia* species (Parami et al., 1997), *Cedrela odorata*. (Gillies et al., 1997), *Fagus sylvatica* (Gallois et al., 1998), *Dipterocarps* species (Rath et al., 1998), *Eucalyptus globulus* (Skabo et al., 1998), *Eugenia dysenterica* (Trindade and Chaves 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Mature leaves and seeds of *C. mopane* (*sensu lato*) were randomly collected from the populations in the Musina and Phalaborwa districts. One individual of *Bauhinia galpinii* N.E. Baker, used as an outgroup, was sampled from the grounds of Polokwane Campus, University of Limpopo. A total of 30 leaf samples were collected each in the Musina and Phalaborwa districts (Figure 5). After collection, the leaf and its petiole were immediately frozen in liquid nitrogen, to maintain DNA stability. The samples were stored at –80°C in a freezer for subsequent genomic DNA isolation.

3.1.2 Reagents

Acetic acid glacial, Chloroform, Ethanol, Ether, Glycerol were obtained from Saarchem, Johannesburg, South Africa; Agarose Bromophenol blue, CTAB, βmecarptoethanol, EDTA, Ethidium Bromide, Isoamylalcohol, NaCl, Phenol, PVP, TRIS were obtained from Sigma, Johannesburg, South Africa; Liquid nitrogen was obtained from the Microscopy and Microanalysis unit, University of Limpopo; Primers were obtained from Qiagen Operon, Germany; RNaseA, PCR components ($MgCl_2$, 5 U *Taq* DNA polymerase, 1×PCR buffer and 2 mM dNTPs) were obtained from Fermentas, Pretoria, South Africa.

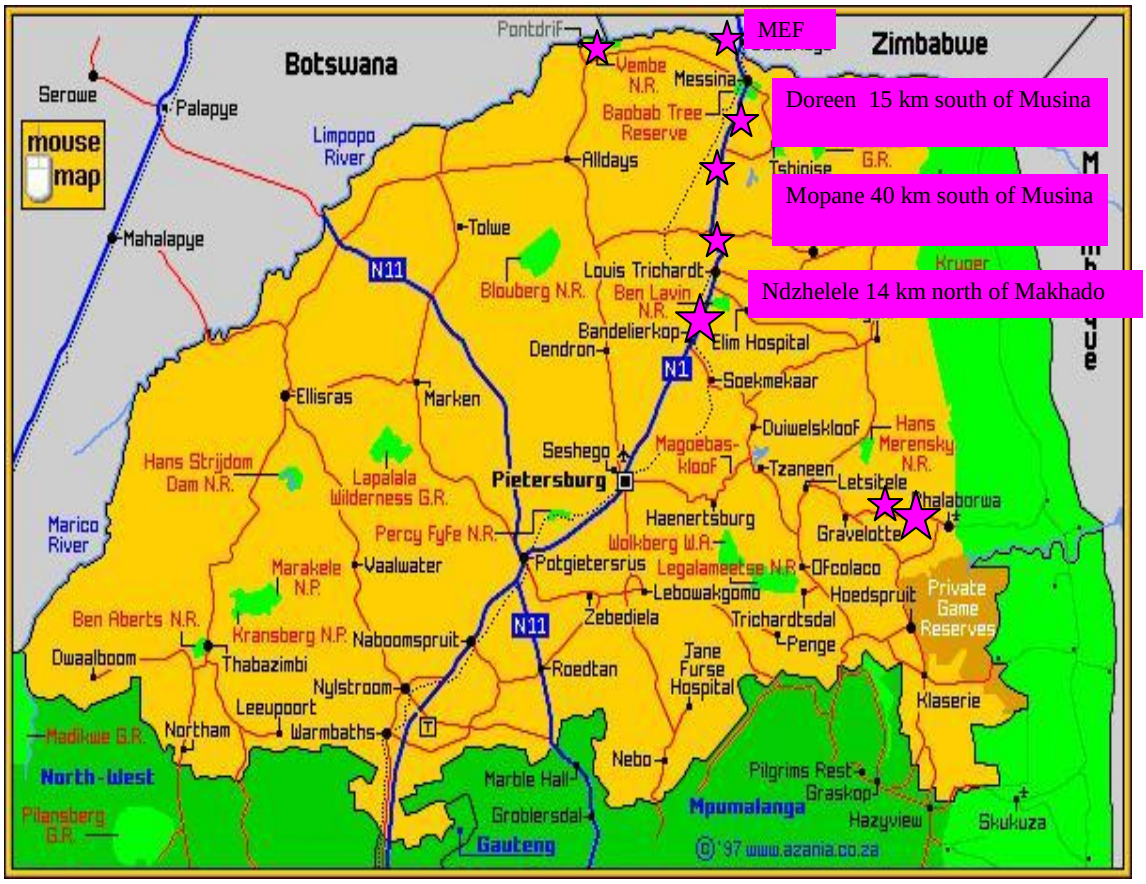


Figure 5: Localities of the samples used. Pink stars indicate collection sites.

3.2 Methods

3.2.1 Seed germination

Four replicates of seeds from each ecotype and *C. mopane* (*sensu stricto*) were germinated in pots with the soil from the collected districts (Musina and Phalaborwa) in the glasshouse of the University of Limpopo for at least six weeks. Leaflets collection was completed after six weeks when the seedlings were at the three to six leaflet stages. A total of 23 leaflets were extracted, two for Alba (Phalaborwa) and five for Alba (Musina), two for Parvispermum, three each for Laevigatum and Acuminata, and four each for Lesliei and Mopane (*sensu stricto*). The leaflets were first washed with sterile autoclaved distilled water and then frozen in liquid nitrogen.

3.2.2 DNA extraction

A modified CTAB procedure described by Doyle and Doyle (1990) was used for the extraction of DNA from the young seedlings and mature leaves of *C. mopane* (*sensu lato*). The CTAB method is relatively simple and is the method of choice for obtaining good quality total DNA from many plant species. The method is applicable to both fresh and dehydrated plant material and was tested on plants with polyphenols and polysaccharides. The procedure uses polyvinylpyrrolidone (PVP) and NaCl to remove contaminations e.g. polyphenols and polysaccharides (Sharma et al., 2002).

In this procedure, between 0.3 to 0.5 g of the leaf samples were weighed and ground into powder while in liquid nitrogen using a pestle and mortar. To the ground leaves, 5 ml of extraction buffer (100 mM Tris-HCl, 1.42 M NaCl, 20 mM EDTA and 2% CTAB, with 2% β-mecaptoethanol) was added. The resulting slurry was poured into a 50 ml

polypropylene centrifuge tube, followed by rinsing of the pestle and mortar with 1 ml of extraction buffer and adding the rinsings (wash off) to the initial extract.

To the leaf slurry 50 mg of PVP was added and then mixed by inverting the tube several times. The tubes were incubated at 60°C for 25 minutes and thereafter left to cool to room temperature. To the tubes, 6 ml of chloroform: isoamylalcohol (24:1) was added and mixed gently by inverting the tubes to form an emulsion. The mixture was centrifuged at 6000 rpm on a MISTRAL 1000 centrifuge fitted with a MSE 935 rotor for 15 minutes at room temperature. A second chloroform: isoamylalcohol extraction was performed on supernatants that were cloudy due to the presence of PVP. The supernatant was collected, and half volume of 5 M NaCl and two volumes of cold (20°C) 95% ethanol were added to precipitate DNA. The precipitated mixture was then centrifuged at 3000 rpm for three minutes followed by 5000 rpm for another three minutes to sediment the DNA pellet on the bottom of the tube. Because CTAB and NaCl sometimes precipitate with DNA, the CTAB/ NaCl residual was removed by a three-time wash with 70% ethanol followed by a short centrifugation run. The supernatant was discarded, followed by washing the pellet with 76% ethanol. The pellet was dried by leaving the tubes uncovered in a water bath at 37°C for 30 minutes.

The dried pellet was resuspended in 300 µl TE buffer (1 mM EDTA and 10 mM Tris, pH 8). In order to remove RNA and protein contamination, the solution was treated with 5 µl RNaseA (10mg/ml) and incubated at 37°C for 1hr. The RNaseA treatment was followed by addition of 300 µl phenol/chloroform/ isoamylalcohol (25:24:1) precipitation

(Cheng et al., 2003) and centrifugation for 15 minutes at 10000 rpm in a Joan microfuge centrifuge. To the supernatant, 100 µl of 5 M NaCl and 400 µl water-saturated ether were added followed by centrifugation for 10 minutes at 10000 rpm. The bottom phase was collected and two volumes of pre-chilled 95% ethanol were added followed by incubation at 4°C until the DNA strand appeared. The precipitated mixture was centrifuged at 5000rpm for three minutes and the formed pellet was washed with 70% ethanol until a colourless pellet was obtained. The pellet was dried by speed vacuum (SC110, New York) and then resuspended in 100 µl of TE buffer. The DNA was kept at -20°C until used.

3.2.3 Quantification of DNA

The determination of DNA quantity and quality was undertaken utilising two methods, spectrophotometric measurements (CARY 1E UV-visible and NanoDrop spectrophotometer, Fermentas) and agarose gel electrophoresis. Using the CARY 1E UV-visible spectrophotometer for each sample, 5 µl of DNA was pipetted and mixed with 995 µl of TE buffer in a 1cm or 1ml quartz microcuvette. The mixture was read at 260 and 280 nm against a TE buffer blank. The actual quantity of the DNA was calculated by converting optical density (OD) readings to µg/ml (reading of 1.0 at OD_{260} is equivalent to 50 µg of DNA/ml). In the NanoDrop method, 2 µl of each DNA sample was pipetted onto the cell of the NanoDrop spectrophotometer. The mixture was precalibrated at A_{260} nm: A_{280} and A_{260} nm: A_{230} nm against TE buffer blank. The DNA sample was run on 0.8% agarose gel electrophoresis to further check the quality of DNA.

3.2.4 RAPD Analyses

For RAPD amplification, 20 arbitrary nucleotide primers (Table 2) were initially screened for amplification and polymorphism, those that gave reproducible and distinct banding patterns were selected. All the primers used were random sequence primers with GC contents of 60 and 70%.

Table 2: The 20 primers screened for amplification and polymorphism.

Primer code	Nucleotides Sequence (5' to 3')	%G+C	Melting/ annealing temperature (T_m) $T_{m=}$ 4(G+C) +2(A+T)
OPA 01	CAGGCCCTTC	70	34
OPA 02	TGCCGAGCTG	70	34
OPA 03	AGTCAGCCAC	60	32
OPA 04	AATCGGGCTG	60	32
OPA 05	AGGGGTCTTG	60	32
OPA 06	GGTCCCTGAC	70	34
OPA 07	GAAACGGGTG	60	32
OPA 08	GTGACGTAGG	60	32
OPA 09	GGGTAACGCC	70	34
OPA 10	GTGATCGCAG	60	32
OPA 11	CAATCGCCGT	60	32
OPA 12	TCGGCGATAG	60	32
OPA 13	CAGCACCCAC	70	34
OPA 14	TCTGTGCTGG	60	32
OPA 15	TTCCGAACCC	60	32
OPA 16	AGCCAGCGAA	60	32
OPA 17	GACCGCTTGT	60	32
OPA 18	AGGTGACCGT	60	32
OPA 19	CAAACGTCGG	60	32
OPA 20	GTTGCGATCC	60	32

RAPD analyses were carried out with varying concentrations of $MgCl_2$ (1.5 mM, 2 mM and 4 mM), *Taq* DNA polymerase (1 U, 1.25 U and 2 U) and DNA template (1 µl, 2 µl, 4 µl and 5 µl) to optimise PCR conditions. Different brands of *Taq* were used. These

included Ex *Taq*, Takara; Ampli gold *Taq*, Applied Biosystems; and Recombinant *Taq* polymerase, Fermentas. In the optimised PCR, RAPD reactions were performed in a total volume of 25 µl in sterile 0.2 ml eppendorf tubes containing 10×PCR buffer (10 mM Tris-HCl, pH 8.0), 2 mM dNTPs, 5 U *Taq* polymerase, (Fermentas), 0.5 µM primers and 2 µl of template DNA. For the initial primer screening, a reaction mixture contained 1× buffer, 1.5 mM, 0.2 mM dNTPs, 0.5 µM primer, 2 U *Taq* and DNA template (Table 3). The negative control mixture (reaction containing all reagents except DNA template whereby the DNA is replaced by sterile water) was included to check for contamination. To reduce the PCR artifact or possibility of cross contamination and pipetting errors for each primer, a reaction master mixture (Table 3) of all the reagents except the genomic DNA was always used and then aliquoted. The manipulation of RAPD reaction was always done on ice.

Table 3: Composition of the PCR reaction mixture (25 µl).

Components	**Stock concentration**	**Final concentration**	**Volume per reaction**
PCR buffer	10×	1×	2.5
$MgCl_2$	25 mM	4 mM	4
***Taq* polymerase**	5 U/ µl	2 U	0.4
dNTPs	2 mM	0.2 mM	2.5
Primer	20 µM	0.5 µM	1
Water			12.6
DNA		50-200 ng	2

The best concentrations of $MgCl_2$ for further analyses were selected based on the number, sharpness and the intensity of bands. Different annealing temperatures (30°C, 37°C, 40°C and 50°C) and alternations of the program were made until both amplification and polymorphism were obtained. The following program was used on a Gene Ampli PCR systems 9700 thermocycler (Applied biosystems, USA): An initial predenaturation step at 94°C for 120 sec, followed by 5 cycles at 94°C for 25 sec, 40°C for 30 sec and 72°C for 90 sec, this was followed by 30 cycles denaturation (94°C for 15 sec), annealing (40°C for 30 sec) and extension (72°C for 80 sec) with final extension (72°C for 420 sec). After completion of the amplification cycles, the reactions were held at 4°C until the products were used. The reaction was repeated at least once to ensure reproducible banding patterns.

3.2.5 Agarose gel electrophoresis

The first step in agarose gel electrophoresis was to combine 12.5 µl of amplified DNA sample with 3 µl of loading buffer (30% glycerol and 0.25% bromophenol blue). The amplification products were separated on 2% (w/v) agarose gel submerged in 1×TAE buffer (100 mM Tris- Acetic Acid and 0.5 M EDTA, pH 8). Ethidium bromide (10mg/ml) was added to the gel for visualisation of DNA bands. A 1kb mass ruler DNA ladder was used to estimate the size of RAPD band. Electrophoresis was conducted at a voltage of 77V for two hours. A photograph of the gel was taken with a Polaroid camera under Ultraviolet (UV) light on a UV transilluminator.

3.2.6 Data analysis

The polymorphism was analysed using RAPD bands. The reproducible, polymorphic and monomorphic bands were visually scored as present (1) or absent (0). Faint reproducible RAPD bands were also scored. The absence and presence of bands were entered in a computer file as a binary matrix. The resulting binary matrix was analysed using the parsimony optimality criterion of the software package PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b10 for Macintosh computer (Swofford 2002). Genotypic relationships based on RAPD data, with respect to *Bauhinia galpinii* selected as outgroup were analysed using both distance and parsimony methods. The database included all RAPD bands obtained. Individual data obtained from the three selected primers and combined data of primers were analysed, to obtain a dendogram that depicts the likely relationships within *C. mopane* (*sensu lato*). The matrix had 24 taxa and 31 characters, formatted as a NEXUS file with RAPD bands represented as 1's and 0's. Question marks were entered for missing data.

Parsimony settings were as follows: the trees were unrooted at the midpoint. All characters were weighted equally, and reversal and convergences were considered equally probable transformations. Character coding was treated as unordered to give rate to any possible transformation between two states (Fitch parsimony, Fitch 1971). MULPARS (Multiple parsimony) and steepest descent options were not in effect. The branches having a maximum length of zero were collapsed to yield polytomies. A topological constraint was not enforced. Branch lengths for trees were calculated using the ACCTRAN (accelerated transformation) character-state optimization, stepmatrix

option allowed assignment of states not observed in terminal taxa to internal nodes (all states in stepmatrix). Because of the large number of taxa used in the parsimony analysis, exact-solution (exhaustive or branch-and-bound) searches were impossible (Schuh 2000). Therefore, parsimony analysis was performed using heuristic search.

3.2.6.1 Construction of parsimonious trees using heuristic search

In order to get the most parsimonious trees, heuristic search was performed because the taxa were beyond 18. The results from this search can often be improved without an increase in time. The data set were subjected to heuristic search with simple sequence additions (reference taxon: *Bauhinia galpinii*) increased automatically by 100 and tree bisection-reconnection (TBR) branch swapping. All multiple equally parsimonious trees were saved to a tree file. The tree length was used to determine the most parsimonious trees. Taxa contained in the parsimony analysis were compared using strict and 50% majority rule consensus analyses (Margush and McMorris 1981).

3.2.6.2 Bootstrapping analysis using Parsimony trees

To test the reliability of certain branches in the evolutionary tree, bootstrapping was performed. The bootstrap proceeds by re-sampling the original data, with replacement, to create a series of bootstrap samples of the same size as the original data. The method used maximum-parsimony as the optimality criterion with 100 replicates. Trees found in parsimony analysis of both primer 03 and combined primers were then used as starting trees for bootstrapping analysis. MAXTREES (maximum trees) were set to 60100 for each replicate, increased automatically by 100 and saving the best trees per replicate.

The tree-bisection-reconnection (TBR) as branch-swapping algorithm was used to test the support of individual clades, starting trees obtained via stepwise addition (a process whereby taxa are added to a developing tree), and one tree held at each step during stepwise addition. To minimize the time spent searching large numbers of trees, a random addition sequence with one replicate was applied for each bootstrapping iterion. This effectively randomized the seed for each bootstrap replicate. The respective dendograms shown in the results section derived from the enforcement of the 50% majority-rule consensus and only those groups of more than 50% frequency were reported. The bootstrap value of a node is the percentage of times that a node is present in the set of trees that is constructed from the new data sets.

3.2.6.3 Distance method

The relationship among individuals was determined using a distance matrix method. In addition, total mean character differences between all pair-wise combinations of the *C. mopane* (*sensu lato*) were calculated using PAUP. Such values (total mean character differences) represents the proportion of different bands between all the possible pairs of the ecotypes. The values were then converted into percentage by taking the total mean value divided by the total number of samples times 100%. Neighbour Joining analyses (Saitou and Nei 1987) were computed from total character mean values using a heuristic search run in PAUP with the settings ADDSEQ (additional sequence) = random, NREPS (Number of replicates) = 1000, and TBR branch swapping.

CHAPTER 4

RESULTS

4.1 DNA yield and quality

The DNA isolated using the CTAB method from the young seedling and mature leaves of *C. mopane* (*sensu lato*) was found to be contaminated with polysaccharides and other contaminants as shown by the spectrophotometer readings which gave A_{260} nm/A_{280} nm ratio of 0.35 to 1.35. These values indicate the levels of contaminants. The DNA sample was also viscous, with some samples concentrating on the wells and not moving into the gel, this being an indication of the presence of polysaccharides (Fig. 6 A). The quantity of DNA obtained from seedlings was generally higher compared to that of mature leaves. Poor and inconsistent PCR amplifications were obtained from DNA extracted using only the CTAB method.

To remove the polysaccharides observed during CTAB extraction, phenol precipitation was introduced as an additional extraction step. This improved the quality of DNA and removed impurities that interfered with the PCR. The DNA obtained was pure white and easily solubilised in TE buffer. Unlike the DNA isolated using the CTAB method, the DNA following electrophoresis migrated into the gel (Fig. 6 B). This DNA was quantified using the NanoDrop spectrophotometer (Fig. 7 and Table 4). The quantity of DNA obtained using phenol precipitation varied from 140.8 ng to 1964 ng per 1 µl. The A_{260} nm/A_{280} nm ratio ranged from 1.56 to 2.58. The ratio of OD_{260} to OD_{280} and OD_{260} to OD_{230} provides some information about the purity of the DNA sample as well as

amount of contamination by proteins and carbohydrates. Pure DNA preparation shows an OD_{260} to OD_{280} ratio of between 1.8 and 2.0. The ratio below 1.8 shows contamination with protein. In a few samples with extremely low contents of DNA, the absorbance ratio was lower than 1.8. These samples were not used for RAPD analyses. The DNA with higher quantities and qualities was used to perform all RAPD analysis, resulting in consistent results.

Figure 6 A: Agarose gel electrophoresis gel (0.8%) of the total DNA prepared from *Colophospermum mopane (sensu lato)* using a CTAB method. For each sample 10 µl of DNA was loaded in each well. Lanes: - 1: Mopane 1; 2: Laevigatum1; 3: Lesliei hek 2; 4: Alba 1 Phala; 5: Parvispermum 1; 6: Alba 5; 7: Lesliei 3; 8: Alba 7 MEF; 9: Alba 6; 10: Parvispermum 2; 11: Alba 11 Phala; 12: Acuminata 2.

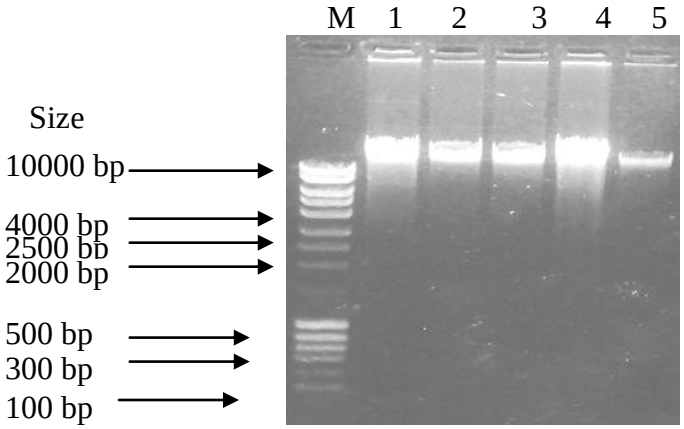


Figure 6 B: Agarose gel electrophoresis gel (0.8%) of the total DNA prepared from *Colophospermum mopane (sensu lato)* using the modified CTAB method. For each sample 10 µl of DNA was loaded in each well. Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Alba 6; 2: Laevigatum 1; 3: Alba 1 Phala; 4: Alba 7; 5: Parvispermum 2.

Table 4: Quantity and quality of selected DNA samples. The measured parameters were obtained using NanoDrop spectrophotometric determination (NanoDrop, technologies Inc).

Sample ID	ng/ µl	A_{260}	$A_{260}/_{280}$	$A_{260}/_{230}$
	(According to the Nanodrop precalibration)			
Alba 6	1269	25.3	2.0	2.6
Acum 2	140	2.8	1.56	0.7
Alba 1	1964	39.2	2.0	2.0
Alba 5	987	19.8	1.9	1.9
Alba 1 Phala	1256	25.1	2.0	1.7
Laevi 1	1501	30.0	2.0	1.7
Alba 7	1213	24.2	2.0	1.3
Parvi 2	156	3.1	1.7	0.8

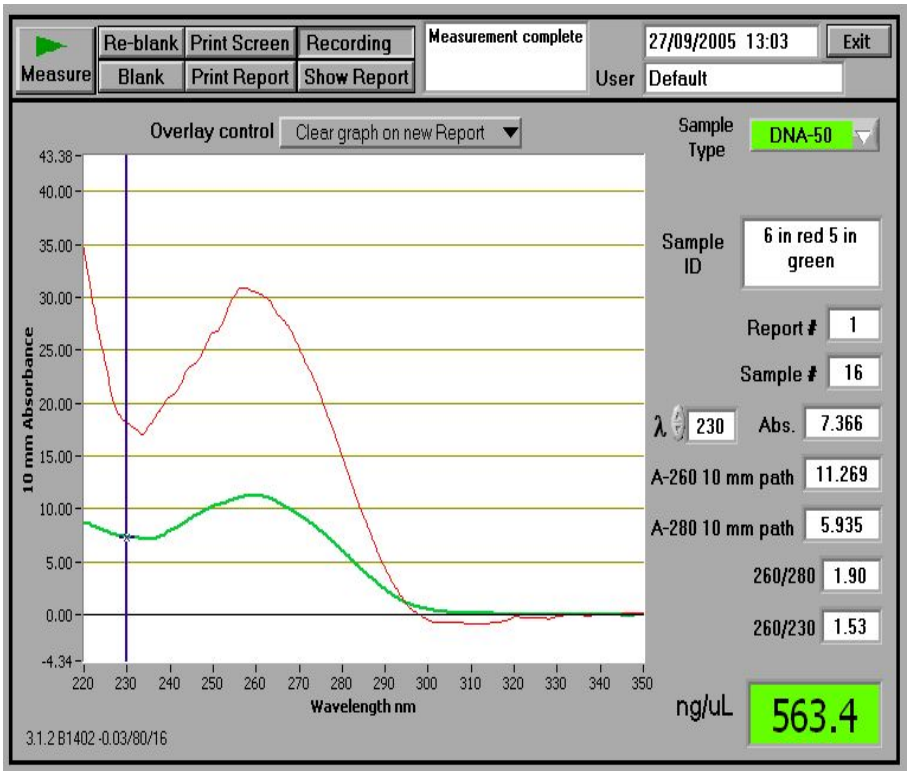


Figure 7: Characteristic UV spectrum of the purified genomic DNA samples of *Colophospermum mopane (sensu lato)* obtained using the NanoDrop DNA quantification (see Table 5). Sample with high DNA concentration-(red line) and low DNA concentration-(green line)

4.2 RAPD analyses

4.2.1 Optimisation of RAPD conditions for amplification

A total of 20 random primers were initially screened for amplification of DNA from *C. mopane (sensu lato)*. The effect of $MgCl_2$, type and amount of *Taq* polymerase, and annealing temperature were examined. Amplifications were obtained using 2 U of recombinant *Taq* DNA polymerase (Fermentas brand). The nucleotide primers amplified at 40°C annealing temperature. Of the 20 primers screened, only nine primers (OPA 01, OPA 02, OPA 03, OPA 08, OPA 09, OPA 10, OPA 13, OPA 18 and OPA 20) produced RAPD amplifications (Figs 8 and 9). The best $MgCl_2$ concentration with OPA 13 was 2 mM, with higher $MgCl_2$ concentrations resulting in several bands that were not easily scorable. For the remaining primers 4 mM $MgCl_2$ was found to be most suitable. The intensity of the fragments produced by OPA 03 (Fig. 8 D) under optimised PCR conditions was low. The primer concentration for this primer was increased from 10 μM to 20 μM with a resultant increase in band intensity (Fig. 10).

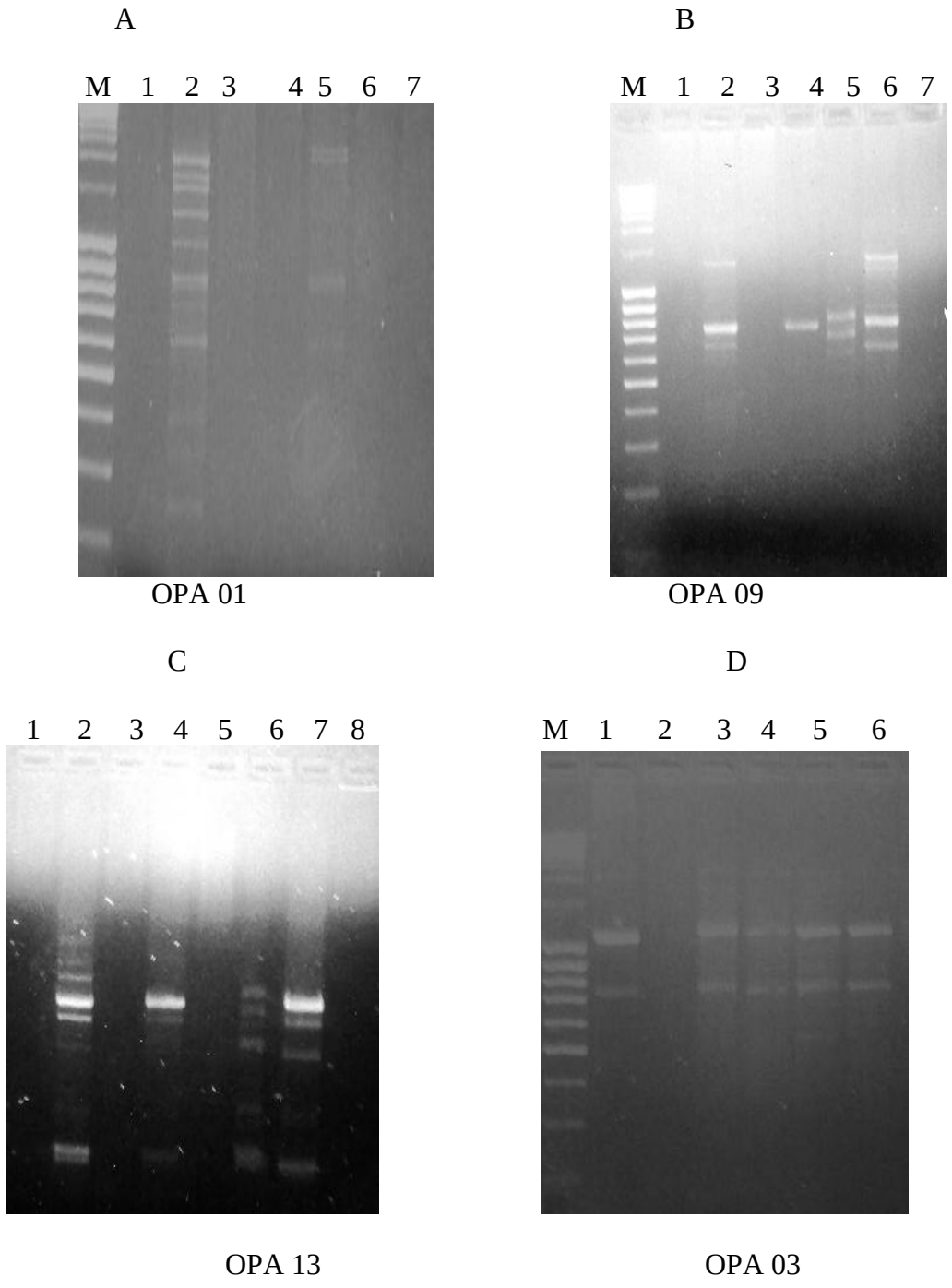
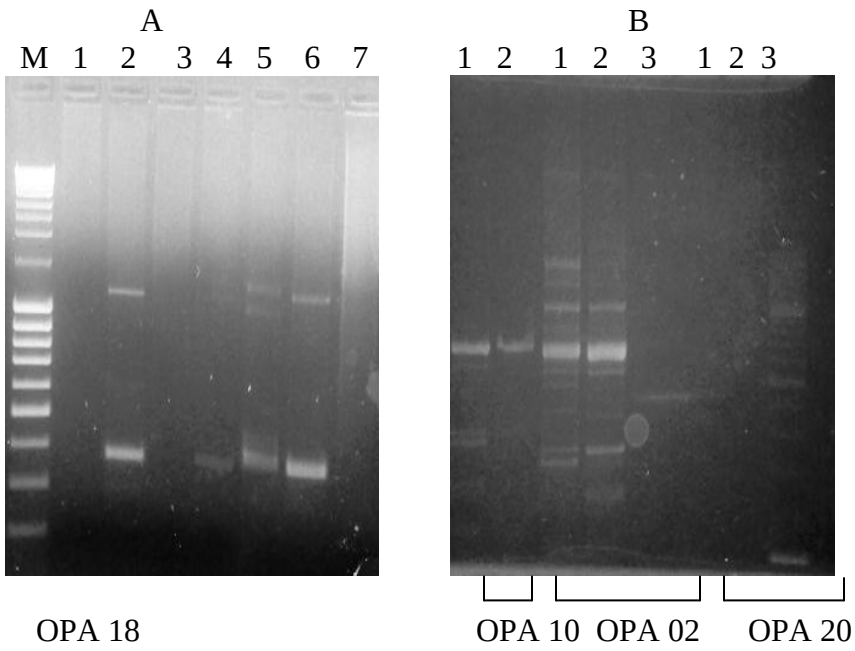


Figure 8: RAPD profile generated using four selected primers on *C. mopane* (s*ensu lato*) DNA. Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Mopane 4; 2: Laevigatum 1; 3: Mopane 2; 4: Parvispermum 1; 5: Lesliei 3; 6: Alba 6; 7: Parvispermum 2; 8: Acuminata 2.



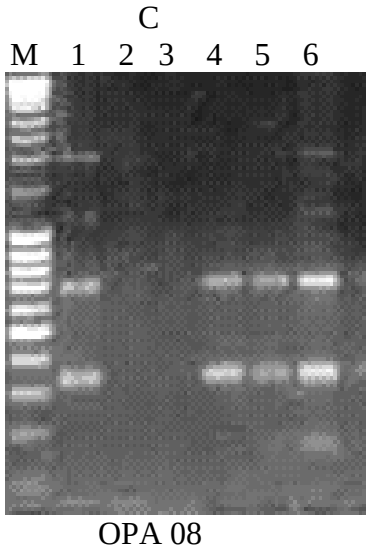


Figure 9: RAPD profile generated using five selected primers on *C. mopane* (*sensu lato*) DNA. Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Mopane 4; 2: Laevigatum 1; 3: Mopane 2; 4: Parvispermum 1; 5: Lesliei 3; 6: Alba 6; 7: Parvispermum 2; 8: Acuminata 2.

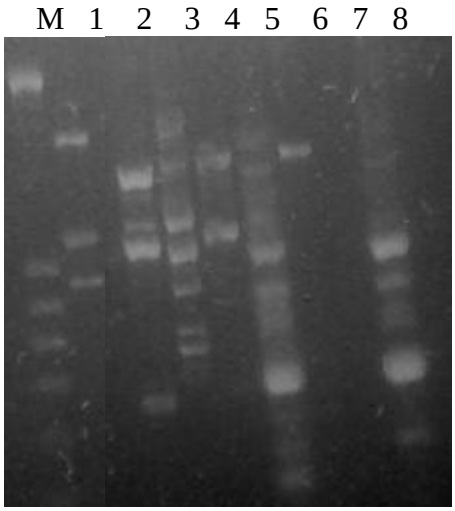


Figure 10: RAPD profile generated using OPA 03 with 20 μM primer concentrations on *C. mopane* (s*ensu lato*) DNA. Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Mopane 4; 2: Laevigatum 1; 3: Mopane 2; 4: Parvispermum 1; 5: Lesliei 3; 6: Alba 6; 7: Parvispermum 2; 8: Acuminata 2.

4.2.2 Optimisation of RAPD primers for polymorphism

Further screening of the primers for polymorphisms was performed using the primers that amplified most DNA samples. Six primers (OPA 02, OPA 03, OPA 08, OPA 10, OPA 13 and OPA 18) were chosen. The primers that showed differentiation were used for RAPD analyses with all the DNA samples (Figs 11-16).

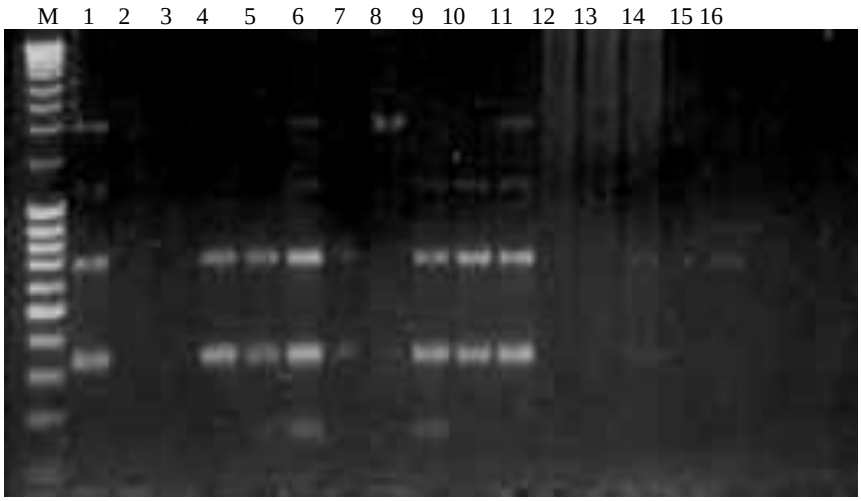


Figure 11: RAPD profile generated using OPA 08 tested during analysis for polymorphism: Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Lesliei 4; 2: Lesliei hek 2; 3: Lesliei 3; 4: Parvispermum 1; 5: Parvispermum 3; 6: Laevigatum 2; 7: Laevigatum 4; 8: *Bauhinia*; 9: Mopane 4; 10: Mopane 3; 11: Mopane 2; 12: Acuminata 2; 13: Acuminata 3; 14: Alba 2; 15: Alba 1 Phala; 16: Alba 6.

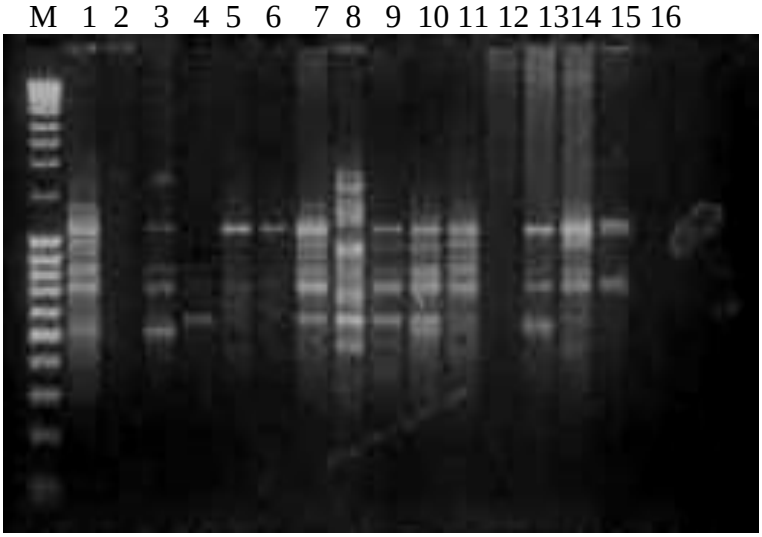


Figure 12: RAPD profile generated using OPA 03 tested during analysis for polymorphism: Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Lesliei 4; 2: Lesliei hek 2; 3: Lesliei 3; 4: Parvispermum 1; 5: Parvispermum 3; 6: Laevigatum 2; 7: Laevigatum 4; 8: *Bauhinia*; 9: Mopane 4; 10: Mopane 3; 11: Mopane 2; 12: Acuminata 2; 13: Acuminata 3; 14: Alba 2; 15: Alba 1 Phala; 16: Alba 6.

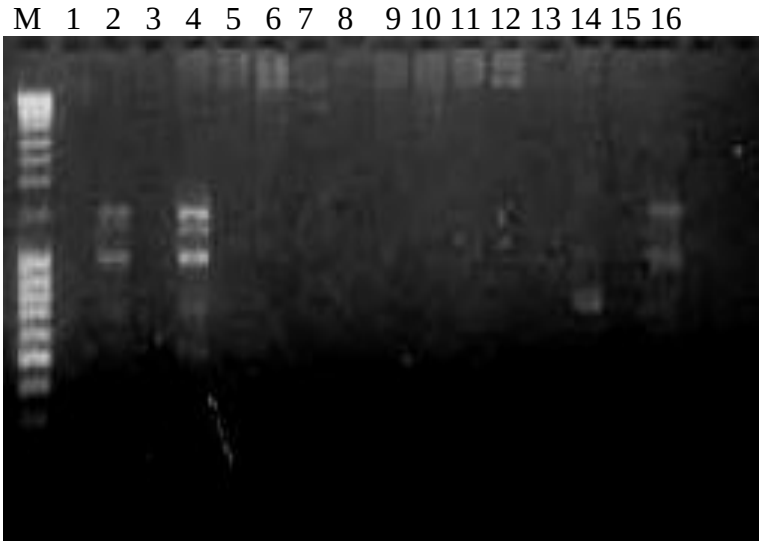


Figure 13: RAPD profile generated using OPA 18 tested during analysis for polymorphism: Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Leslie 4; 2: Lesliei hek 2; 3: Lesliei 3; 4: Parvispermum 1; 5: Parvispermum 3; 6: Laevigatum 2; 7: Laevigatum 4; 8: *Bauhinia*; 9: Mopane 4; 10: Mopane 3; 11: Mopane 2; 12: Acuminata 2; 13: Acuminata 3; 14: Alba 2; 15: Alba 1 Phala; 16: Alba 6.

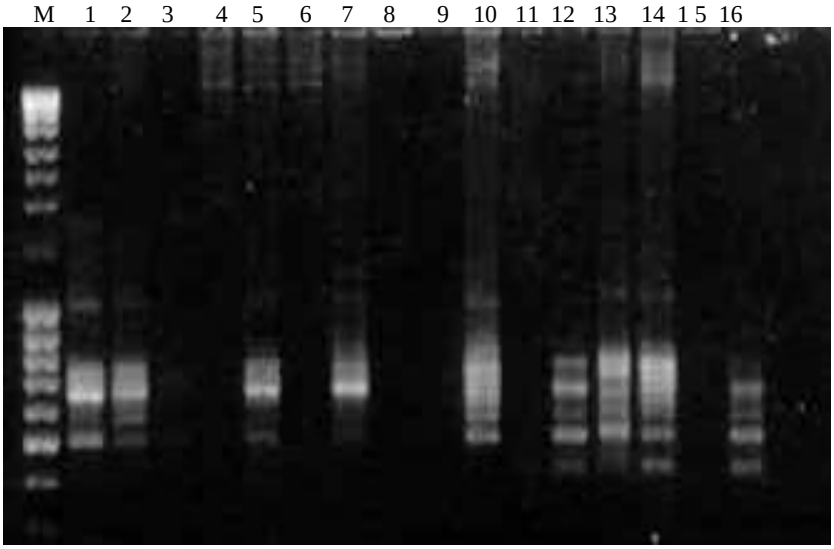


Figure 14: RAPD profile generated using OPA 09 tested during analysis for polymorphism: Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Lesliei 4; 2: Lesliei hek 2; 3: Lesliei 3; 4: Parvispermum 1; 5: Parvispermum 3; 6: Laevigatum 2; 7: Laevigatum 4; 8: *Bauhinia*; 9: Mopane 4; 10: Mopane 3; 11: Mopane 2; 12: Acuminata 2; 13: Acuminata 3; 14: Alba 2; 15: Alba 1 Phala; 16: Alba 6.

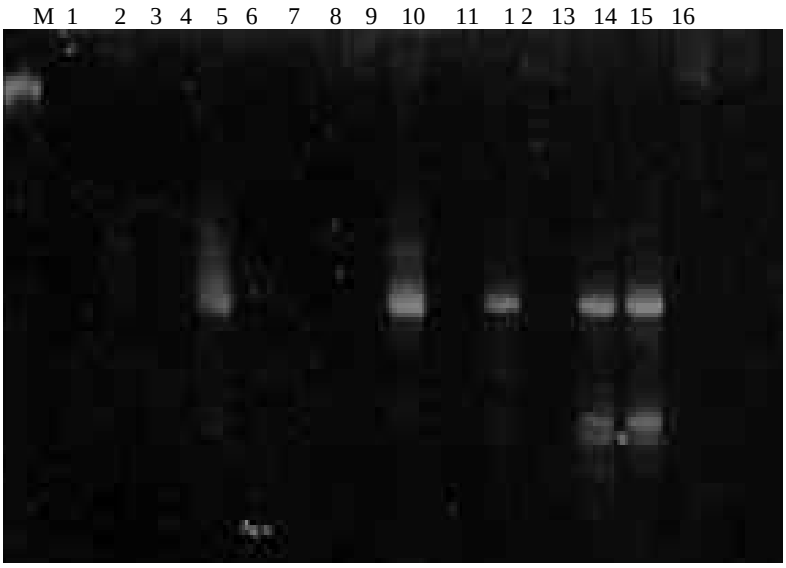


Figure 15: RAPD profile generated from OPA 02 tested during analysis for polymorphism: Lanes: - M: (1 kb Mass ruler DNA ladder mix); 1: Lesliei 4; 2: Lesliei hek 2; 3: Lesliei 3; 4: Parvispermum 1; 5: Parvispermum 3; 6: Laevigatum 2; 7: Laevigatum 4; 8: *Bauhinia*; 9: Mopane 4; 10: Mopane 3; 11: Mopane 2; 12: Acuminata 2; 13: Acuminata 3; 14: Alba 2; 15: Alba 1 Phala; 16: Alba 6.

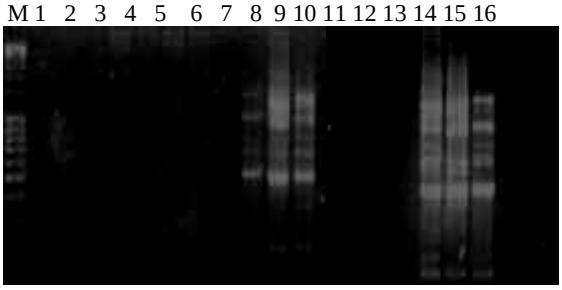


Figure 16: RAPD profile generated from OPA 13 tested during analysis for polymorphism: Lanes:- M: (1 kb Mass ruler DNA ladder mix); 1: Lesliei 4; 2: Lesliei hek 2; 3: Lesliei 3; 4: Parvispermum 1; 5: Parvispermum 3; 6: Laevigatum 2; 7: Laevigatum 4; 8: *Bauhinia*; 9: Mopane 4; 10: Mopane 3; 11: Mopane 2; 12: Acuminata 2; 13: Acuminata 3; 14: Alba 2; 15: Alba 1 Phala; 16: Alba 6.

4.2.3 RAPD analysis of *Colophospermum mopane (sensu lato)*

Based on results for polymorphism, three primers (OPA 03, OPA 08 and OPA 09) were chosen for RAPD analysis of *C. mopane* (*sensu lato*) (Figs 11-16). The replication reactions showed reproducible banding patterns for these primers. The three polymorphic primers produced DNA band ranged from 180 bp to 2000 bp (Figs 17-19). The amplified fragments generated from the each amplified primer varied between seven (OPA 09) and thirteen (OPA 03). OPA 03 amplified most of the samples tested whereas OPA 08 and OPA 09 amplified 50% of samples.

Mopane 2 had a fragment of 1300 bp and 1500 bp when using primer 09. The fragments of 500 and 600 bp were the most prevalent in all taxa except Lesliei hek 2 and *Bauhinia* (Fig. 17). Primer 08 produced monomorphic bands of 600 bp and 280 bp in addition to polymorphic bands (Fig. 18). Fragments of 1500 and 1300 bp differentiate Lesliei 4 from the other ecotypes and Mopane. Mopane 3 was the only taxon which had a 180 bp band. The 600 bp band was amplified in Laevigatum 4 (Fig. 18).

RAPD profile obtained from OPA 03 yielded several common bands and a few polymorphic bands. The fragment of 700 bp was produced in all samples except *Bauhinia* and Acuminata 4. *Bauhinia* was the only sample that produced 1500 and 690 bp fragments (Fig. 19). The 2000 bp fragments were present in *Bauhinia* and Lesliei 4 which clearly separates them from the other ecotypes and Mopane (Fig. 19).

Accordingly, all samples isolated from *C. mopane* (*sensu lato*) yielded several common RAPD bands, and when the unique bands for each primer were considered, it was observed that there were two distinct bands, namely 180 bp (OPA 08); 1900 (OPA 03). Although the primers produced some polymorphic bands, there was not a single primer that could clearly distinguish *C. mopane* (*sensu lato*). RAPD banding pattern when using all the primers on *C. mopane* (*sensu lato*) is similar for most samples (Figs 17-19).

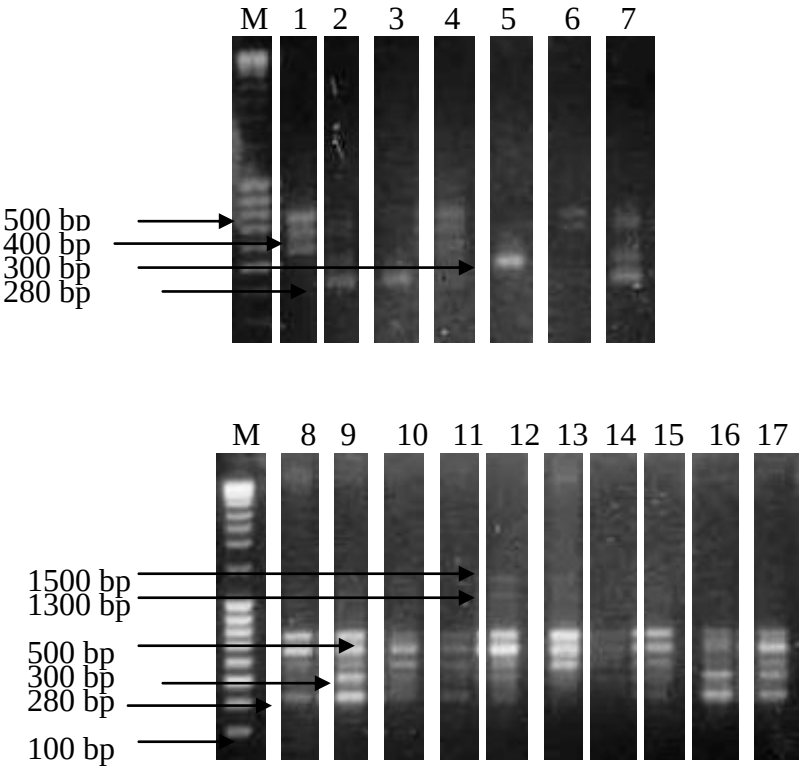


Figure 17: RAPD profiles generated from *C. mopane* (s*ensu lato*) using OPA 09. The amplification products were separated on 2% Agarose gel, stained with ethidium bromide and photographed under UV light using Polaroid camera: Lanes: - M: Molecular marker; 1: Lesliei 4; 2: Lesliei 3; 3: Lesliei hek 2; 4: Parvispermum 3; 5: *Bauhinia*; 6: Acuminata 4; 7: Laevigatum 5; 8: Laevigatum 4; 9: Mopane 3; 10: Mopane 1; 11: Mopane 4; 12: Mopane 2; 13: Alba 6; 14: Alba 1 Phala; 15: Alba 2; 16: Alba 7; 17: Alba 2 Phala.

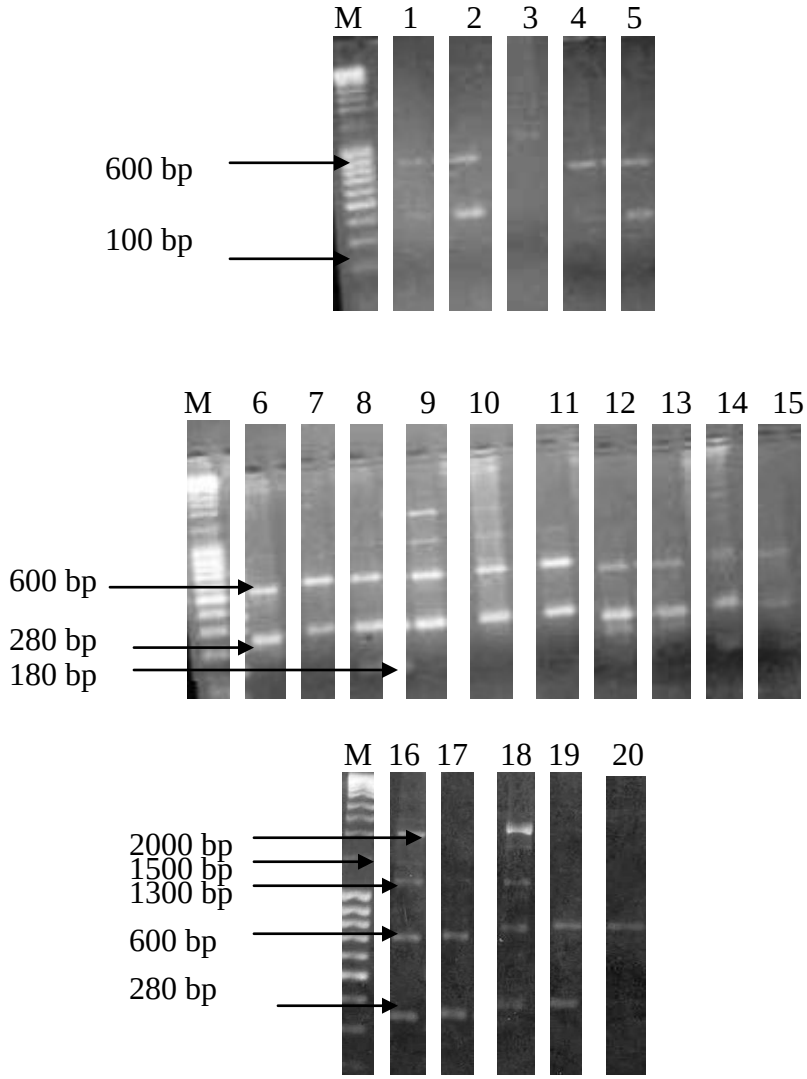


Figure 18: RAPD profiles generated from *C. mopane* (*sensu lato)* using OPA 08. Lanes: - M: Molecular marker; 1: Lesliei hek 2; 2: Parvispermum 1; 3: Acuminata 4; 4: Acuminata 3; 5: Laevigatum 5; 6: Laevigatum 2; 7: Mopane 1; 8: Mopane 3; 9: Mopane 2; 10: Alba 6; 11: Alba 2; 12: Alba 7; 13: Alba 2 Phala; 14: Alba 4; 15: Alba; 16: Lesliei 4; 17: Lesliei 3; 18: *Bauhinia*; 19: Laevigatum 4; 20: Mopane 3.

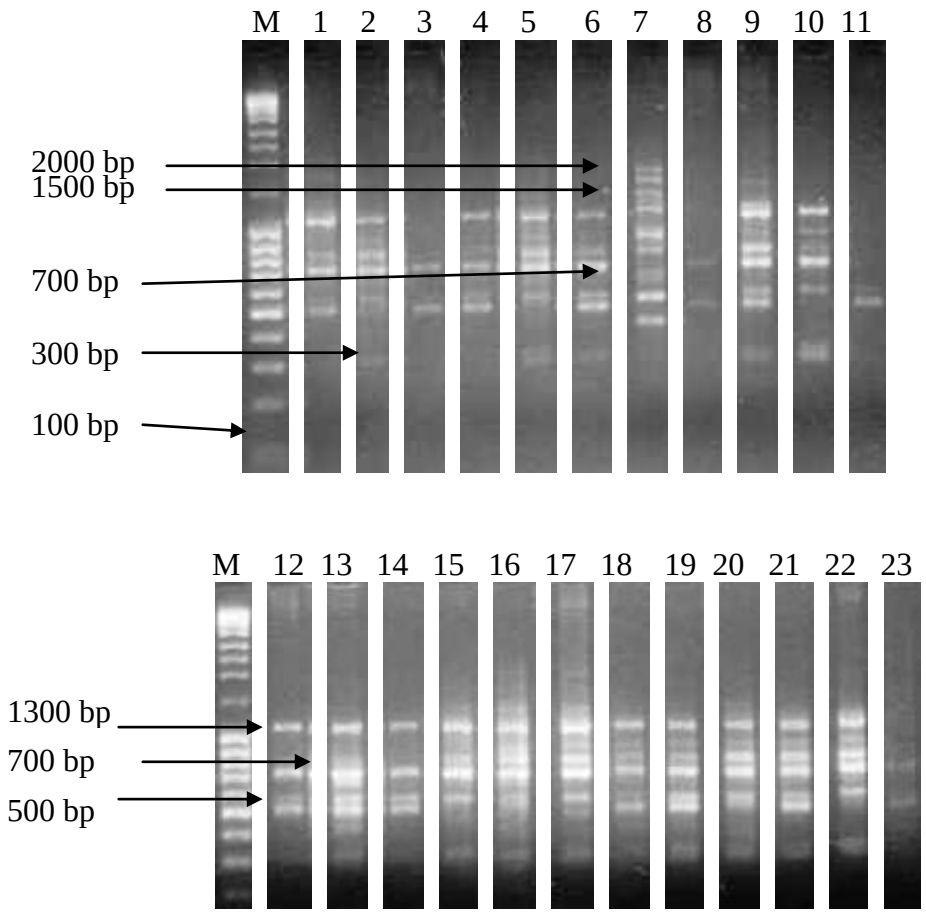


Figure 19: RAPD profiles generated from *C. mopane* (*sensu lato*) using OPA 03. The amplification products were separated on 2% Agarose gel, stained with ethidium bromide and photographed under UV light using Polaroid film: Lanes:- M: Molecular marker, 1: Lesliei 4; 2: Lesliei 3; 3: Lesliei turn 2; 4: Lesliei hek 2; 5: Parvispermum 3; 6: Parvispermum 1; 7: *Bauhinia*; 8: Acuminata 1; 9: Acuminata 4; 10: Acuminata 3; 11: Laevigatum 5; 12: Laevigatum 4; 13: Mopane 3; 14: Mopane 4; 15: Mopane 3; 16: Mopane 2; 17: Alba 6; 18: Alba 1 Phala; 19: Alba 2; 20: Alba 7; 21: Alba 2 Phala; 22: Alba 4; 23: Alba 1.

4.3 Phylogenetic analysis

4.3.1 Parsimony analysis with heuristic search

Parsimony analysis with 24 parsimony informative characters constructed the shortest tree by simple stepwise addition of taxa and character. A dendogram generated from RAPD data for most parsimonious tree of each primer and combination of the primers is shown in Figure. 20. PAUP yielded 60100 most parsimonious trees for combined data. Each had a tree length of 59, a consistency index (CI) of 0.508, retention index (RI) of 0.580 and a homoplasy index of 0.492. The RI values indicate that the RAPD characters are highly congruent, that the homoplasy is low and support the use of RAPD. Most of the homoplasy is due to variable characters.

In the combined data (Fig. 20 A) the dendogram does not reveal a high separation of *C. mopane* (*sensu lato*). The dendogram shows Lesliei 4 as a sister group to the remaining taxa (100%). Mopane 2 and Alba 6 (70%) were unresolved but can be seen as a sister group to a monophyletic group which divided into two clades. The data provided form the monophyletic grouping, which is poorly resolved, and others resulted in the position of basal polytomy. In Clade I (54%), Mopane 1 is the sister group of Lesliei turn 2, Acuminata 2, Laevigatum 5 and Alba 1. The polytomy has 56%, with Alba 1 as a sister group to a polytomy grouping (100%). Clade II (60%) forms a small clade (group) consisting of Alba 1 Phala, Alba 7 and Alba 2 Phala, with Alba 1 Phala as a sister group.

Parsimony with OPA 03 (Fig. 20 B) yielded 76 most parsimonious trees, each had a tree length of 23, and a consistency index (excluding uninformative characters) of 0.152.

Figure 20 B shows 63% support of Lesliei 4 as a sister group to a monophyletic group containing 2 clades with the rest unresolved taxa. Clade I (100%) is a sister group to clade II. Mopane I (100%) in Clade II is a sister group to polytomy grouping which consists of Parvispermum1, Laevigatum 4 and Mopane 3.

OPA 08 and OPA 09 parsimony analyses show the same dendogram with 22 trees. Each tree had a tree length of 17 and a CI of 0.235. The dendogram for *C. mopane* (*sensu lato*), resulted in an extreme poorly resolved tree with Lesliei 4 (100%) as a sister group to a monophyletic group with one small clade of Alba 1 Phala and Alba 7, the rest are unresolved taxa resulting in polytomy (Figs 20 C and D). These unresolved trees of OPA 08 and 09 are also supported by RAPD banding which shows a similar RAPD profile.

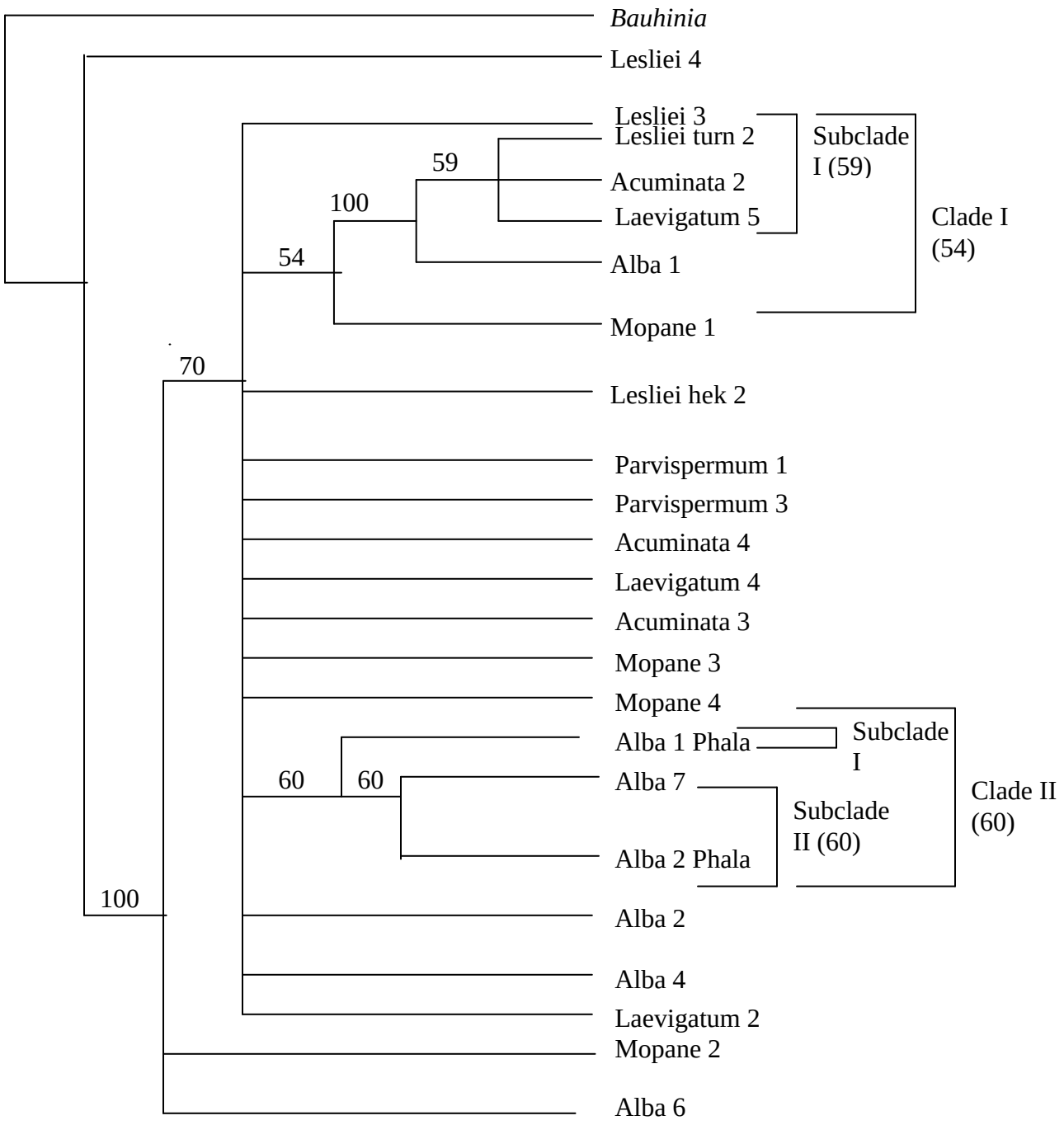


Figure 20 A: Dendogram obtained from RAPD data obtained for *C. mopane (sensu lato)* by parsimony analysis of combined primers.

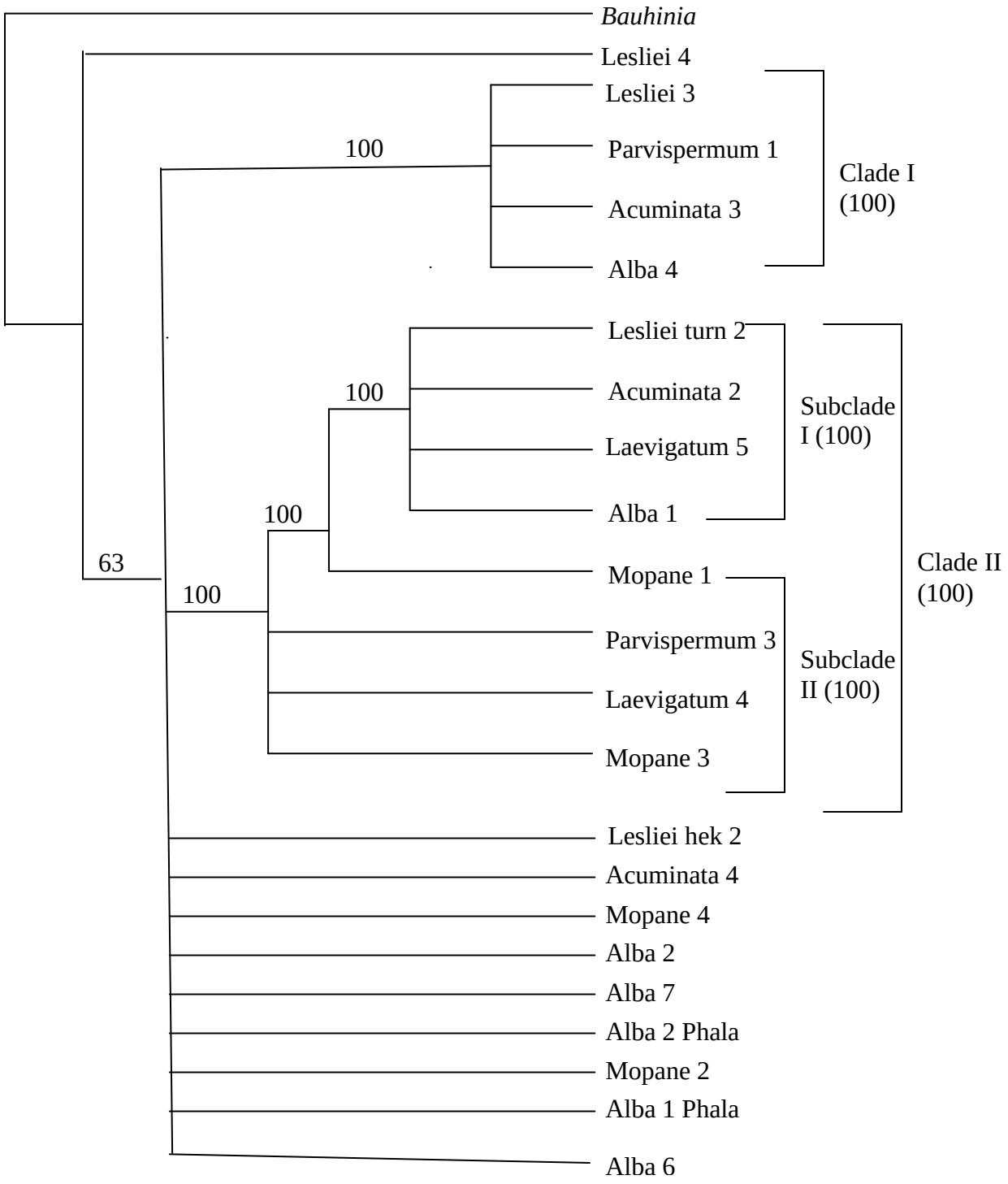


Figure 20 B: Dendogram obtained from RAPD data for *C. mopane (sensu lato)* by parsimony analysis of primer 03.

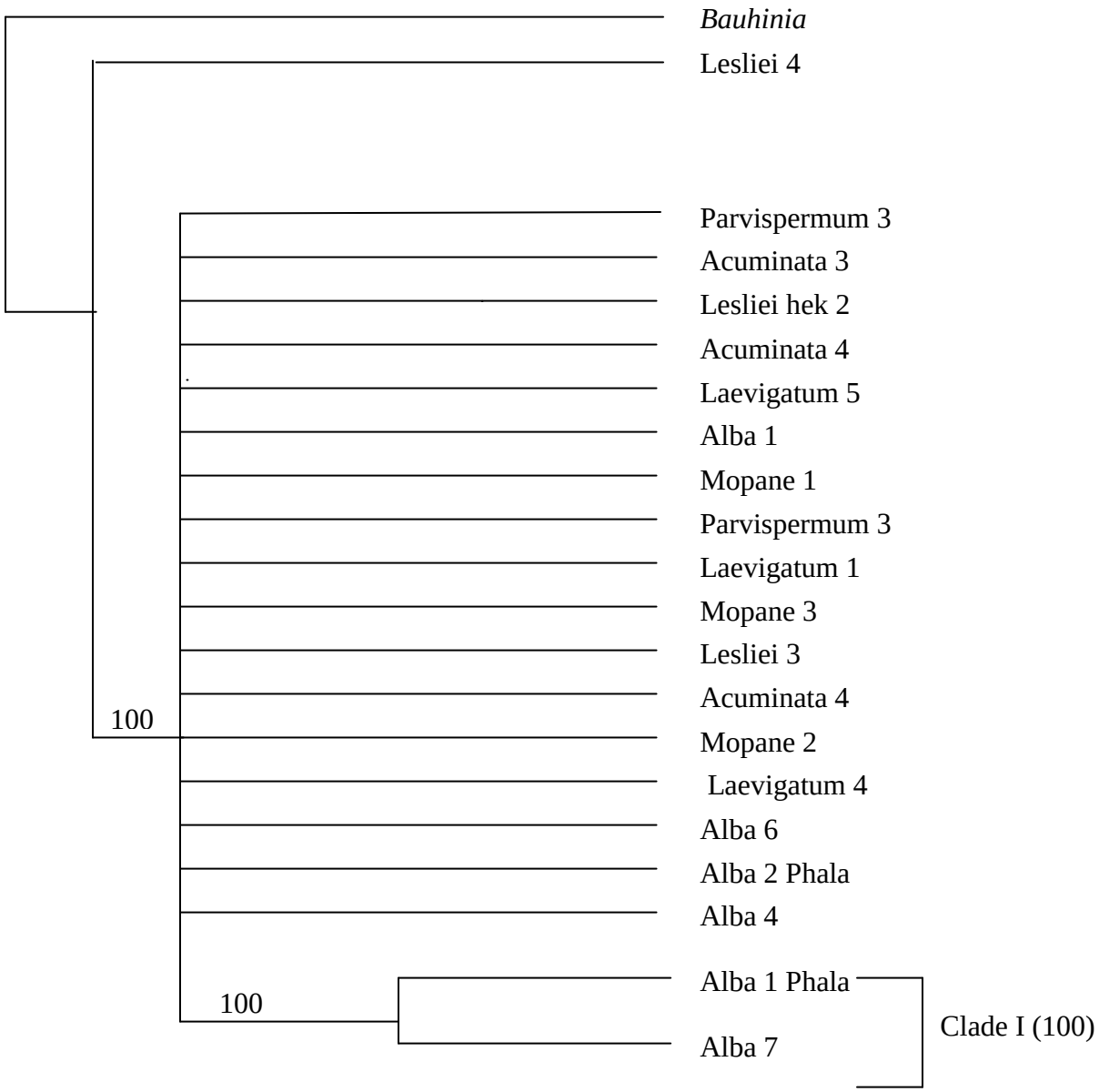


Figure 20 C: Dendogram obtained from RAPD data obtained for *C. mopane (sensu lato)* by parsimony analysis of primer 09. Numbers indicate the percentage values of a 50% majority consensus tree.

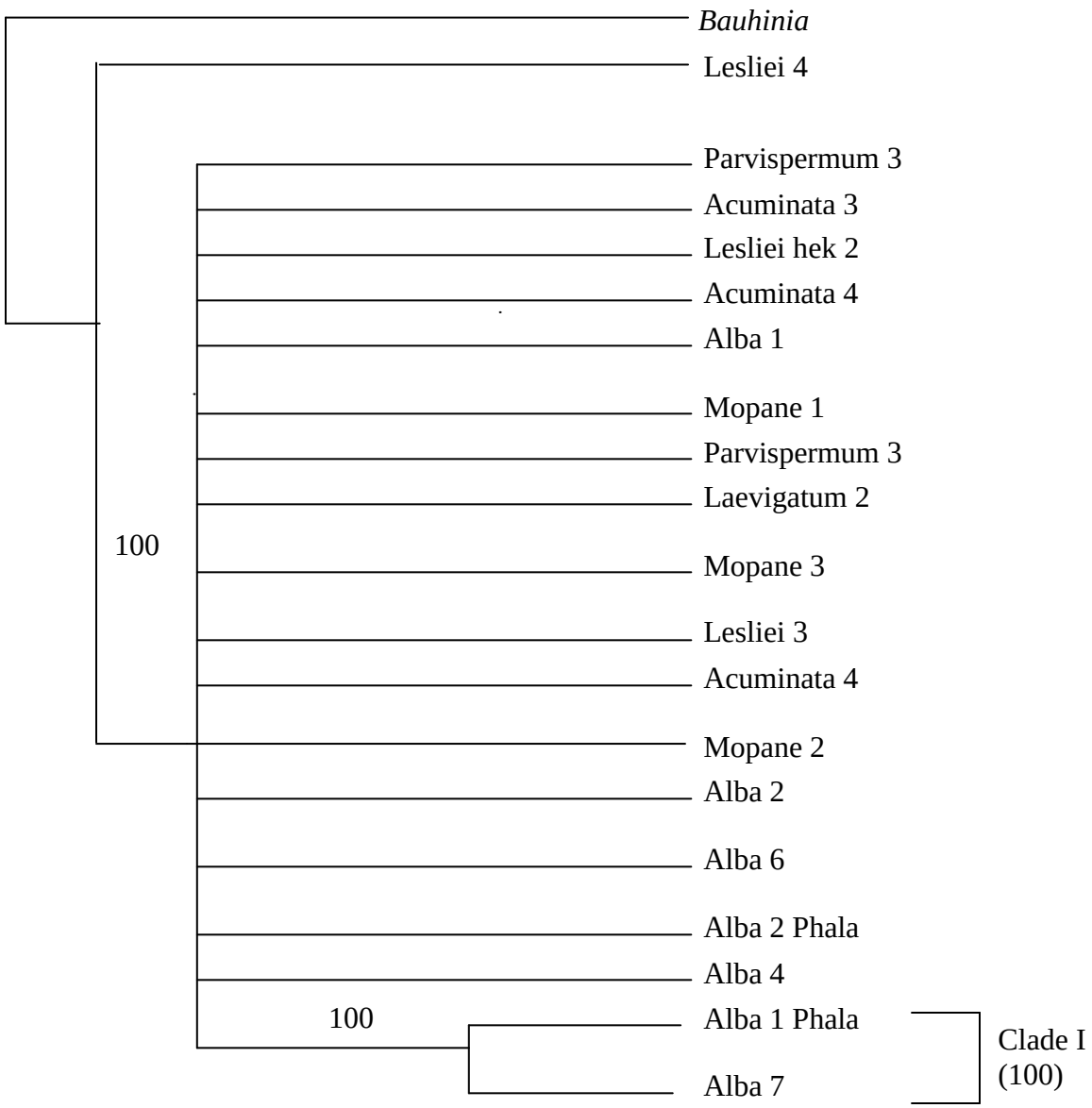


Figure 20 D: Dendogram obtained from RAPD data obtained for *C. mopane (sensu lato)* by parsimony analysis of primer 08. Numbers indicate the percentage values of a 50% majority consensus tree.

4.3.2 Bootstrapping using Parsimony tree

When the RAPD data was subjected to a bootstrap test, 5990 trees were produced (Fig. 21 A). Bootstrap analysis of the data set indicates a wide range of support values, revealing strong support (> 70%) and weak support (<70%) for most nodes. A branch of 90% separates Alba 4 from the remaining taxa on the tree. A branch of 56% show Lesliei 3 and Lesliei hek 2 poorly resolved which is a sister group to monophyletic group that also form a three clades which further divides into subclades. Clade I (56%), the position of Parvispermum 1, Mopane 3 and Laevigatum 4 were unresolved, at branch 81%. Mopane 1 was a sister group to the polytomy group of branch 87%. In clade II (90%), Acuminata 4 was a sister group of Mopane 4. The three representatives of Alba (Alba 1 Phala, Alba 7 and Alba 2 Phala) were grouped together at a branch 70%, with Alba 1 Phala as a sister group to Alba 7 and Alba 2 Phala (77%). Figure 21 B shows one small clade with a support of 61% and the remaining of the taxa poorly resolved and not supported resulting in a basal polytomy.

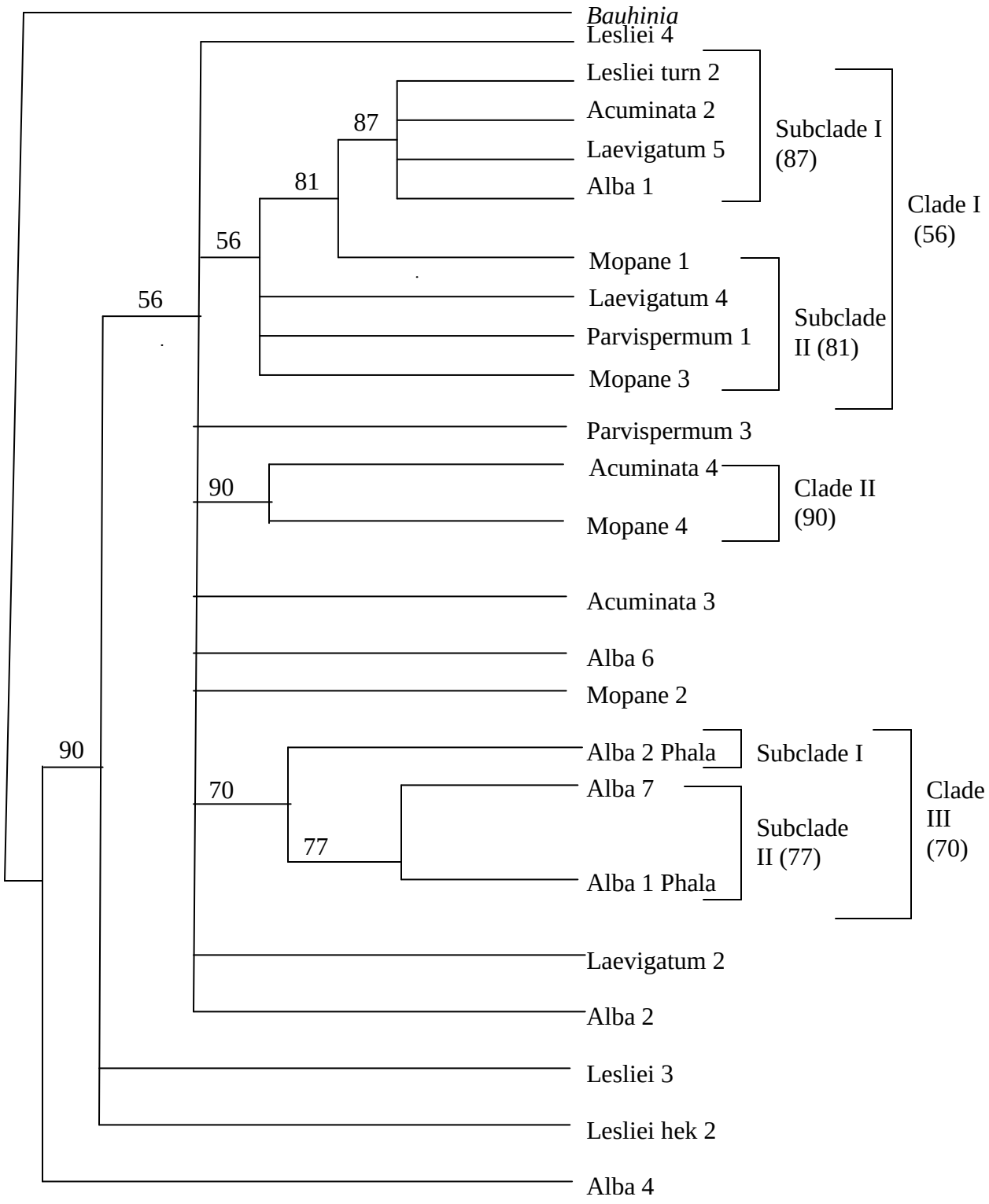


Figure 21 A: Dendogram for RAPD data obtained for *C. mopane (sensu lato)* by parsimony bootstrapping analysis of combined primers data. Numbers on each node indicate the percentage values of a 50% majority consensus tree obtained from 100 bootstrap replicates.

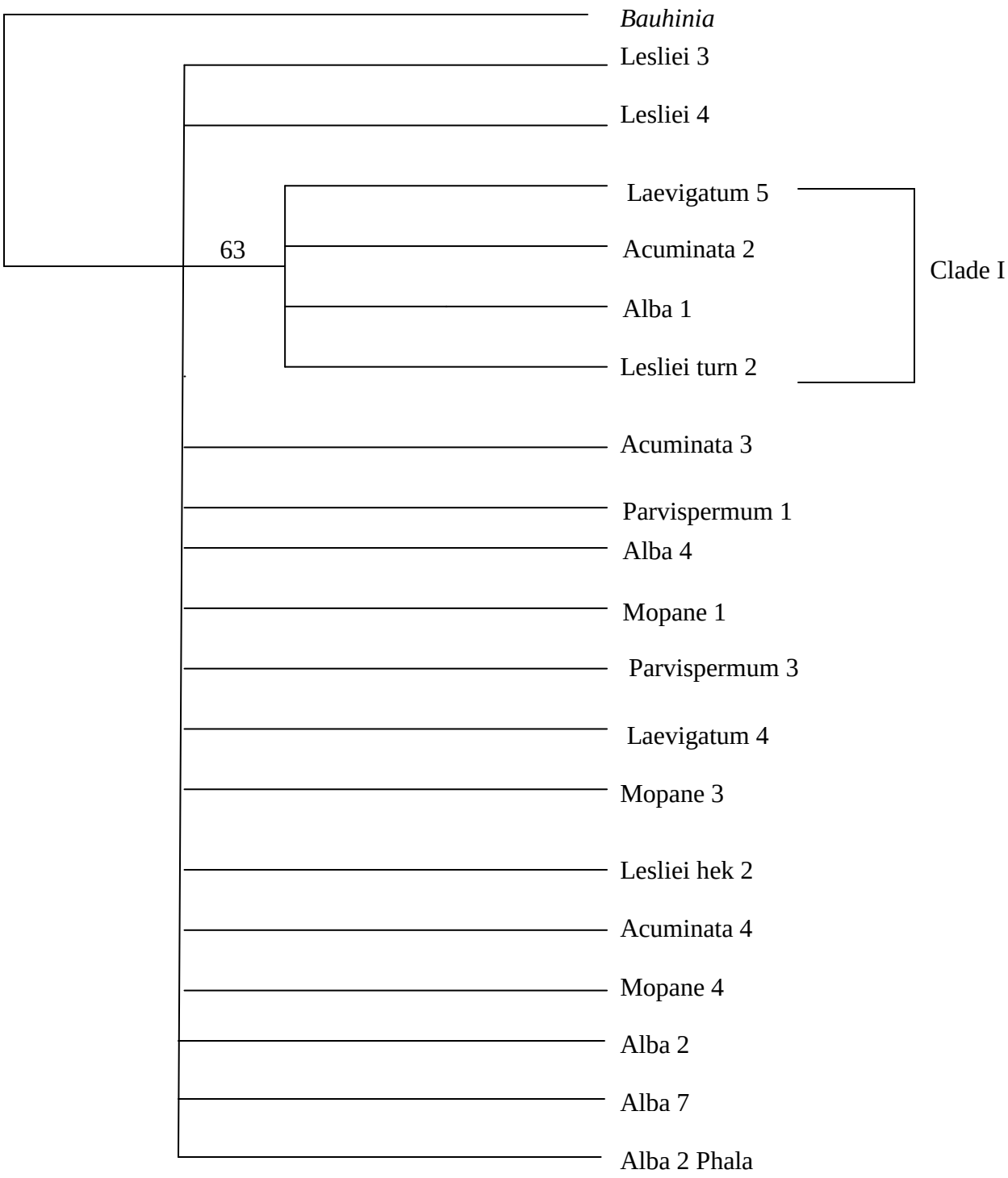


Figure 21 B: Dendogram for RAPD data obtained for *C. mopane (sensu lato)* by parsimony bootstrapping analysis of primer 03 data. Numbers on each node indicate the percentage values of a 50% majority consensus tree obtained from 100 bootstrap replicates.

4.3.3 Genetic similarity and differences

4.3.3.1 Total mean differences

RAPD binary data was converted into a total mean character difference matrix (Tables 5 A and B). The total mean character difference matrix was used to generate the Neighbour Joining dendogram consisting of the combined data and OPA 03 (Fig. 22). The genetic distance between 24 individuals of *C. mopane* (s*ensu lato*) were calculated from the total mean character difference values (Table 6A) and converted into percentages. The highest intra ecotypic similarity (95.5%) was observed for Alba (Phalaborwa), while the lowest intra ecotypic similarity (72.2 %) occurred in Lesliei (Table 6). For *C. mopane* (*sensu lato*) a similarity percentage value of 84.1% was obtained.

Alba has a high (91.3%) genetic similarity with Parvispermum and a low (74.4%) similarity with Lesliei (Table 7 A). Acuminata exhibit its highest (86.1%) genetic similarity with Parvispermum and its lowest (76.1%) similarity with Lesliei (Table 7 B). Lesliei show its highest (79.7%) genetic similarity with Parvispermum and its lowest (64.9%) similarity with Mopane (Table 7 C). *Colophospermum mopane* (*sensu stricto*) has an 86.5% genetic similarity with Parvispermum (its highest similarity to any other ecotype) and a 64.9% genetic similarity with Lesliei (its lowest similarity to any other ecotype) (Table 7 D). Laevigatum has its highest (90%) genetic similarity with Parvispermum and its lowest (73.7%) similarity with Acuminata (Table 7 E).

Table 5 A: Pair-wise genetic distance matrix of *C. mopane* (*sensu lato*) with outgroup included, based on RAPD data of the combined primers used and computed using the character mean difference calculated by the PAUP.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1 *Bauhinia*																								
2 Les 4	16																							
3 Les 3	18	10																						
4 Les turn 2	12	6	6																					
5 Les hek 2	18	10	4	4																				
6 Parvi 3	19	9	5	6	7																			
7 Parvi 1	10	4	2	4	2	2																		
8 Acum 2	12	6	6	6	4	6	4																	
9 Acum 4	19	9	7	7	7	6	3	7																
10 Acum 3	16	8	1	6	2	0	2	6	4															
11 Laevi 5	19	13	11	2	9	8	4	2	10	7														
12 Laevi 4	13	5	6	4	7	2	0	4	5	2	5													
13 Mop 3	13	7	6	5	7	4	1	5	7	3	7	2												
14 Mop 1	21	11	9	3	9	4	1	3	8	4	6	1	3											
15 Mop 4	19	11	9	5	9	4	1	5	6	4	10	1	3	4										
16 Mop 2	18	14	10	8	12	7	4	8	11	4	15	7	5	9	9									
17 Alba 6	17	9	9	6	9	4	2	6	6	4	10	2	4	4	4	5								
18 Alba 1 phala	14	6	5	4	6	3	2	4	6	2	6	3	3	4	2	6	3							
19 Alba 2	20	8	6	5	6	1	1	5	5	1	7	1	3	3	3	8	3	2						
20 Alba 7	20	10	6	5	6	3	1	5	7	2	9	2	2	5	5	8	5	1	2					
21 Alba 2 phala	20	10	6	5	6	3	1	5	7	2	9	2	2	5	5	8	5	1	2	0				
22 Alba 4	15	9	2	6	3	1	2	6	5	1	8	2	3	5	3	5	5	2	2	3	3			
23 Alba 1	19	9	6	1	3	5	3	1	7	5	4	3	4	3	7	9	7	5	4	5	5	6		
24 Laevi 2	7	5	2	0	1	1	0	0	2	1	2	0	0	0	2	1	1	0	1	2	2	2	1	

Table 5 B: Pair-wise genetic distance matrix of *C. mopane* (*sensu lato*) with outgroup included, based on RAPD data of OPA 03 used and computed using the character mean difference calculated by the PAUP**.**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1 *Bauhinia*																								
2 Les 4	6																							
3 Les 3	8	4																						
4 Les turn 2	12	6	6																					
5 Les hek 2	8	2	2	4																				
6 Parvi 3	8	4	0	6	2																			
7 Parvi 1	10	4	2	4	2	2																		
8 Acum 2	12	6	6	0	4	6	4																	
9 Acum 4	7	3	3	7	3	3	3	7																
10 Acum 3	8	4	0	6	2	0	2	6	3															
11 Laevi 5	10	6	6	2	4	6	4	2	7	6														
12 Laevi 4	10	4	2	4	2	2	0	4	3	2	4													
13 Mop 3	9	5	3	5	3	3	1	5	4	3	5	1												
14 Mop 1	11	5	3	3	3	3	1	3	4	3	3	1	2											
15 Mop 4	9	3	1	5	1	1	1	5	2	1	5	1	2	2										
16 Mop 2	6	6	2	8	4	2	4	8	3	2	8	4	3	5	3									
17 Alba 6	8	4	2	6	2	2	2	6	1	2	6	2	3	3	1	2								
18 Alba 1 Phala	10	4	2	4	2	2	2	4	3	2	4	2	3	3	1	4	2							
19 Alba 2	9	3	1	5	1	1	1	5	2	1	5	1	2	2	0	3	1	1						
20 Alba 7	9	3	1	5	1	1	1	5	2	1	5	1	2	2	0	3	1	1	0					
21 Alba 2 Phala	9	3	1	5	1	1	1	5	2	1	5	1	2	2	0	3	1	1	0	0				
22 Alba 4	8	4	0	6	2	0	2	6	3	0	6	2	3	3	1	2	2	2	1	1	1			
23 Alba 1	11	5	5	1	3	5	3	1	6	5	3	3	4	2	4	7	5	5	4	4	4	5		

Table 6: Intra ecotypic similarity.

Ecotype	Similarity percentage
Alba (Phalaborwa)*	95.5%
Parvispermum*	91.7%
Laevigatum	90.3%
Alba (Phalaborwa and Musina)	85.9%
Mopane (*sensu stricto*)	77.1%
Acuminata	76.4%
Lesliei	72.2%

*Note: These values are based on only two individuals each. For other values see Materials and Methods, section 3.2.1.

Table 7 A: Inter ecotypic percentage difference and similarity: Alba.

Alba vs:	Difference	Similarity
Lesliei	25.6	74.4
Laevigatum	20.0	80.0
Mopane (*sensu stricto*)	19.1	80.9
Acuminata	18.2	81.8
Parvispermum	8.7	91.3

Table 7 B: Inter ecotypic percentage difference and similarity: Acuminata.

Acuminata vs:	Difference	Similarity
Lesliei	23.9	76.1
Mopane (*sensu stricto*)	23.6	76.4
Alba	18.2	81.8
Laevigatum	15.2	84.8
Parvispermum	13.9	86.1

Table 7 C: Inter ecotypic percentage difference and similarity: Lesliei.

Lesliei vs:	Difference	Similarity
Mopane (*sensu stricto*)	32.1	64.9
Alba	25.6	74.4
Acuminata	23.9	76.1
Laevigatum	22.5	77.5
Parvispermum	20.3	79.7

Table 7 D: Inter ecotypic percentage difference and similarity: *C. mopane* (*sensu stricto*).

C. mopane (*sensu stricto*) vs:	Difference	Similarity
Lesliei	32.1	64.9
Acuminata	23.6	76.4
Alba	19.1	80.9
Laevigatum	18.0	82.0
Parvispermum	13.5	86.5

Table 7 E: Inter ecotypic percentage difference and similarity: Laevigatum.

Laevigatum vs:	Difference	Similarity
Acuminata	26.3	73.7
Lesliei	22.5	77.5
Alba	20.0	80.0
Mopane (*sensu stricto*)	18.0	82.0
Parvispermum	10.0	90.0

4.3.3.2 Neighbour Joining

Laevigatum 2 was the sister group to all other taxa, making Lesliei 4 a sister group to the remaining taxa except Laevigatum 2. Parvispermum was also a sister group to remaining taxa except for Laevigatum 2 and Lesliei 4. Acuminata 2 was a sister group to a monophyletic group, that clustered into three large clades which further divided into subclades. Clade I was a sister group to Clade II and clade III and vice versa. Subclade I was a sister group to subclade II, with subclade III a sister group to subclade IV (Fig. 22 A)

Lesliei 4 was a sister group to all other taxa, with Acuminata 4 a sister group to the remaining taxa. Mopane 2 was a sister group to all other taxa, except Acuminata 4 and Lesliei 4. Mopane 2 was a sister group to a monophyletic group that divided into two clades and three subclades. However, the remaining taxa of subclade II are unresolved. Clade I was a sister group to clade II and III. Clade II is divided into three subclades (Fig. 22 B).

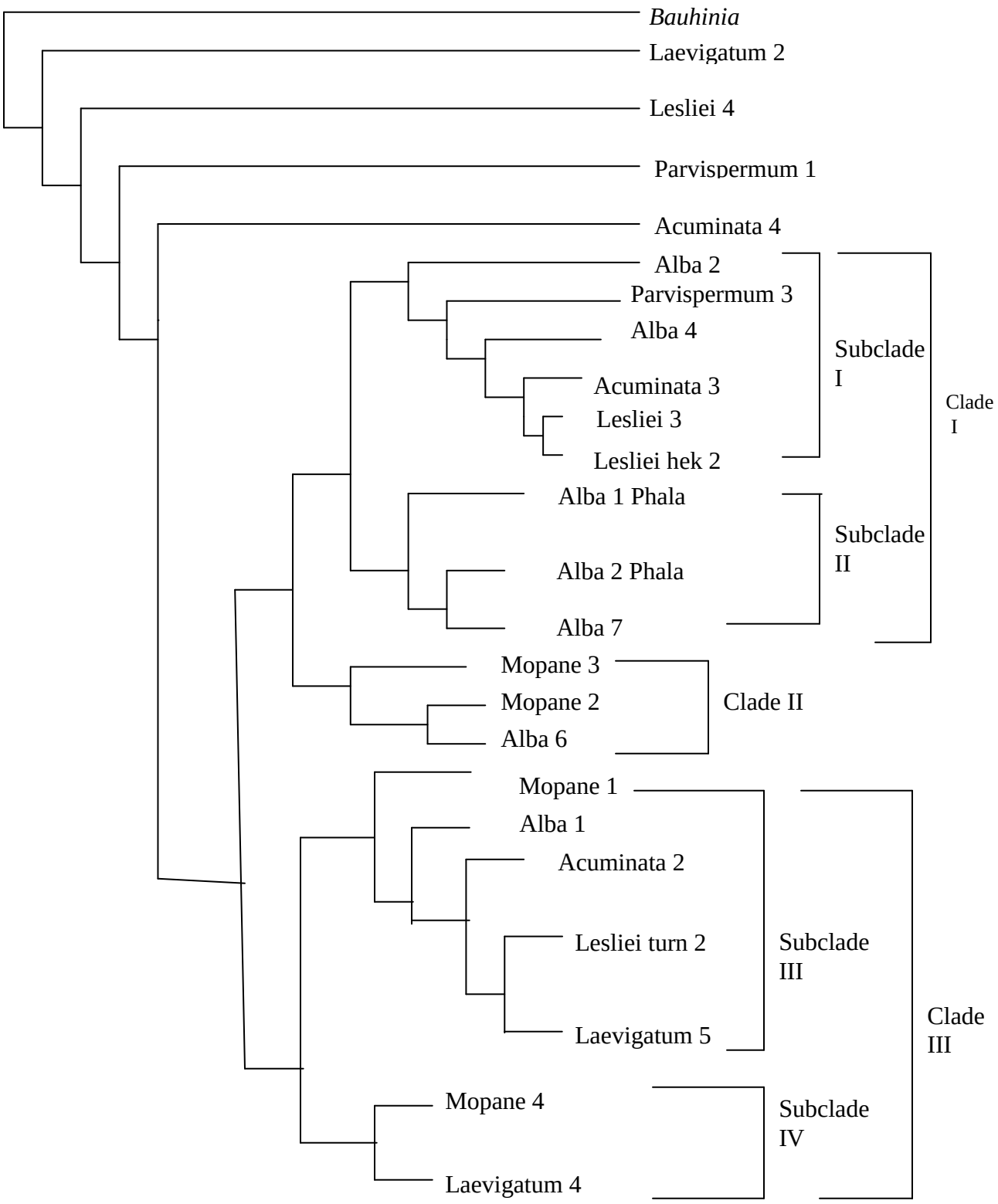


Figure 22 A: Dendogram for RAPD data obtained for *C. mopane (sensu lato*) by Neighbour Joining trees of combined primers based on the total character difference matrix. The branch length indicates relative similarity and difference of the taxa, the closer the cluster, the more similarity.

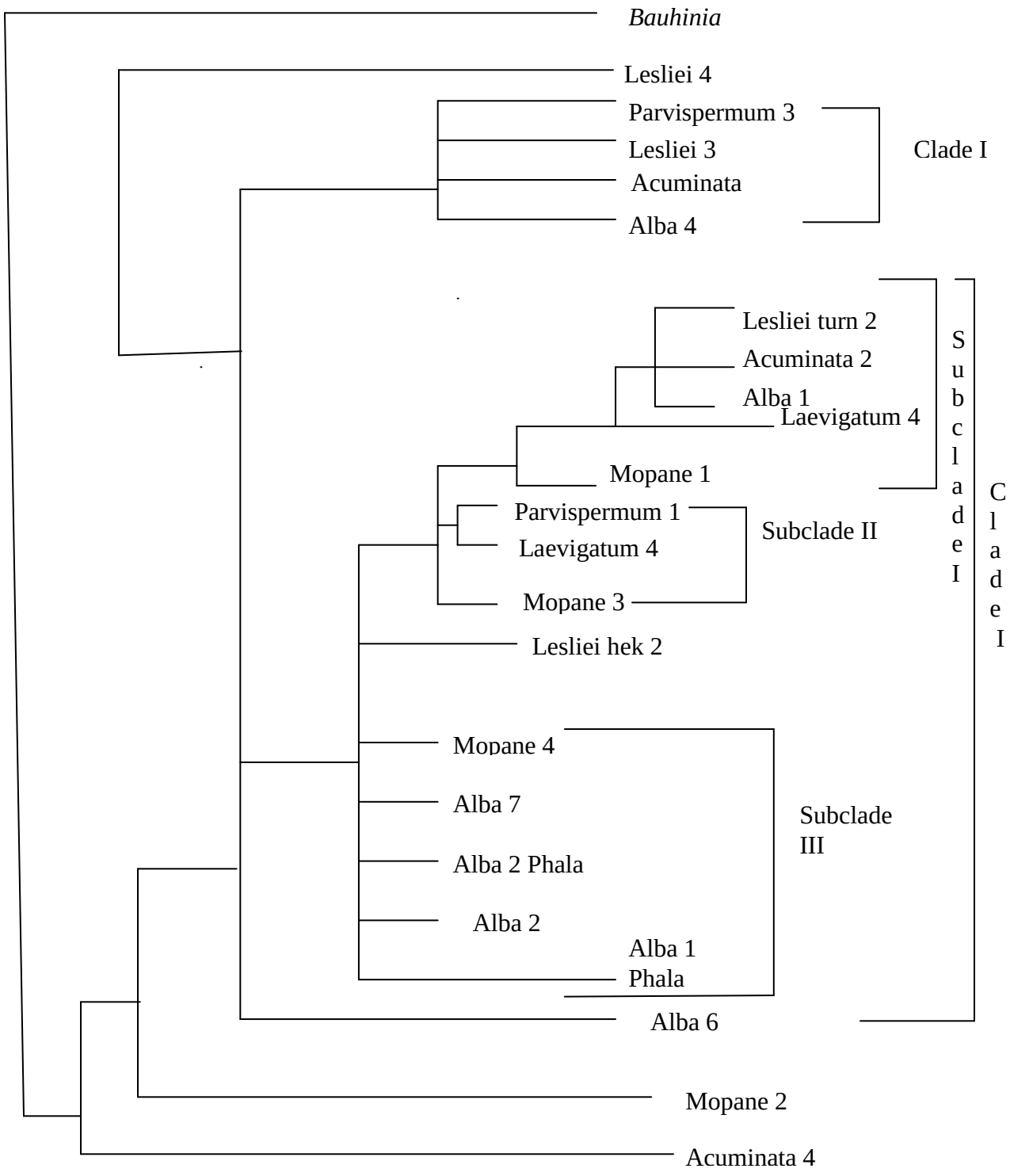


Figure 22 B: Dendogram for RAPD data obtained for *C. mopane* (s*ensu lato*) by Neighbour Joining trees of OPA 03 based on the total character difference matrix. The branch length indicates relative similarity and difference of the taxa. The closer the cluster, the more similarity.

CHAPTER 5

DISCUSSION

5.1 DNA extraction method and removal of contaminants

Extracting genomic DNA from mature leaves of *C. mopane* (*sensu lato*) presented a number of difficulties, due to high levels of polysaccharides and phenolics, which interfered with DNA isolation. A previous study, by Legodi and Mahlo (2003) using DNA extraction method described by Edwards et al. (1991), showed the presence of polyphenolics in DNA samples. In an attempt to genotype the present study of *C. mopane* (*sensu lato*), a few published nuclear isolation protocols (Murray and Thompson 1980; Dellaporta et al., 1983; Doyle and Doyle 1990; Rogers and Benedich 1994) were investigated for the isolation of pure, PCR-ready DNA from the leaves. The CTAB method by Doyle and Doyle 1990 was found to be the most appropriate. This method is known to avoid the problem of DNA degradation, contamination and low yield due to binding or co-precipitation with starches and polysaccharides from both young seedlings and mature leaves (Sharma et al., 2002). However, for *C. mopane* (*sensu lato*), the DNA extracted with the CTAB method was of low yield, quality and viscous, possibly due to the presence of polysaccharides.

It was found that the DNA yield from young seedling leaves was higher than from mature leaves. This is because using young seedlings reduce nucleic acid plant metabolites that interfere with the solubility of precipitated DNA as compared to mature leaves (Scott and Playford 1996). However, notable amounts of polysaccharides were still present.

Polysaccharides are visually evident in DNA extracted by their viscous, glue-like texture, making the DNA unmanageable during pipetting. Polysaccharides are also known to affect manipulation and inhibit enzyme reactions (Schlink and Reski 2002). Fang et al. (1992) found that more than 1 M of NaCl can facilitate the removal of polysaccharides, using its solubility in ethanol, thereby preventing co-precipitation with DNA. Sodium chloride at a concentration of more than 0.5 M together with CTAB is also known to remove polysaccharides (Paterson et al., 1993). The concentration of NaCl ranges mentioned in the literature varies between 0.7 M (Clark 1997) and 6 M (Aljanabi et al., 1999). NaCl can be replaced by KCl (Thompson and Henry 1995).

It is assumed that using a higher concentration of NaCl (5 M) can result in a more efficient removal of polysaccharides in plant leaves, but the removal of polysaccharides in mature and seedling leaves of *C. mopane* (*sensu lato*) did not improve. Extraction of high quality DNA from plants with high phenol and polysaccharides levels such as apples, grapes, pears and conifers species are known to be difficult. This is because there is no described extraction method for obtaining large quantities of high quality of DNA from plants with a high phenolic and polysaccharide content (Kim et al., 1997; Porebski et al., 1997). However, a modification of the various different extraction methods could conceivably solve this problem. For this study phenol precipitation and water-saturated ether steps was introduced to the extraction procedure to reduce the levels of polysaccharides in the DNA extracted samples. These steps ultimately solved the problem of polysaccharide contamination in the DNA extracted sample.

5.2 Optimisation of PCR conditions

The amplification profile for nucleotide primers are largely dependent on specific conditions of reactions, with banding patterns varying extensively due to inconsistencies in a number of reaction parameters (Williams et al., 1993). A necessary precondition for any RAPD analysis is the establishment of PCR conditions that ensure reliable and reproducible results (Ramser et al., 1996). This refers mainly to the choice of appropriate $MgCl_2$ concentrations, which differ among primers and plant species (Bousquet et al., 1990), *Taq* polymerase units (Barcaccia 1994), reproducible primers and the correct cycles (Carlson et al., 1991).

To obtain good results for each primer, $MgCl_2$ concentration were chosen from the results where the bands were clear, easy to read and suitable for data analysis. The $MgCl_2$ concentration used in this study (4mM) was higher than 1.5 mM and 2.0 mM used by authors such as Claros et al., (2000) and Kafkas (2002). Choosing the appropriate $MgCl_2$ concentrations minimized errors caused by *Taq* polymerase in the PCR. However, amplicons from a too high concentration of $MgCl_2$ may appear as streaks. This results from fragments of DNA with differences in length which are too small to permit their separations during electrophoresis. In this investigation, this phenomenon has been observed with OPA 13 where the resulting bands were not easily scorable using 4 mM $MgCl_2$. According to Bousquet et al. (1990), a too low concentration of $MgCl_2$ (1.0 mM to 1.5 mM) may result in absence of amplification in the gel.

Other problems that can be associated with RAPD markers include the appearance and disappearance of minor bands with different runs and variability between thermocycler and *Taq* polymerase obtained from different manufactures (Ellsworth et al., 1993). This has been shown with OPA 02, which did not give consistent amplification during optimization.

Variation in fragment intensity and number are also problematic with RAPD analyses. The variation in intensity of some fragments observed in OPA 03 with 10 μM and 20 μM concentrations of primer might be due to a more efficient amplification at some target sites, or to a greater complementary action between the primer and the DNA. Variation in fragments intensity and number of fragments produced with 10 μm of primer concentration could be due to a low amount of primer concentration, which becomes limiting and results in few amplification products. Variation of fragments may appear in keeping with target sites present at highly repetitive sequences (Tyler et al., 1997).

5.3 Genetic relatedness

The dendograms were constructed using the PAUP program with combined and individual data using parsimony and Neighbour Joining analysis. Combination of primer data normally results in finding the most parsimonious tree, enhancing the resolution, and increasing the internal support for clades (Olmstead and Sweere 1994). Combining of data sets also increases the rate of resolution among species (Soltis et al., 1998). Conflict between individual data sets indicates that there are evolutionary processes that results in different patterns of relationships (Wiens 1998).

Nei (1991) has shown Neighbour Joining as the most efficient method in establishing the correct topology as proposed by Saitou and Nei (1987). Distance methods are less stringent than parsimony methods, but have the advantage of being less demanding computationally for the analysis of large data sets (Dagenbach and Endow 2004). The Neighbour Joining trees (Figs 22 A and B) showed the same two clades, as the maximum parsimony trees, but some groups include ecotypes that were previously not classified as members of the same group. For example, Mopane 4 and Laevigatum 4 did not fall into one group in the parsimony (Fig. 20 A), but was included in the group in the Neighbour Joining trees. However, the parsimony trees were not as well resolved as compared to Neighbour Joining trees. Therefore, the discussion will focus mainly on the parsimony data as this is considered as the most representative of the evolutionary relationships, and the best estimate of phylogeny of *C. mopane* (*sensu lato*). The other parsimony dendogram trees were used as additional information (Figs 20B-D).

Bootstrapping of parsimony trees (Figs 21A and B) was done as support of the data set for the clades of each node on the parsimony analysis tree is necessary, to determine if the phylogenetic relationship within and between ecotypes is supported from (100-1000) bootstrap replicates. The higher the bootstrap value the more likely the relationship in the parsimony tree is true. Conversely, low bootstrap values suggest less confidence in the various relationships. Bootstrap values of 70-100% are generally considered to be highly supportive of the designated clades, as opposed to values of 50-69% that shows weak support.

The combined primer data (Fig. 21 A) shows unexpected high support (90%) for *C. mopane* (*sensu lato*) to be monophyletic. Lesliei 4 is shown to be a sister group to the monophyletic group. The topology of the dendogram in the first and second clades in Figure 21A was unexpected, as those ecotypes are morphologically different. Individuals of the same ecotypes are expected to cluster together because of their similar genetic make-up. A possible reason for first and second clades in Figure 21A might be that some of the ecotypes, thought to be different, were in fact not.

The remaining individuals in Figure 21A also show no support and collapse in basal polytomy. Trees that have polytomy are poorly resolved, indicating uncertainties or lack of resolution. A polytomy in a dendogram indicates that all taxa connected to that node have the same degree of similarity. Figure 21A did not clearly show the relationships of the ecotypes with one another. More studies should be done on these ecotypes in order to classify them (see recommendation for future research, section 5.7).

5.4 Genetically differentiated species

Genetic species is a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups. The focus on genetic isolation rather than reproductive isolation distinguishes the Genetic Species Concept from the Biological Species Concept (Baker and Bradley 2006). Morsolais et al. (1993) suggest that for a species to be similar, the genetic similarity should range between 61 to 99% (same entity).

5.5 Comparison of RAPD data with morphological characteristics

5.5.1 Intra ecotypic variation

5.5.1.1 Lesliei

Lesliei shows the lowest genetic similarity (72.2%) between its four sampled trees of all the ecotypes. This similarity value is very close to the similarity value of 74.15% obtained by Potgieter (2006), based on 40 characters derived from the leaves, seeds, fruits, flower and pollen from five Lesliei trees. Thus it would seem that the genetics of this ecotype strongly supports the morphology exhibited in the field. A possible reason for this close correlation between the genetics and morphology might be that Lesliei is a very coherent group or taxon that could eventually lead it to speciation.

5.5.1.2 Alba

Alba shows a very high (85.5%) genetic similarity between its seven sampled trees. This similarity value is, however, not close to the similarity value of 53.25% obtained by Potgieter (2006), based on 40 characters derived from the leaves, seeds, fruits, flower and pollen from five trees. This shows that the genetics of Alba do not strongly support the morphologically differences exhibited in the field. A possible reason might be that the phenotypes are governed by a relatively small number of genes which were not detected by RAPD analyses, due to the use of random sampling of genes.

5.5.1.3 Laevigatum and Parvispermum

Laevigatum and Parvispermum exhibit very high (90.3% and 91.7%, respectively) genetic similarity between the sampled trees. These high similarities should, however, be viewed with

caution as it was derived from only three trees in the case of Laevigatum and two tree in the case of Parvispermum. Although no morphological similarity value is available for these ecotypes, it is postulated that the genetics will strongly support the morphology exhibited in the field. To this end it is recommended that a full morphological investigation be conducted on these two ecotypes.

5.5.1.4 Acuminata

Acuminata show high (76.4%) genetic similarity between the three sampled trees. This similarity value lies fairly close to the 66% similarity value obtained by Potgieter (2006), based on five trees. This means that the genetics partially support the morphology of this ecotype.

5.5.1.5 Mopane (*sensu stricto*)

Mopane (*sensu stricto*) has a fairly high genetic similarity value of 77.1%. This genetic value do not support the morphological similarity value of 55.8% obtained by Potgieter (2006), based on five trees. This might be indicative that the taxon is not a coherent entity and as such could lend itself to ecotypic speciation. Thus, it would seem that Mopane (*sensu stricto*) can change morphologically and remain unchanged genetically.

5.5.2 Inter ecotypic relationship

5.5.2.1 Acuminata

- Acuminata vs Mopane (*sensu stricto*) (parental material)

Potgieter (2006) found a very low correlation (35% difference, 65% similarity) between Acuminata and Mopane (*sensu stricto*), based on the morphology. This low correlation is corroborated by the genetics, where a difference of 23.6% was found (Table 7B). Thus the genetics and morphology indicate a fairly high similarity between Acuminata and Mopane (*sensu stricto*). This would imply that Acuminata has not genetically drifted far from the parental material.

- Acuminata vs Lesliei

The most distant relative (lowest similarity) of Acuminata was found to be Lesliei. Potgieter (2006) found a 50.0% difference/similarity between Acuminata and Lesliei, based on the morphology of the fruit, seed, leaves and pollen. This morphological difference does not agree to a large extent with the genetics, where a 23.9% difference was obtained (Table 7B) between Acuminata and Lesliei. This indicates that a closer genetic relationship (i.e. a higher similarity), in terms of the genetics, exists between Acuminata and Lesliei than is the case for their morphology.

Smith and Smith (1989) suggest that the use of phenotypic traits is not always the best way to evaluate genetic distance. This is because of the degree of divergences between genotype at the phenotypic level is not necessarily correlated with a similar degree of genetic differences.

Molecular markers provide better coverage of the genome, resulting in a better determination of relationships (Hamrick and Godt 1989).

The discrepancies between RAPD and morphological data have been reported in various taxa such as *Eriastrum densifolium* (Brunell and Whitkus 1997), *Fragari*a species. (Harrison et al., 1997), *Hordeum vulgare* (Papa et al., 1998), *Rhenum* species (Persson et al., 2000), *Anacardium occidentale* (Samal et al., 2003), and *Alpinia* species (Saritnum and Sriuamsiri 2003).

- Acuminata vs Parvispermum

The lowest genetic difference (13.9%) was found between Acuminata and Parvispermum (Table 7B). This high correlation indicates that Acuminata has not genetically diverged far from Parvispermum. This was not expected since these two taxa grow in different habitats. Acuminata is usually found on the banks of non permanent streams, in sandy soils. In contrast, Parvispermum is found far from water bodies on calcareous soils. It would therefore seem that this close relationship between Acuminata and Parvispermum is not the result of habitat, but an inherent genetic condition. A low diversity could exist in terms of the available alleles which would lead to a situation where the lack of enough alleles would lead to a potential lack of expression. Potgieter (2006) did not include Parvispermum in his study, and as such no correlation can be drawn between the genetics and the morphology of these two ecotypes.

5.5.2.2 Alba

- Alba vs Mopane (*sensu stricto*) (parental material)

Potgieter (2006) found a high difference (55.0%) between Alba and Mopane, based on the morphology. This high difference is not supported by the genetics, where it was found to have a difference of only 19.1% (Table 7A). Thus the genetics does not support morphology, indicating a fairly genetic high similarity between Alba and Mopane. This would imply that Alba has not genetically drifted far from the parental material. A possible reason for this could be that the two entities grow in the same environmental parameters.

- Alba vs Lesliei (highest)

Based on the morphology, Potgieter (2006) found a high difference (77.5% difference) between Alba and Lesliei. This high morphological difference is not in agreement with the genetical difference of 25.6% (Table 7A). Thus the genetics indicate a fairly high similarity between Alba and Lesliei. This would mean that Alba is isolated from Lesliei, but the distance is not far.

- Alba vs Parvispermum

The lowest genetic difference (13.9%, 86.1% similarity) was found between Alba and Parvispermum (Table 7A). This low difference indicates that Alba has not genetically diverged far from Parvispermum. This is unexpected since these two taxa have different fruit size and colour. Alba has whitish brown fruits and a small mean weight of 0.4g, as opposed to Parvispermum having a brown fruit and a mean weight of 0.5g. In addition, Alba is generally found on sandy soils as opposed to the calcareous soils on which Parvispermum is found. This

dichotomy has been illustrated in two *Acer* species (*saccharum* and *nigrum*) that were very distinctive morphologically and genetically not isolated (Skepner and Krane 1998).

Potgieter (2006) did not include Parvispermum in his study, and as such no correlation can be drawn between the genetics and the morphology of these two ecotypes.

5.5.2.3 Lesliei

- Lesliei vs Mopane (*sensu stricto*) (parental material)

Potgieter (2006) found a high (62.16%) difference (37.84 % similarity) between Lesliei and Mopane (*sensu stricto*), based on the morphology. This high difference does not compare very well with genetics, where these two entities were found to have a difference of 32.1% (Table 7C). This indicates that a closer relationship, in terms of the genetics, exists between Lesliei and Mopane (*sensu stricto*) than is the case for their morphology. See Smith and Smith (1989) explaination in Acuminata vs Lesliei for the discussion on the difference between morphology and genetics.

- Lesliei vs Mopane

Lesliei also shows the highest difference (32.1%) with Mopane, compared to all the other ecotypes (Table 7A-7E). See paragraph above for discussion on the relationship between Lesliei and Mopane (*sensu stricto*).

- Lesliei vs Parvispermum

The lowest genetic difference (13.9%) was found between Lesliei and Parvispermum (Table 7C). This low correlation indicates that Lesliei has not genetically diverged far from Parvispermum. This is unusual since these two taxa do not occur in the same habitat. Lesliei is generally found along the banks or short distances away from non-permanent streams and rivers, as opposed to Parvispermum which is found in dry habitats. In addition, Lesliei is generally found on sandy soils as opposed to the calcareous soils on which Parvispermum is found. It would therefore seem that this close relationship between Lesliei and Parvispermum is not the result of habitat, but an inherent genetic condition.

5.5.2.4 Laevigatum

- Laevigatum vs Mopane (*sensu stricto*) (parental material)

The data shows that Laevigatum has a low (18.0%) genetic difference with Mopane (Table 7E). Thus indicate that Laevigatum has not genetically drifted from Mopane. Potgieter (2006) did not investigate these ecotypes, and therefore no comparison can be drawn between the genetics and the morphology of these ecotypes.

- Laevigatum vs Acuminata

Laevigatum reveals its highest (26.3%) genetic difference with Acuminata (Table 7E). However, this difference genetically indicates that Acuminata has not diverged far from Laevigatum.

- Laevigatum vs Parvispermum

Laevigatum shows its lowest (10.0%) genetic difference with Parvispermum (Table 7E). However, this difference genetically indicates that Parvispermum has not diverged far from Laevigatum. This indicates that the genetics support the morphological characters exhibited in the field, as these two taxa do not differ morphologically very much, except the smooth bark texture of Laevigatum, and the rough texture of Parvispermum.

5.5.2.5 Parvispermum

- Parvispermum vs Mopane (*sensu stricto*) (parental material)

Parvispermum reveals low (13.5%) genetic difference with Mopane (Table 7A). Genetically, thus indicate that Parvispermum has not genetically drifted far from the parental material.

5.6 Taxonomic recommendation

It is well documented from the study of Potgieter (2006) that *C. mopane* (*sensu lato*) exhibits a mosaic of variation. Within this mosaic of variation some entities exist with unique characters - the ecotypes. Potgieter (2006) therefore postulates that value exists to recognize *C. mopane* (*sensu lato*) as a taxonomical complex that can and should be subdivided into subunits (e.g., subspecies, varieties etc.).

According to Potgieter (2006), Acuminata (7 trees) has the highest intra ecotypic variation of Alba and Lesliei and Mopane (*sensu stricto*) in terms of the 40 investigated morphological characters and the lowest mean separation (48%) from Alba and Lesliei and Mopane (*sensu stricto*). In view of the proposed "statistical classification" by Potgieter (2006), it is clear that the

statistical data assign Acuminata to the rank of variety.

Potgieter (2006) state that Alba and Lesliei show high levels of morphological separation, with means of 64% and 63%, respectively, from the other types (Alba vs Acuminata, Lesliei and Mopane – *sensu stricto*; Lesliei vs Acuminata, Lesliei and mopane – *sensu stricto*). Potgieter (2006) thus recommend that these two types (Alba and Lesliei) be formally recognized as subspecies. The wide geographical range of Alba (from the Musina area to the Phalaborwa area) is indicative that this type is more than a local variant occurring in and around the Musina area where it was first noticed (Potgieter 2006). Lesliei has not been reported from outside the Messina Experimental Farm. However, this does not mean that it is just a local variant as this type is not easily spotted among the cluster of *C. mopane* (*sensu lato*). This type may be more widespread than is currently known, an aspect that needs investigation before a formal classification can commence (Potgieter 2006).

More studies should be done on the ecotypes as they are not genetically isolated. No formal classification based on the genetics of Alba, Acuminata, Lesliei, Laevigatum and Parvispermum can be proposed. A possible reason for the perceived close relationship between Mopane (*sensu stricto*) and the other ecotypes, as well as between the various ecotypes is that the primers used highlighted similarities more than differences. It is thus proposed that future studies use primers that showcase differences.

5.7 Conclusions

The overall results of *C. mopane* (*sensu lato*) show high (84.1%) genetic similarity. No ecotypic marker was obtained. Most of the ecotypes have not diverged genetically far from one another or from the parental material (Mopane (*sensu stricto*). The genetic results partially support the perceived morphological differences. In this study the RAPD technique has established its value as an additional tool to express the genetic variability in *C. mopane* (s*ensu lato*). No formal classification can be drawn from the genetic results since the ecotypes did not genetically diverge far from each other. More studies should be done on the ecotypes.

5.8 Recommendations for future research

a. Higher yields of DNA were obtained from seedlings as opposed to mature leaves and it is recommended for future work that young plants should be used when DNA from *C. mopane* (*sensu lato*) is required.

b. Overall, the ecotypes show high similarity from each other and from Mopane (*sensu stricto*) and a formal classification of the ecotypes cannot be done as the results are not conclusive. Therefore, the following recommendations:

    - An additional RAPD marker which would probably be enough to permit a more precise way to differentiate the ecotypes by using more primers.
    - Further extensively studies are needed to determine if more sensitive methods like AFLP and RFLP can be used to study the genetic diversity and genetic relationship of *C. mopane* (*sensu lato*).

c. In terms of taxonomy of *C. mopane* (*sensu lato*), it is suggested that:

- Both phenotypic and DNA analyses be used. This will also establish an identification key of *C. mopane* and its ecotypes.
- Finally, more research is necessary to determine the genetic differentiation of *C. mopane* (*sensu stricto*) from populations in countries neighbouring South Africa where this species occur. This includes enlargement of the number of markers used by other molecular techniques in order to have a deeper insight into molecular polymorphisms.

CHAPTER 6

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APPENDIX

Table 8: Character state data matrix for PAUP analysis of the combined data with 24 taxa and 31 characters.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1 *Bauhinia*	1	1	1	1	1	1	1	0	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	1	0
2 Les 4	0	0	0	0	1	1	1	1	0	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1
3 Les 3	0	0	0	0	0	0	0	1	0	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
4 Les turn 2	0	0	0	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
5 Les hek 2	0	0	0	0	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	1	1	0
6 Parvi 3	0	0	0	0	1	0	1	1	0	1	1	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
7 Parvi 1	1	1	0	0	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	1	0	1	0	0	0	0	1	1	1	0	0
8 Acum 2	0	0	0	0	0	0	0	1	0	0	1	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
9 Acum 4	0	1	0	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0
10 Acum 3	0	0	0	0	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	?	?	?	?	?	?	?
11 Laevi 5	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	1	0
12 Laevi 4	0	0	0	0	1	0	1	1	0	1	1	0	1	?	?	?	?	?	?	?	?	?	?	?	0	0	1	1	1	1	0
13 Mop 3	0	0	0	0	1	0	1	1	0	1	1	1	1	?	?	?	?	?	?	?	?	?	?	?	0	0	1	1	1	1	1
14 Mop 1	0	0	0	0	1	0	0	1	0	1	1	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	1	1	0
15 Mop 4	0	0	0	0	1	1	1	1	0	1	1	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	1	1	1	1	0
16 Mop 2	0	0	0	1	1	1	1	1	0	1	0	1	1	1	0	1	0	0	0	1	0	1	0	0	1	1	1	1	1	1	1
17 Alba 6	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	0	0	0	1	0	1	0	0	0	0	1	1	1	1	0
18 Alba 1 Phala	0	0	0	0	0	1	1	1	0	1	1	0	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
19 Alba 2	0	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	1	1	1	1	0
20 Alba 7	0	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	1	1
21 Alba 2 Phala	0	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1	1	1	1
22 Alba 4	0	0	0	0	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	?	?	?	?	?	?	?
23 Alba 1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	?	?	?	?	?	?	?
24 Laevi 2	?	?	?	?	?	?	?	?	?	?	?	?	?	0	0	1	0	0	0	1	0	1	0	0	?	?	?	?	?	?	?

0: absence of data

1: presence of data

?: missing data