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An alternative hypothesis for explaining anomalies in the fine scale distribution patterns of *Colophospermum mopane*: Are shrub and tree forms genetically distinct?

Submitted in partial fulfilment of the requirements of the systematics module component of the Plant Ecology honours degree in the Botany Department of the University of Cape Town.

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Abstract

Abiotic and top down control hypotheses do not adequately explain the fine scale distribution patterns of shrub and tree *Colophospermum mopane* (Caesalpinioideae). Genetic distinctiveness between growth forms is investigated as an alternative hypothesis. Tree and shrub *C. mopane* from the riparian and inland savanna zones were sampled at four sites in the northern Kruger National Park. Molecular DNA sequences were obtained for four plastid and one nuclear region, and the inter simple sequence repeat (ISSR) technique used to fingerprint individuals. Very low levels of sequence divergence were observed. The ISSR technique revealed no genetic structure between plants when grouped by growth form or by habitat in an analysis of molecular variance (AMOVA). Soil profile and xylem pressure potential data also did not explain the distribution of growth forms. A principle component analysis and a discriminant analysis of five leaf and branching characters identified a significant difference in the shape of shrub and tree *C. mopane* leaves. It is concluded that the *C. mopane* growth forms are not genetically distinct and that their fine scale distributions may be due to top down controls such as large mammals and fire.

Introduction

Colophospermum mopane (Kirk ex Benth.) J. Léonard belongs to the legume subfamily Caesalpinioideae and is a well known species that dominates the vegetation in much of the hot, low lying areas of tropical southern Africa (Coates Palgrave 2002). Its range extends from Angola into northern Namibia, Zambia and Botswana and through Zimbabwe into Malawi, Mozambique and the north eastern region of South Africa (Mapaure 1994). Its predominance in the vegetation types in which it occurs is reflected in the classification of much of this area as Mopane veld (*sensu* Acocks 1975) within which at least seven major plant communities have been recognised (Siebert *et. al.* 2003).

Two growth forms of *C. mopane* are commonly recognised, a tall 'tree' form generally taller than 10m that is often referred to as cathedral mopane, and a short, frequently multi-stemmed, 'shrub' form usually < 4m tall and variously known as scrub, bonsai or hedged mopane (Figure 1). Tree *C. mopane* is regularly associated

with riparian zones while shrub *C. mopane* is generally encountered in the open areas away from drainage lines (Mapaure 1994, Styles & Skinner 2000). The distribution of the two growth forms are however not mutually exclusive, with occasional tall individuals often occurring in areas of shrub *C. mopane*, and shrub *C. mopane* also often being interspersed among the tall individuals in the riparian zones. The factors attributed to determining the occurrence and distribution of these two forms include bottom up abiotic controls (Mapaure 1994) as well as top down disturbance controls (Styles & Skinner 2000, Kennedy & Potgieter 2003).

Central to the abiotic control argument is that the underlying geology is the major determinant for *C. mopane* growth form (Van Rooyen *et. al.* 1981, Fraser *et. al.* 1987, O'Connor 1992, Siebert *et. al.* 2003). The shrub form of *C. mopane* appears to be found on fine textured soils, while more sandy soils tend to support single stemmed trees (O'Connor 1992, Fraser *et. al.* 1987). Soil depth is also purported to be an important determinant for *C. mopane* growth form, with shallow soils with impeded drainage resulting in *C. mopane* remaining in a shrub form, while on deeper soils the height of *C. mopane* has been considered an indicator for soil depth (Mapaure 1994). The rooting structure of the shrub form of *C. mopane* has been described as 'decisively shallow' (highest mean root concentration in top 60cm of soil; Smit & Rethman 1998) in excavations on relatively fine textured but deep soils. The rooting structure of the tall form of *C. mopane* is not apparent in the literature.

Associating growth form with soil depth suggests that access to soil water is an important determinant of the height to which *C. mopane* will grow. This would potentially explain the occurrence of a tall, riparian growth form, with the shrub growth form occurring away from drainage lines (Mapaure 1994, Styles & Skinner 2000). This hypothesis does not, however, satisfactorily explain the occurrence of tall *C. mopane* interspersed amongst shrub *C. mopane*, unless these occasional tall individuals are sited on isolated patches of deeper soils, or have somehow managed to access deeper soil water. The tendency for shrub *C. mopane* to be shallow rooted on deep soils (Smit & Rethman 1998) suggests that this is an unlikely scenario.

An alternative or potentially overlaying hypothesis for the development of shrub *C. mopane* is that it results from top down disturbance controls such as large mammals (Styles & Skinner 2000, Smallie & O'Connor 2000) and fire (Kennedy & Potgieter 2003). Smallie & O'Connor (2000) describe the active selection by elephants of previously utilised *C. mopane* individuals as a preference for the prolific coppice growth produced by *C. mopane* in response to damage. This has the effect of producing a 'hedged' 1.5m tall *C. mopane* growth form, and potentially preventing recruitment of individuals into taller height classes. Styles & Skinner (2000), however, highlight the importance of year round eland browsing at a nearby study site, where elephant only arrive after the pre-spring flush of new leaves, and attribute the resulting 'hedged' shrub *C. mopane* growth form as largely being due to eland browsing. Nonetheless, there is evidence to suggest that large mammals can exert a top down control on *C. mopane* growth form.

In the experimental burn plots in the Kruger National Park, trees growing in the no burn treatment, where fire has been excluded for 50 years, are significantly taller (± 2.5m) than those on the biennial mid dry- (± 1.56m) and early wet-season (±1.82m) burns respectively (Kennedy & Potgieter 2003). This height difference is attributed to the unburnt individuals having a single dominant stem, a feature which was generally lacking in the individuals subjected to biennial fires (Kennedy & Potgieter 2003). In a separate study in the same burn treatment plots, elephant herbivory was found to be reduced by burning (Kennedy 2000). The nature of the interaction between elephants and fire and their top down disturbance effects on *C. mopane* is still not fully understood. It does however seem unlikely that fire is the primary determinant for the development of vast areas of shrub *C. mopane,* since grass biomass is often low in mopane veld (Mapaure 1994, Guy 1981).

The available evidence does not satisfactorily explain the current distributional patterns of tall and short *C. mopane* growth forms. While abiotic controls may be providing a template for the development of *C. mopane* stands which subsequently are modified by top down controls such as fire and herbivory, various anomalies give the impression that the variation in *C. mopane* growth forms has not been adequately explained. These anomalies include the seemingly random occurrence of occasional tall *C. mopane* individuals in a sea of shrub *C. mopane*, as well as the apparent lack of

further development of the shrub form of *C. mopane* growing amongst tall form *C. mopane*. An alternative hypothesis is that there are genetic differences between tall and short *C. mopane* growth forms.

The hypothesis of genetic distinctiveness between tall and shrub *C. mopane* is based on unpublished data (E. February) from dendrobands measuring growth of both tall and shrub *C. mopane* on alluvial riparian soils. Shrub *C. mopane* growing amongst tall *C. mopane* at the study site have shown no growth over five years of observation, giving rise to the notion that the growth forms of these shrubby individuals are genetically predetermined, rather than being environmentally induced.

The degree of genetic divergence between the tree and shrub forms of *C. mopane* has not been assessed. In the only published study assessing genetic variation in *C. mopane*, five widely distributed *C. mopane* populations in South Africa are compared using allozyme variation (Villeon *et. al.* 2003). Based on the distance between sampled populations (up to 400km), and also the phenotypic plasticity of *C. mopane*, Villeon *et. al.* (2003) hypothesise that regional genetic variants would be encountered. However, only very low levels of inter population genetic variation were observed, with only the northern most population showing any notable genetic divergence from the remainder. This was attributed to it being separated by a tributary to the Limpopo River, potentially forming a barrier to pollinators. This is however unlikely, as *C. mopane* is wind pollinated (Coates Palgrave 2002), and little variation was observed between populations 400km apart. The colonisation pattern of *C. mopane* from the north into South Africa is also offered as a potential explanation for the genetic patterns observed (Villeon *et. al.* 2003), but this has not yet been substantiated.

The present study takes a systematic approach to assessing the existence of a genetically distinct tree and shrub *C. mopane* growth form, the discovery of which may suggest the delimitation of two distinct species. DNA sequencing and the inter simple sequence repeat or ISSR technique (Zietkiewicz *et. al.* 1994) are used to assess genetic variability among *C. mopane* individuals. The ISSR technique requires no prior sequence knowledge, and is thus a quick method for determining genetic diversity (Godwin *et. al.* 1997). Its efficacy in assessing plant genetic diversity has

been shown in a number of studies (e.g. Li & Ge 2001), and it compares favourably with other microsatellite techniques such as using RFLP and RAPD markers (Godwin et. al. 1997).

Combined with the genetic approach the present study also examines the various abiotic hypotheses for the two growth forms of *C. mopane*. To this end, soil samples were collected to determine whether or not there is a difference in soil under the two growth forms. Plant water stress was determined to assess whether or not the two growth forms exhibit differences in water availability, while leaf and branching morphology was also measured. The aim of this study is thus to use systematic tools to assess whether there is any genetic distinctiveness between the tall and short *C. mopane* growth forms, while at the same time taking into account the abiotic and top down control factors previously hypothesised as being responsible for their occurrence.

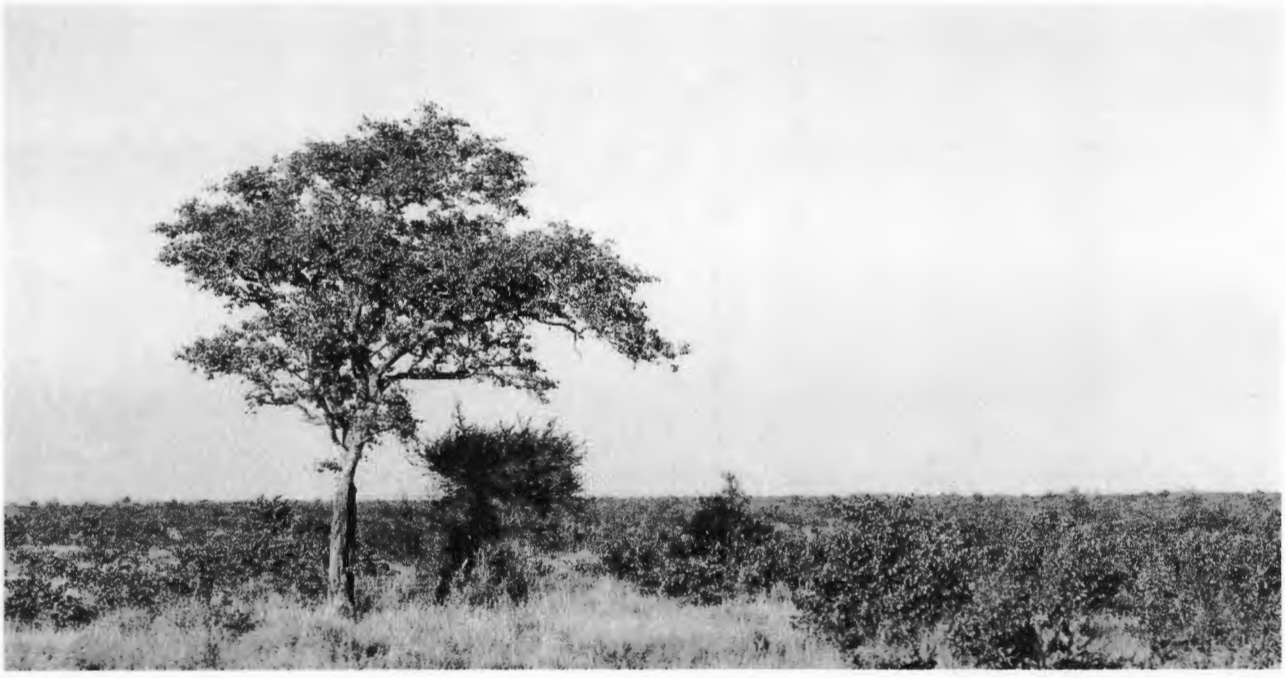

Figure 1: A lone tree form specimen of *Colophospermum mopane* stands out in a plain of shrub form *C. mopane* in the northern Kruger National Park.

Methods

Study site

Fieldwork for the study was carried out in the Shingwedzi region of the Kruger National Park, South Africa, from the 7-11 August 2004. Data was collected from four sites (Figure 2), each associated with a major drainage line of the region. Two sites were located on the northern bank of Shingwedzi River; the first in the west in the vicinity of the Joao water point (S 23° 14'49" E 31° 35'41"), and the second to the east, 1km south of the Shingwedzi rest camp (S 23° 10' 93" E 31° 45' 36"). The Shingwedzi River is the most permanently flowing of the rivers in the region. The remaining two sites were located on the northern bank of the seasonal Phugwane River (S 23° 01' 79" E 31° 29' 44") and the eastern bank of the Mpongolo River (S 23° 01' 56" E 31° 31' 92") respectively. These two sites were located within 5km of each other, both being upstream of the confluence of these rivers.

The mean annual rainfall at the Shingwedzi rest camp during the period 1961 – 1990 was 400mm, with December being the wettest month (84mm) and July the driest (3mm; Venter *et. al.* 2003). This area would thus be described as xeric savanna (Bond 1997). Mean maximum daytime temperatures peak in January at 34.1°C with the

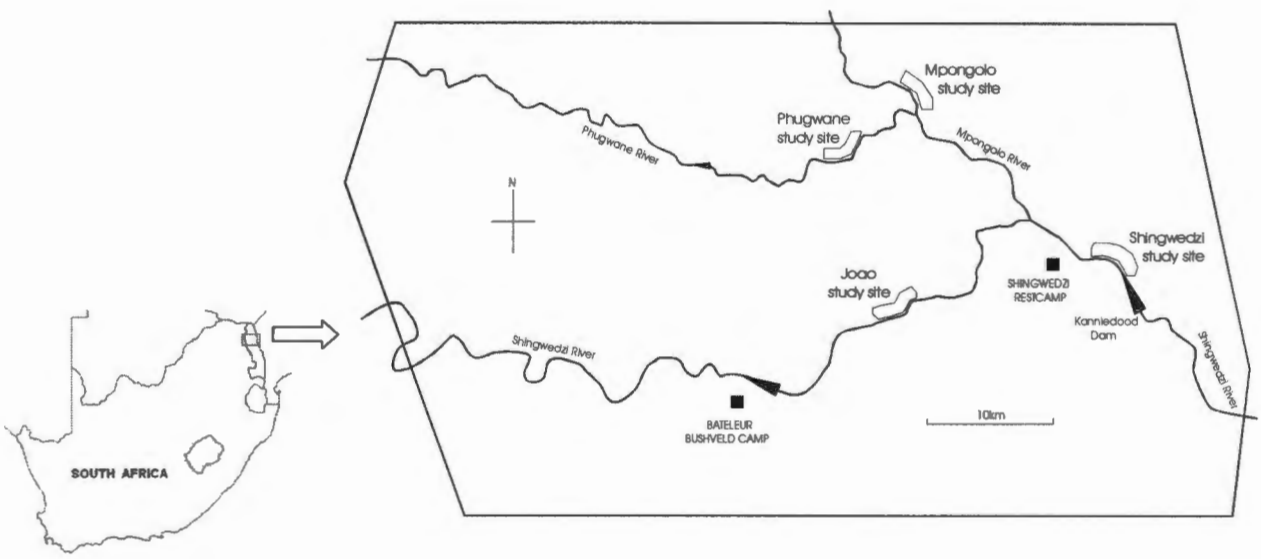

Figure 2: Map showing the location of the four study sites in the northern Kruger National Park, South Africa.

lowest mean monthly minimum temperatures of 7.4°C being recorded in June (Venter *et. al.* 2003). The geology underlying all four study sites is of the olivine rich Letaba basalt formation in the Karoo sequence, and fall within the Letaba land system (Venter *et. al.* 2003).

Fieldwork

Sampling at each site was conducted in two lines, one running along the river bank within the riparian zone, and the second running parallel to it, 100 - 300m inland beyond the edge of the riparian zone. Ten trees were sampled on each line, five short (< 5m) and five tall (> 9m), with each individual being at least 100m from the next. Tree height was recorded for each individual included in the study, and its position marked with a GPS. Xylem pressure potentials were determined for each individual using a Scholander Pressure Chamber (PMS Instruments). Measurements were taken between 11h00 and 15h00 when the trees would exhibit most moisture stress (Lambers *et. al.* 1998). Leaf samples (for DNA extraction) and voucher specimens were collected for each tree. Voucher specimens are housed in the Bolus Herbarium. The heights of the five *C. mopane* individuals closest to the study tree were also recorded to give an indication of the height class frequency distribution of *C. mopane* along each sampling line. A hand auger was used to collect soil samples to a maximum of 1.2m from either end of each sampling line. Soil core samples at 10cm depth intervals were laid out and photographed with a digital camera for later assessment of the colour change with depth through the profiles. Soil samples were also collected from three depths within the profile; the top 10cm and bottom 10cm of the profile, and also from an intermediate depth (50 - 60cm). These samples were used to assess soil texture using the flow chart in Tongway & Hindley (1995).

Total DNA extraction

C. mopane leaves were collected and stored in silica gel in zip lock plastic bags until extraction. Total DNA was extracted using a modification of the protocol described by Gawel & Jarret (1991). Dried leaf material was ground with liquid nitrogen, 0.05 - 0.10mg of polyvinylpyrrolidone (PVP) and purified sand in a mortar and then added to 700µl of 2x CTAB extraction buffer in a 1.5ml Eppendorf tube containing 0.14% mercaptoethanol which had been pre-heated to 65°C. This was incubated at 65°C for a minimum of 30 minutes in a water bath. After incubation, 600µl of chloroform:

isoamyl alcohol (24:1, v/v) was added and the product mixed by inversion for 5 minutes. Samples were then centrifuged at 13 000rpm for 10 minutes and the resulting supernatant pipetted into a clean 1.5ml Eppendorf tube. An equal volume of ice cold isopropanol was added to the supernatant, and the sample briefly mixed by inversion before being placed in a freezer for at least 1 hour to precipitate DNA. The DNA was recovered as a pellet by centrifuging the chilled sample at 13 000 rpm for 5 minutes, after which the isopropanol was discarded and the sample washed with 250µl 75% ethanol and centrifuged at 13 000 rpm for 3 minutes before being allowed to air dry. DNA was resuspended in 50µl 1x TE buffer. DNA quality and quantity were checked in a 1% agarose gel and visualised with ethidium bromide.

Sequencing

A shrub and a tree form *C. mopane* specimen from the river sampling line of each study site were used for initial plastid molecular sequence screening. One shrub and one tree *C. mopane* specimen from the inland lines of the Phugwane and Mpongolo study sites and both lines of the Joao and Shingwedzi study sites were screened for nuclear molecular sequence variation. Four plastid regions and one nuclear region were amplified. The chloroplast *trn*L-F intron-spacer region was amplified using the forward primer 'c' and the reverse primer 'f' (Taberlet *et. al.* 1991), and the *psb*A-*trn*H region using the *psb*AF forward primer and the *trn*HR reverse primer (Sang *et. al.* 1995). Amplification of the *trn*S-*trn*G intergenic spacer region was achieved using the *trn*S forward primer and the *trn*G reverse primer (Hamilton 1999). The *rps*16 intron was amplified using the *rps*F forward primer and *rps*R2 reverse primer (Oxelman *et. al.* 1997). The 5*S*-rRNA nuclear spacer region was amplified using the p3 and p4 forward and reverse primers respectively (Cox *et. al.* 1992).

The respective regions were amplified in a 50µl reaction volume comprised of 33.5µl autoclaved PCR water, 5µl of 50mM $MgCl_2$, 5µl of 10x DNA polymerase buffer (SuperTherm, Eisenberg Bro. Co., Givat Schmuel, Israel), 2µl of dNTP (10mM), 1µl of each primer (10µM), 0.5µl of SuperTherm *Taq* DNA polymerase (Eisenberg Bro. Co., Givat Schmuel, Israel) and 2µl of template DNA. The thermal profile for amplification of chloroplast regions involved an initial 2 min denaturation period at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 52°C and 2 minutes at 72°C, with a final extension period of 7 minutes at 72°C. The thermal profile for the

nuclear 5*S* region started with 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute, and terminating with 72°C for 6 minutes. The products of the PCR reaction were visualised on 1% agarose gels stained with ethidium bromide. Where the PCR amplification was successful, products were cleaned using a QIAquick®Qiagen™ PCR Purification Kit (Qiagen GmbH, D-40724, Hilden, Germany).

Cycle sequencing was performed using an ABI PRISM®BigDye™Terminator Cycle Sequence Kit (Applied Biosystems, Warrington, UK). Reactions were carried out in a 10µl volume made up of 2.84µl of autoclaved PCR water, 2µl of sequence terminator BigDye™ vers. 3.0, 3µl 2.5x cycle sequence buffer, 0.16µl of the respective primer and 2µl DNA product. The cycle sequencing thermal profile consisted of 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Initial amplification and cycle sequencing were carried out on a GeneAmp® PCR System 2700 (ABI). Cycle sequencing products were realised on an ABI PRISM® 3100 Genetic Analyser (ABI) by the core sequencing facility at the University of Stellenbosch, South Africa.

ISSR PCR amplification

Nine primers from the University of British Columbia (UBC) SSR Primer Oligonucleotide Set 100/9 were screened for successful PCR amplification of sample DNA using two GeneAmp® PCR Systems 2700 (ABI). The thermal profile commenced with 1.5 minutes at 94°C, followed by 35 cycles of 1 minute at the annealing temperature (Table 1), 1 minute at 72°C and 30 seconds at 94°C, and ended with 2 minutes at 2°C below the annealing temperature and 3 minutes at 72°C. Reactions were initially carried out in 2.5µl 10x Buffer (Eisenberg Bro. Co., Givat Schmuel, Israel), 1.5µl $MgCl_2$ (50mM), 1µl dNTP (10mM), 1µl of 10mM primer or 0.67µl of 15mM primer, 0.15µl SuperTherm *Taq* DNA polymerase (Eisenberg Bro. Co., Givat Schmuel, Israel) and 2µl of template DNA, with the total volume being made up to 25µl with autoclaved PCR water. Electrophoresis was used to resolve the amplification products on 25ml 2% agarose gels, stained with 1.6µl of ethidium bromide and run at 50V in 0.5 x TBE. These were photographed under ultraviolet

light at a range of exposures to increase the information obtained from the gel Zietkiewicz et. al. 1994). Product sizes were estimated using a Lambda/Eco RV ladder.

Table 1: List of nine primers from the University of British Columbia (UBC) SSR Primer Oligonucleotide Set 100/9 that were screened for use in ISSR fingerprinting. The superscript [a] denotes inclusion of primer fragment data in the 'full' dataset, and [b] indicates inclusion in the conservative dataset. The final annealing temperature at which each primer was amplified is also shown.

Primer	Sequence of primer	Annealing temperature (°C)
812[ab]	$(GA)_8A$	48
813[a]	$(CT)_8T$	50
825[ab]	$(AC)_8T$	50
834[ab]	$(AG)_8YT$	53
835[ab]	$(AG)_8YC$	50
841	$(GA)_8YC$	55
851	$(GT)_8YG$	55
853	$(TC)_8RT$	50
858	$(TG)_8RT$	50

Analysis of molecular sequence data

Sequences were assembled, edited and aligned in SeqMan II™ ver 2.04 (Lasergene software, DNASTAR Inc., Madison, WI, USA), where they were visually inspected for sequence variation, using a Macintosh iMac computer.

Analysis of ISSR data

The bands produced by ISSR amplification were scored as present (1) or absent (0). The ISSR analysis was split into two sets of results, the first being the full set of scorable fragments and the second being a very conservative assessment of the banding pattern designed to remove any potentially ambiguous scoring situations from the dataset. The primers ultimately contributing to each dataset are shown in Table 1. The pairwise banding similarity between samples was calculated using the Jaccard coefficient in the NTSYS program (Rohlf 2000). Dendrograms were produced from cluster analyses using the unweighted paired group method with arithmetic mean (UPGMA).

An analysis of molecular variance (AMOVA) was performed on both the full and conservative ISSR datasets using the program Arlequin (Schneider *et. al.* 2000). Two AMOVAs were performed on each dataset. In the first AMOVA the variation between all tall and short trees was assessed relative to that within each growth form category with sites set as populations. The second AMOVA assessed the variation between trees growing on rivers and those growing inland relative to that within each habitat, with sites again set as populations.

Leaf and branching morphology

The morphological variation in the leaves and branching of those trees from which DNA was extracted was quantified using a range of measurements obtained from the pressed herbarium specimens. Four leaf dimensions were calculated for the three largest leaves of each specimen (Figure 3):

1. Leaf length from tip to pulvinus.
2. Sum of the maximum perpendicular length between the inner (B1) and outer (B2) leaf margin and the respective lines joining tip to pulvinus (inner) and tip to corner of lower leaf lobe (outer). In order to eliminate the effect of leaf size on this variable the sum of the leaf margin widths (B1 & B2) was divided by leaf length (A), which is hereafter referred to as margin width.
3. Angle between lines joining tip to pulvinus and pulvinus to corner of lower leaf lobe.
4. Petiole length. This was also divided by leaf length (A) to eliminate the effect of leaf size on petiole length.

One measurement describing the branching pattern of specimens was calculated. The lengths of branches diverging off the end 40cm of the longest branch were summed with the 40cm of longest branch to give the 'total branch' length. It was necessary to divide the total branch length by the length of the longest branch to standardise measurements across specimens because a 40cm 'longest branch' was not available for each specimen.

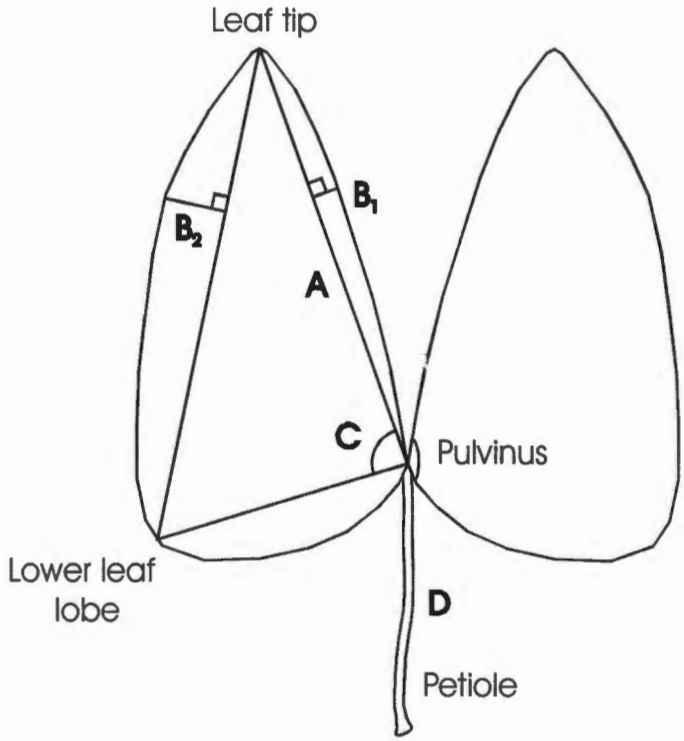

Figure 3: Schematic diagram of the measurements made to quantify leaf morphology

The five leaf and branching morphology descriptors were analysed in Statistica version 6.1 (Statsoft Inc. 2003) using a principal component analysis (PCA) as well as a discriminant analysis. All measurements were standardised prior to analysis. The factor coordinates for each variable and the percentage variation described by each factor were calculated in the PCA. The factor scores for each specimen were also calculated and the factor 1 scores plotted against factor 2 scores.

Two discriminant analyses were performed on the data, the first grouping the specimens by growth form (i.e. Tall versus Short) and the second grouping the specimens by habitat (i.e. River versus Inland). Wilk's Lambda was calculated for the five variable model, as well as for each of the five variables in the model. A canonical analysis was performed on the model and the scores of each specimen on the canonical axis were calculated. The canonical axis was divided into intervals of 0.5 for which the number of scores of each group was counted and plotted in a histogram.

Results

Soil

The descriptions of the soil profiles for each sampling line are shown in Table 2. The inland soils were finer textured than those along the rivers and were also more predictably structured. The inland soils generally had an orthic A horizon overlaying a red apedal B horizon, which is likely to place them in the Hutton form soils (MacVicar *et. al.* 1977). The depth, structuring and texture of the soils of the inland lines did not vary significantly between samples taken from the same line, nor did they vary significantly between the lines of each site. The riparian soils displayed a less predictably structured soil profile. This is likely to reflect the strong depositional influence in their formation, which gives them a stratified alluvial appearance, typical of Dundee form soils (MacVicar *et. al.* 1977). The river soils were over 110cm deep at all but one sampling point.

Table 2: Description of the soil profiles of the inland and river lines at each study site.

	Inland			River		
	Depth	Colour	Texture	Depth	Colour	Texture
Joao						
A horizon	± 0 – 20	Light grey - Grey brown	Silt loam – Sandy clay	± 0 – 25	Grey – Grey brown	Loamy sand – Sand clay loam
B horizon	± 20 – 70	Dark red brown	Sandy clay loam – Sandy clay	± 25 – 60	Dark brown – Red/grey brown	Loamy sand – Sand clay loam
C horizon	± 70 – 110+	Light red brown	Loamy sand – Sandy loam	± 60 – 110+	Pink red – Grey brown	Sand – Loamy sand
Mpongolo						
A horizon	± 0 – 30	Grey – Grey brown	Sandy loam	± 0 – 15	Grey brown – Light grey	Sandy loam – Loamy sand
B horizon	± 30 – 75	Dark brown – Dark red brown	Sandy clay loam – Sandy clay	± 15 – 35	Dark grey – Red grey	
C horizon	± 75 – 110+	Light grey brown – Light red brown	Sandy clay loam – Sandy loam	± 35 – 110+	Red brown – Dark brown	Silt clay loam – Sandy clay loam
Phugwane						
A horizon	± 0 – 20	Light grey	Loamy sand	± 0 – 20	Light red/grey – Grey brown	Silt clay loam – Sandy loam
B horizon	± 20 – 50	Red brown – Light red brown	Sandy clay loam	± 20 – 70	Grey red – Dark brown	Loamy sand – Silt loam
C horizon	± 50 – 110+	Dark red brown – Red brown	Sandy clay loam	± 70 – 110+	Dark brown – Light brown	Loamy sand – Silt clay loam
Shingwedzi						
A horizon	± 0 – 40	Grey – Grey brown	Sandy loam – Loamy sand	± 0 – 30	Grey – Grey/red brown	Loamy sand – Silt clay loam
B horizon	± 40 – 80	Red brown – Dark brown	Sandy loam – Loamy sand	± 30 – 80	Dark brown – Red brown	Sandy loam – Silt clay loam
C horizon	± 80 – 110+	Pink/red brown – Light brown	Sand/Loamy sand – Sandy clay	± 80 – 110+	Red brown – Light red brown	Loamy sand – Silt clay loam

Water stress

Xylem pressure potentials for all the plants sampled were compared between habitats (riparian – inland), growth form and site (Table 3). Trees growing closer to the river were significantly less water stressed at midday than those growing further inland (Table 3; $t_{78} = 3.352$, $p = 0.001$). Despite the significance of this difference, the mean water tension of the inland trees was only 0.04MPa greater than that of the river trees. There was no significant difference in xylem pressure potential between the short and tall *C. mopane* when compared across all sites and sampling lines ($t_{78} = 0.815$, $p = 0.418$). Trees at the Phugwane site had the highest pressure potentials and those at the Shingwedzi site the lowest. A two-way ANOVA analysis of habitat and growth form did not show a significant interaction between these groupings ($F_{1, 76} = 3.71$; $p = 0.058$).

Table 3: Mean and standard deviation of xylem pressure potentials (MPa) for a range of tree groupings. The p-levels were calculated using an analysis of variance (habitat: $F_{1, 78} = 11.239$; growth form: $F_{1, 78} = 0.664$; site: $F_{3, 78} = 3.497$). The means of rows sharing the same letter (A or B) do not differ significantly from one another (site means compared using a Tukey-Kramer Honestly Significant Difference (HSD) test; $q^* = 2.627$, $p < 0.05$).

	Mean ± standard deviation		p-level
Habitat			
Inland	-0.455 ± 0.047	A	0.001**
River	-0.415 ± 0.060	B	
Growth form			
Short	-0.440 ± 0.067	A	0.418
Tall	-0.430 ± 0.045	A	
Site			
Phugwane	-0.457 ± 0.047	A	0.020*
Joao	-0.451 ± 0.032	AB	
Mpongolo	-0.420 ± 0.070	AB	
Shingwedzi	-0.410 ± 0.062	B	

Tree morphology

The mean heights of all trees measured on river lines (n = 238) and inland lines (n = 240) were not significantly different when compared using a t-test (t_{476} = -1.438, p = 0.151). Riparian trees were however significantly taller than inland trees when the thirty tallest trees measured on river and inland lines were compared (Figure 4; t_{58} = -4.187, p < 0.000). A similar comparison of the thirty shortest trees was not significant (Figure 5; t_{58} = -0.031, p = 0.976). There was no significant variation in tree height between sites when analysed using a one-way ANOVA ($F_{3, 474}$ = 0.579, p = 0.629).

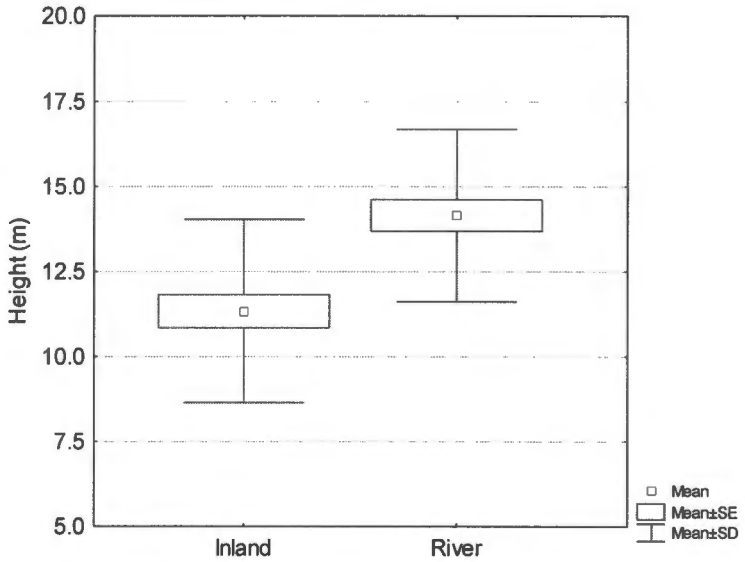

Figure 4: Boxplot comparing the heights of the 30 tallest trees from all inland and river lines.

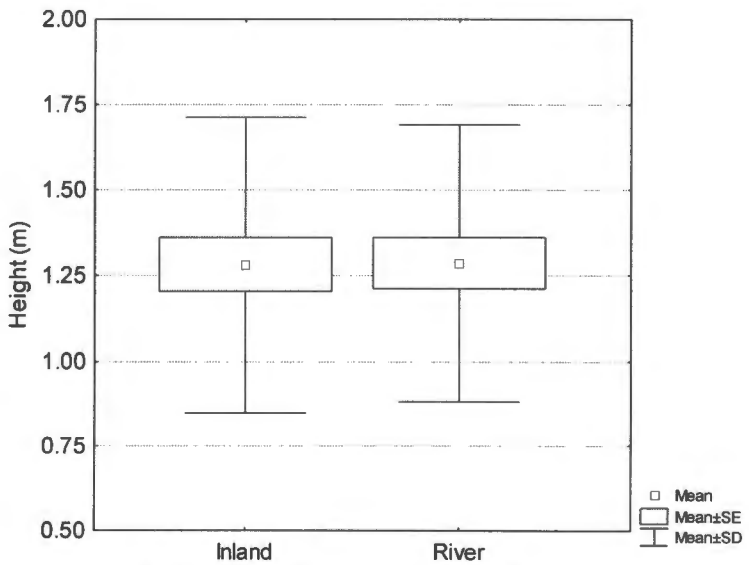

Figure 5: Boxplot comparing the heights of the 30 shortest trees from all inland and river lines.

DNA sequencing

There was no variation in the *trn*C (951 base pairs), *psb*AF (456 base pairs) and *rps*16F (913 base pairs) plastid sequences of the samples which were screened (8, 7 and 6 samples respectively). Two haplotypes were observed in the *trn*S plastid sequences (7 samples), which differed by a 16 base pair insertion (-AATTAATAAATATTGT-) situated between base positions 307 and 322 in the 963 base pair sequence. The two individuals which shared the insertion haplotype were a tree form *C. mopane* on the Mpongolo river line and a shrub form *C. mopane* on the Joao river line. The haplotype lacking the insertion was shared by shrubs on the Phugwane, Mpongolo and Shingwedzi river lines and trees on the Shingwedzi and Joao river lines. There was no variation in the 5*S* nuclear spacer region sequences (572 base pairs, 8 samples).

ISSR polymorphism

The five primers included in the full ISSR dataset produced 39 scorable fragments ranging in size from 3500 to 250 base pairs in length. Of these fragments, 13 were polymorphic among 41 individuals giving a percentage polymorphism value of 33.3%. Four of the nine primers screened produced banding patterns which were included in the conservative dataset. These four primers produced 23 bands of which 9 (39.1%) were polymorphic. The sampled individuals did not group according to site, height or habitat when the similarities of their banding patterns were assessed by producing dendrograms for the full dataset (Figure 6) or the conservative dataset (Figure 7).

The AMOVA results for both the conservative and full ISSR datasets show that very little of the observed variation can be explained by combining *C. mopane* samples into growth form or habitat groups (Table 4). Effectively none of the variation in the conservative and full datasets is distributed amongst growth forms, while habitat groupings only account for ~1 – 4% of the variation. Most of the variation when plants were grouped by growth form lay within the site defined populations (~93 – 98%), although this was only significant in the conservative dataset ($p = 0.03$).

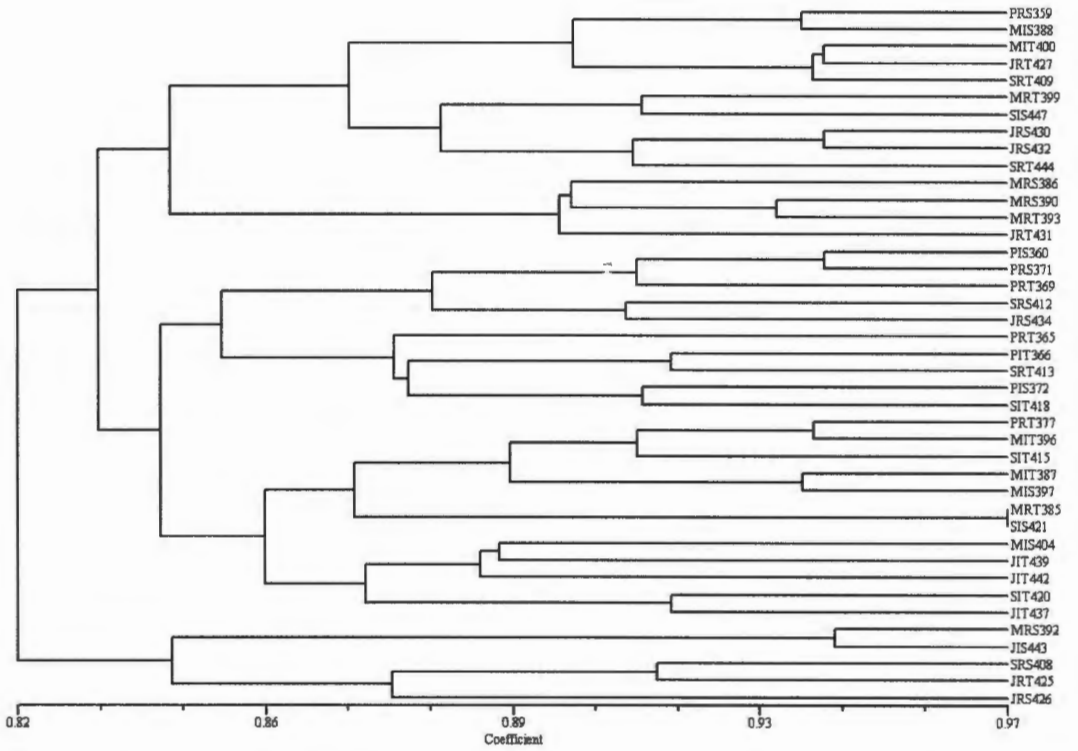

Figure 6: Dendrogram showing relationships between samples (n = 41) based on the full ISSR dataset. Sample codes consist of a site letter (J = Joao, M = Mpongolo, P = Phugwane, S = Shingwedzi), a line letter (I = inland, R = river), a growth form letter (S = shrub/short, T = tree/tall) and a unique number code.

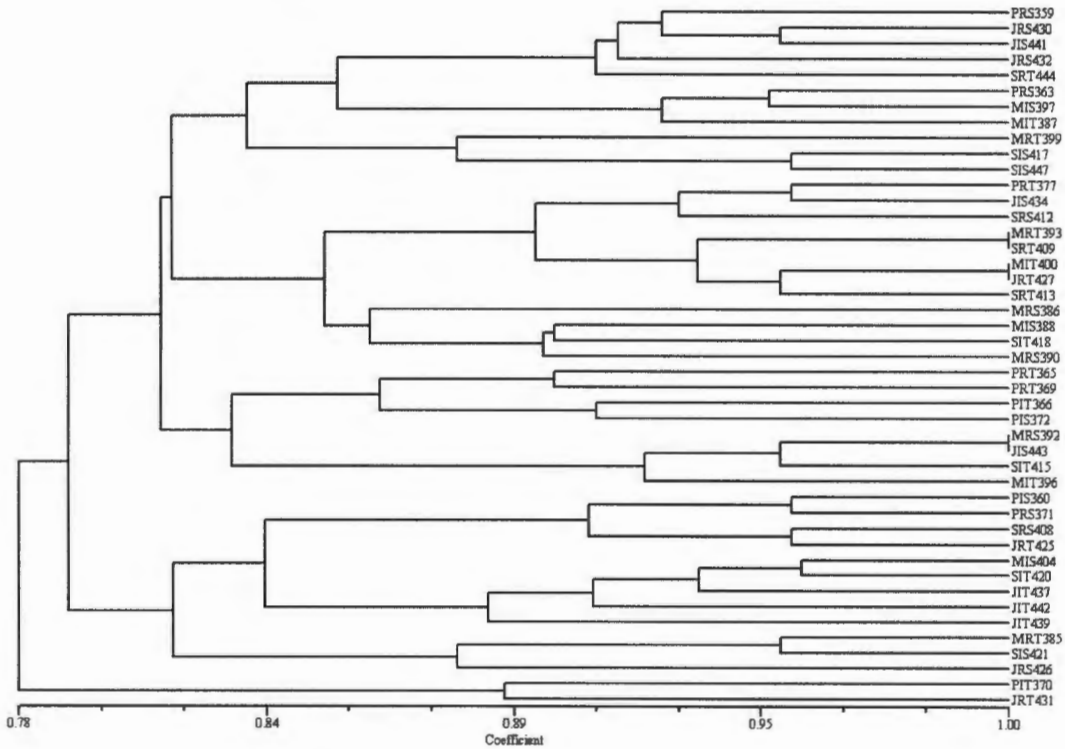

Figure 7: Dendrogram showing relationships between samples (n = 45) based on the conservative ISSR dataset. Sample codes consist of a site letter (J = Joao, M = Mpongolo, P = Phugwane, S = Shingwedzi), a line letter (I = inland, R = river), a growth form letter (S = shrub/short, T = tree/tall) and a unique number code.

Similarly, a significant amount of the variation (~89 – 92%) in both datasets lay within the site defined populations when plants were grouped by habitat (full: p = 0.01, conservative: p = 0.01). Approximately 5 – 7% of the ISSR fragment variation when plants were grouped by growth form was attributable to differences between sites, and ~4 – 9% when plants were grouped by habitat.

Table 4: Results of the analysis of molecular variance (AMOVA) for both the full and conservative ISSR datasets. The percentage values in the group and population rows refer to the percentage variation in the datasets within the respective levels of the analysis.

		Among groups	Among populations within groups	Within populations
Full dataset				
Group	Tall vs. short	-2.54%	5.30%	97.24%
Population	Sites			
p-level		0.792 ±0.013	0.083 ±0.007	0.120 ±0.010
Group	River vs. Inland	3.76%	4.53%	91.71%
Population	Sites			
p-level		0.098 ±0.010	0.061 ±0.009	0.012 ±0.003
Conservative dataset				
Group	Tall vs. short	-0.35%	7.01%	93.33%
Population	Sites			
p-level		0.476 ±0.013	0.034 ±0.006	0.029 ±0.005
Group	River vs. Inland	1.92%	8.41%	89.67%
Population	Sites			
p-level		0.242 ±0.012	0.015 ±0.004	0.008 ±0.003

Leaf and branching morphology

Leaf margin width contributed most to factor 1 in the PCA analysis (-0.810), followed by angle (-0.747) and petiole length (0.679). The contributions of branching structure (0.393) and leaf length (0.042) were the least influential on the orientation of factor 1. Factor 1 accounted for 36.63% of the total variance. Factor 2 accounted for a further 23.30% of the variation, and was most influenced by branching structure (-0.757) and leaf length (0.669). The factor 1 and factor 2 scores for each specimen did not separate samples into distinct groups when plotted against one another (Figure 8). Growth forms were weakly separated by factor 1, with tall specimens generally having higher scores.

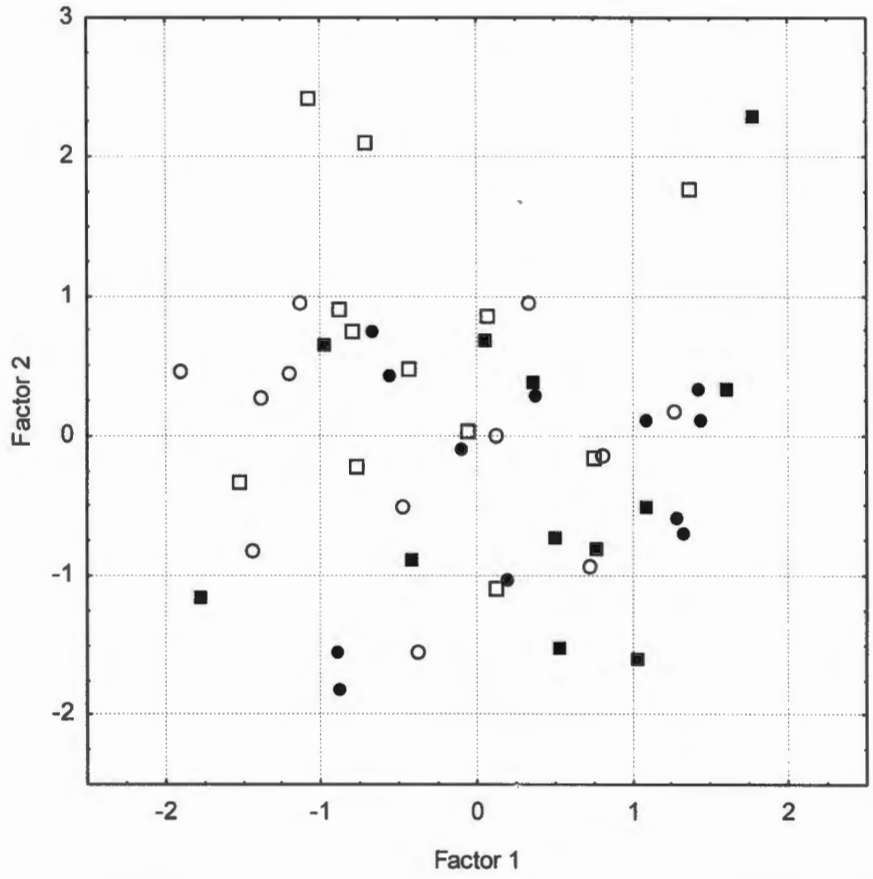

Figure 8: Plot of sample scores on factor 1 versus factor 2 from the principle component analysis of five leaf and branching morphology variables (□ river short; ■ river tall; ○ inland short; ● inland tall). Factor 1 accounts for 36.63% of the variation in the dataset and factor 2 a further 23.30%.

Discriminant analysis based on the full set of leaf and branching characters identified a significant difference between the growth forms (Wilk's Lambda = 0.648; $F_{5, 42}$ = 4.560; $p < 0.002$). Leaf margin width ($p < 0.001$) and angle ($p < 0.049$) both contributed significantly to this discrimination. The histogram of specimen scores on the canonical axis illustrates the difference between growth forms, with short trees grouping towards the negative end of the axis (Figure 9). The discriminant analysis model of specimens grouped by habitat (i.e. river versus inland) was not significant (Wilk's Lambda = 0.918; $F_{5, 42} = 0.754$, $p < 0.588$), with the result that the grouped canonical scores overlap completely when plotted in a histogram (Figure 10).

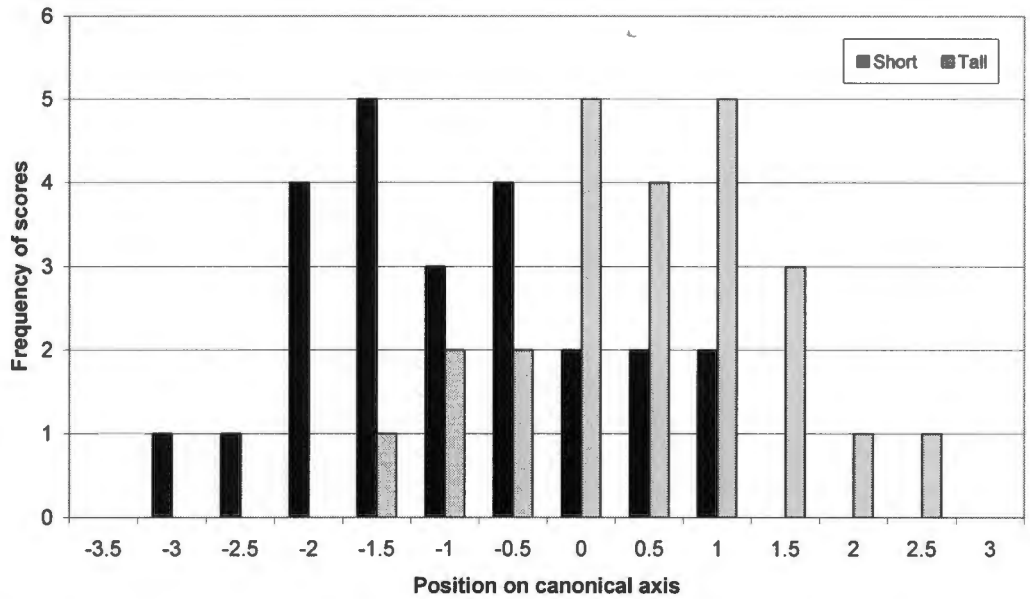

Figure 9: Histogram showing the frequencies of sample scores on the canonical axis when grouped by growth form (tall versus short) in a discriminant analysis.

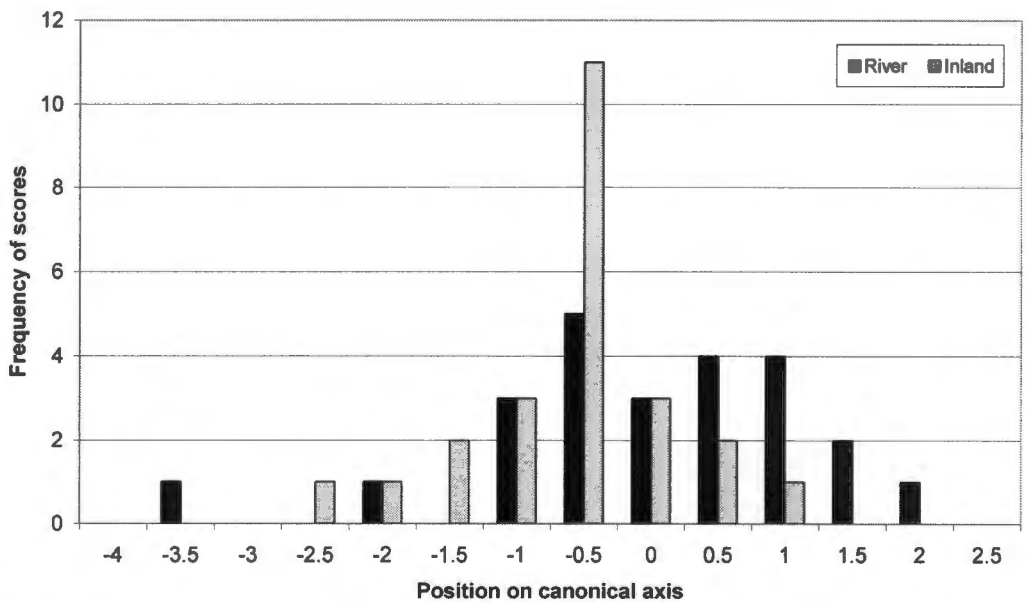

Figure 10: Histogram showing the frequencies of sample scores on the canonical axis when grouped by habitat (river versus inland) in a discriminant analysis.

Discussion

Neither the molecular sequence data nor the ISSR fingerprinting results support the hypothesis that *C. mopane* growth forms are genetically distinct entities. Very little variation was observed in the plastid and nuclear regions that were sequenced. Both haplotypes that were encountered in the *trn*S-*trn*G intergenic spacer region spanned the growth form boundary, and thus do not support the notion of genetic divergence between *C. mopane* trees and shrubs.

The sampling of multiple independent loci is important, as lineage sorting at any single locus may cause alleles in different species to be more closely related than the species are (Page & Holmes 1998). Thus, tokogenetically distinct lineages (e.g. the hypothesised *C. mopane* trees and shrubs) may be genetically indistinct at one or more neutral markers. The ISSR technique amplifies fragments from across the entire nuclear and plastid genome, thereby sampling multiple loci that have the potential to assort independently. This technique thus limits the potential for the process of lineage sorting to mask genetic variation attributable to growth form.

The dendrograms illustrating the similarity of ISSR fragment banding patterns between individuals do not identify any groupings that correspond to the two growth forms. Consequently, the analysis of molecular variance of the ISSR data indicates that effectively none of the genetic structure in *C. mopane* is distributed among growth forms. It can only be concluded from the genetic analysis that *C. mopane* growth forms are not genetically distinct.

ISSR fingerprinting is a very useful tool for assessing clonal diversity among plants (Li & Ge 2001), as samples of vegetatively produced entities should show identical ISSR profiles. This study, while designed to limit the sampling of apparent individuals linked by root suckering (i.e. by sampling individuals >100m apart), found no identical profiles across the individuals sampled, confirming that none of the plants assessed were vegetatively produced clones. Variation in the polymorphisms evident in the ISSR profiles was found by the analysis of molecular variance to occur largely within study sites. This supports the conclusion of Villeon *et. al.* (2003) that *C. mopane* has the potential for relatively long distance and effective outcrossing.

The very low growth rates of *C. mopane* were initially interpreted as evidence that a genetically determined limit to plant size had been achieved in individuals of both tree and shrub growth forms. The lack of genetic distinctiveness between growth forms, however, suggests that these low growth rates may be an intrinsic feature of *C. mopane* at a wide range of developmental stages and across a range of habitats. Low growth rates are a common feature of plants occurring in environments with low water availability (Kramer & Boyer 1995), and may not be free to vary in *C. mopane*.

C. mopane growth form does not appear to be abiotically determined at the scale of the sampling in this study. While soil samples were not taken alongside each individual included in the study, there was no significant difference in the depth, structuring or texture of soil horizons at either ends of the four inland sampling lines. The structuring of the soil profiles along the river lines was more variable as a result of their depositional origin (MacVicar *et. al.* 1977), but soil depth at all but one point was greater than 110cm. Shrub and tree *C. mopane* were encountered arbitrarily along the full length of all sampling lines, despite the general homogeneity of soils. Soil parameters are not able to predict the distribution of *C. mopane* growth forms.

The xylem pressure potentials of *C. mopane* suggest a small but significant difference between river and inland sampling lines, which is likely attributable to greater water availability along rivers, and possibly the depth at which inland trees are sourcing water. There was, however, no significant difference between the pressure potentials of the *C. mopane* growth forms, even when assessed between riparian and inland sites using the multiple-factor ANOVA. This suggests that access to soil water is not a major determinant for the distribution of shrub and tree form *C. mopane*.

The significant difference in the heights of the thirty tallest river and inland *C. mopane* trees may be a response to a greater availability of water along the rivers than further inland. The generally coarser texture of the river soils may contribute to this effect, as finer soils have a greater internal surface area which can reduce plant-available water (Kramer & Boyer 1995). The lower mean xylem pressure potentials of trees at the Shingwedzi site is likely to be an artefact of a raised ground water table due to the presence of the Kanniedood dam a few kilometres downstream.

The analysis of leaf and branching morphology revealed the existence of semi-distinct leaf morphologies between the *C. mopane* growth forms. Leaf margin width and angle were identified by the principle component analysis and the discriminant analysis as being the major leaf morphology characters separating growth forms. These are the only parameters of the five measured that describe leaf shape. In their assessment of *C. mopane* leaf morphology, Potgieter & Wessels (1998) do not describe variation in leaf shape parameters, and also limit their analysis to tree form *C. mopane*. Their work shows that *C. mopane* produces larger leaves during the middle of its growth season, the growth period to which the leaves measured in this study should thus principally correspond (Potgieter & Wessels 1998).

The significant distinction between shrub form *C. mopane* having more rounded leaflets than tree form *C. mopane* is not readily explained. Genetic and potential abiotic factors have been shown to not have any role in distinguishing between growth forms, and thus cannot be used to explain the variation in leaf shape between trees and shrubs. It may be that the pattern is simply an artefact of the sampling strategy, with shrub *C. mopane* leaves having been collected from the top of the plants, and tree *C. mopane* leaves being collected at approximately a quarter to half the height of the plants. Alternately, it may be a response to taller *C. mopane* individuals having access to a more permanent water supply deeper in the soil profile.

The evidence from the plastid and nuclear molecular sequence data as well as the ISSR fingerprinting technique suggest that there is no genetic distinctiveness between the tree and shrub *C. mopane* growth forms. A broader landscape scale distribution of *C. mopane* growth forms that correlates with geology is well documented (van Rooyen *et. al.* 1981, Fraser *et. al.* 1987). At the scale of this study, however, the distribution of tree and shrub *C. mopane* growth forms can not be predicted by the soils or the degree of water stress. The stochasticity of the distribution of tree and shrub *C. mopane* at this finer scale could thus be as a result of top down controls such as large mammals and fire (Styles & Skinner 2000, Smallie & O'Connor 2000, Kennedy & Potgieter 2003).

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