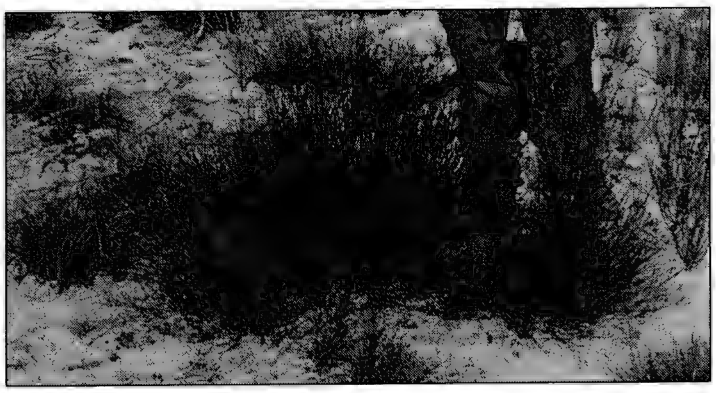
MORPHOLOGICAL AND MOLECULAR ANALYSES FOR THE CHARACTERIZATION OF *ASPALATHUS LINEARIS* DAHLGR. (FABACEAE; 'ROOIBOS')

Alastair Potts

Supervisors: Tony Verboom and Timm Hoffman

University of Cape Town



Submitted in fulfilment of Botany Honours (Plant Ecology), UCT 2006

KD POTT

MORPHOLOGICAL AND MOLECULAR ANALYSES FOR THE CHARACTERIZATION OF *ASPALATHUS LINEARIS* DAHLGR. (FABACEAE; 'ROOIBOS')

Abstract:

I investigated the infraspecific diversity among individuals of *Aspalathus linearis* from five localities in the Cederberg Fynbos region using morphological traits and two DNA-based methods: inter-simple sequence repeats (ISSR) and a plastid marker. A principle component analysis (PCA) based on seven morphological variables grouped the samples into three different growth forms, specifically an erect-form, a prostrate-form and a shrub-form. The genetic variability of four plastid markers and one nuclear marker was determined for seven individuals selected from the populations. The trnL$'^{UAA}$F-trnF^{GAA} plastid marker was used to amplify the remaining samples and revealed four haplotypes, with a basal haplotype fixed in both the prostrate form and a single population of the erect form, a unique haplotype fixed in the shrub form, and a mix of two other haplotypes in the remaining erect forms. The congruence between haplotypes and regeneration strategy suggests a longstanding pattern of restricted gene flow. However, a small sample size associated with the plastid marker data limits or reduces the certainty of these findings.

Introduction:

Aspalathus (Fabaceae, Tribe Crotalarieae) consists of 272 species and is the second largest genus of vascular plants in the Cape Floristic Region (Goldblatt and Manning 2002). The genus is and is endemic to South Africa and most of the species are concentrated in the Cape Floristic Region, with only six extending to southern KwaZulu-Natal. *Aspalathus linearis* (BURM. F) DAHLG. is an important commercially cultivated crop known as rooibos. The leaves are processed and used to make rooibos tea. *Aspalathus linearis* is endemic to South Africa and extends through most of the Western Cape and into the Northern Cape. This species plus three other species form a distinct group within *Aspalathus* that have simple, needle-like leaves, in contrast to the rest of the genus that have trifoliate leaves. It is a species that displays extraordinary variation in morphology, with distinct geographic growth forms. Dahlgren (1968) gives a detailed account of the morphology and distribution of wild growth forms, splitting the species into three subspecies based on this variation. However, the uncertainties surrounding the natural groupings within the species are reflected in his later revision, where the subspecies taxonomy is abandoned (Dahlgren 1988). The species occurs across a wide geographic range, where it exists as a series of partially allopatric populations (van der Bank 1999) which differ in a number of respects, specifically growth form, fire-survival strategy, vegetative and reproductive morphology, isozyme patterns and flavonoids (Dahlgren 1968; van der Bank *et al.* 1995, 1999).

Wild populations of *A. linearis* have been noted to contain both resprouting and reseeding individuals (Schutte *et al.* 1995; van der Bank 1999). Fynbos is a fire driven system in which plant reproduction is largely centred around recurrent fires (Cowling 1987). In general, resprouting and reseeding are mutually exclusive strategies (Vesk *et al.* 2004) used by plants in response to periodic natural disturbances that result in loss of above ground biomass, such as fire. Resprouters are able to regenerate from subterranean lignotubers after a fire, whereas reseeders are killed by fire and must re-establish through seeds. There is a tendency for seeds of reseeders to germinate more rapidly than those of resprouting species (Schutte *et al.* 1995). Reseeding is the dominant strategy found in most fynbos legumes, with the resprouter/reseeder ratio being reported as less than 25% (Le Maitre & Midgley 1992). Van der Bank *et al.* (1999) note that all populations of *A. linearis* can be classified as reseeding or resprouting and that there are no known intermediates.

The rooibos tea industry is based exclusively on the cultivation of a single 'Rockland's' type which occurs naturally in a small geographical area. This cultivated form is a reseeder with an erect and densely branched growth form. This 'Rockland's' type has been planted far beyond its original distribution because rooibos cultivation has gained major economic importance. Farmers tend to plant close to wild populations to ensure optimal abiotic and biotic environmental conditions. If the wild and cultivated forms are interfertile, the genetic integrity of the wild populations may thus be at risk from the cultivated type, an issue first raised by van der Bank *et al.* (1995). If new or locally rare alleles persist in wild populations, gene flow between numerically dominant cultivated populations and wild populations may lead to evolutionary changes in the latter. There are many reports of hybridisation occurring between cultivated species and their wild relatives (reviewed in Ellstrand *et al.* 1999) yet the consequences of hybridisation are still to be established. These could range from species extinction (through swamping and outbreeding depression) to beneficial effects (e.g. the introduction of favourable alleles).

Despite the detailed work by Dahlgren (1968, 1988) there is still no satisfactory infraspecific classification system and the affinities of the various forms remain poorly understood. In this study, both morphological and molecular techniques are used to evaluate the infraspecific diversity of *Aspalathus linearis* within a relatively small geographic area. Both morphological and molecular data are used because they are complementary, and most likely non-linked, sources of phylogenetic information.

The morphological data is assessed within the framework of a morphometric analysis. A morphometric analysis is the quantitative examination of biological diversity and has been used extensively in plant systematics (reviewed in Henderson 2006). I use the multivariate principle component analysis (PCA), which is a commonly used method in morphometrics (Henderson 2006). Two DNA-based techniques are used, specifically the inter-simple sequence repeats (ISSRs; Ziętkiewicz *et al.* 1994) and plastid nucleotide sequence data, to try to clarify any taxonomic groupings and metapopulation coherence within *A. linearis* populations occurring in the Northern Cederberg.

The ISSR method has been widely used to evaluate genetic diversity in plants (e.g. Tsumura *et al.* 1996; Godwin *et al.* 1997; Wolfe & Randle 2001; Barth *et al.* 2002; Yasodha *et al.* 2004), determine systematic relationships between species (e.g. Wolfe & Randle 2001) and facilitate cultivar identification (e.g. Rotandi *et al.* 2003, Sica *et al.* 2005). ISSRs sample loci that are interspersed throughout the genome and result in multiple products of anonymous loci. The primers bind to numerous sites in the genome and any sequence that is within a suitable distance of two primers bound in opposite orientation will be amplified. The lengths of the amplified loci determine the banding patterns for each individual. If bands are found to be polymorphic, the absence of a band is treated as mutational change/s at the primer annealing sites, but could also be due to structural chromosomal rearrangements (Wolfe & Liston 1998). PCR primers consisting of a core sequence of repeated di-, tri-, tetra-, or pentanucleotide motifs are used to generate ISSR markers (Barth *et al.* 2002). The loss or gain of binding sites and insertion/deletion events (indels) within the genome will be detected as polymorphic bands (Yang *et al.* 1996). The ISSR method has a number of advantages (Barth *et al.*, 2002): (i) low development and overall costs, (ii) no prior information or mapping studies are required, and (iii) the method can easily be used on any plant species.

Plastid DNA sequences have been widely used in plant phylogenetic, phylogeographic and population level studies (Álvarez & Wendel 2003). DNA sequences are used to estimate the variation within gene regions. Non-coding gene regions, such as the ones used in this study, are presumed to offer more resolution at lower taxonomic levels as they are not under adaptive selective pressures and thus evolve (accumulate mutations) relatively fast. Neutral markers therefore provide more phylogenetically informative characters relative to coding regions of the same length (Clegg *et al.* 1994). The mutations in the sequence data give rise to haplotypes which can be used to infer relationships between populations in a species complex. Plastid DNA is maternally inherited and does not undergo recombination, and this means that effective population size is reduce to one quarter of its actual size. Because of this, plastid DNA tends to detect divergence between lineages earlier than nuclear DNA.

ISSRs represent an indirect comparison of DNA variation, since it has the inherent assumption that bands of the same length are homologous. In contrast DNA sequencing provides the means for direct comparison by detecting polymorphic loci where nucleotide changes occurred. Different parts of a single organism's genome may have different evolutionary histories due such effects as incomplete lineage sorting and introgression.

Using many gene regions from the different genomes can reduce the effects of separate evolutionary histories of specific gene on the results. As the cost for such a study is prohibitive and there are only a limited number of loci that can be amplified given the current availability of primers, this study will use ISSRs to confirm or contest the findings using a single gene region.

The objectives of this study are to evaluate infraspecific morphological variation in *A. linearis* within the Cederberg region in a quantitative manner, and to make use of two molecular techniques to determine whether any morphological groupings identified are supported by variation in molecular characters.

Method:

Sampling

Aspalathus linearis populations were sampled from five localities in the Cederberg mountain range (Figure 1). Four of the localities were geographically close (< 20 km apart). These localities were selected to minimise the effects of population isolation on any patterns observed. Seven populations were sampled from five localities as two identifiable growth forms found at Witbank were sample separately, and wild and cultivated populations were sampled at Landskloof. For each growth form within the separate localities, 8-11 individual plants were selected for morphological analyses, and leaf material was collected from the same individuals for genetic analyses. Between 2 and 5 of these individuals were used in the genetic analyses. Two representative herbarium voucher specimens were taken for each growth form found in each locality, and accurate locality data were record by GPS. A bioclimatic envelope based on climatic parameters has been generated for *A. linearis* in the region of the Northern Cederberg and Bokkeveld plateau region (Louw *et al.* unpublished; Figure 1). The bioclimatic envelope distinguishes between suitable and unsuitable areas for wild populations of a species.

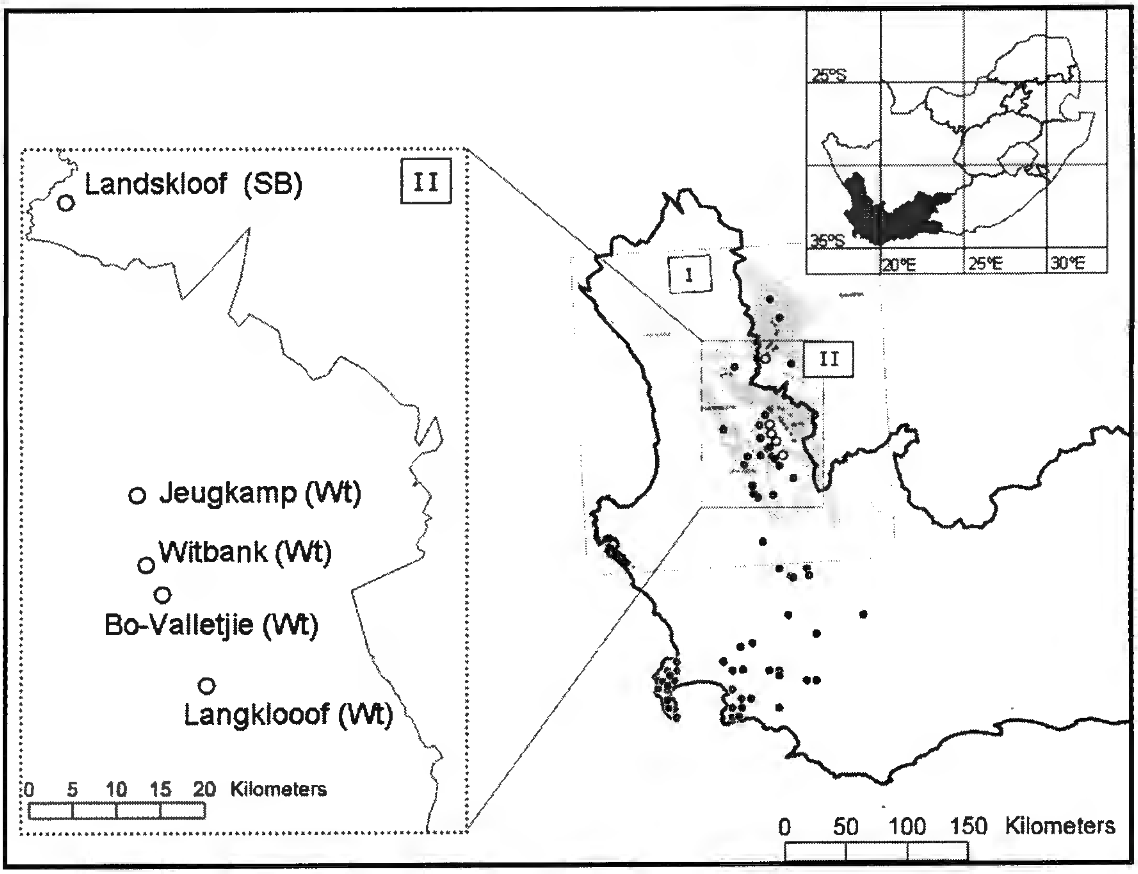


Figure 1: A distribution map of *Aspalathus linearis* with the localities (○) sampled in this study (Box II; Wt = Wupperthal, SB = Suid Bokkeveld). Collection sites reported by Dahlgren (1968) are shown as solid circles (●). A bioclimatic envelope, which is the potential distribution, for *A. linearis* in the northern Cederberg and Bokkeveld plateau is shown as the shaded area in Box I (Louw *et al.* unpublished).

Morphometric analysis

For the morphometric analysis, between 8 and 11 plants were sampled for each growth form found in the five localities. The morphological characters that were measured are summarised in Table 3. The height to diameter ratio was calculated as the pre-harvest height divided by the average of the length and breadth of a plant.

All morphological data were tested for normality using the Lilliefors Test, and then analysed by means of an analysis of variance (ANOVA) and a post-hoc Tukey HSD test at a 5% level of significance using the STATISTICA 7.0 computer package (STATISTICA for

Windows, StatSoft Inc.). The morphological variables were then standardized and subjected to a PCA (Joliffe 1986) performed in NTSYS-PC v2.10 (Rholf 2000). PCA is an ordination technique that captures the maximum variance within a dataset in as few dimensions as possible (James & McCulloch 1990). The data was standardized because PCA results are sensitive to units of measurement, which are often chosen arbitrarily.

DNA extractions

Genomic DNA was extracted from dried leaf material stored in silica using a modified version of the extraction method of Gawel and Jarret (1991). Polyvinylpyrrolidone-40 (PVP) was added when grinding the leaf material in liquid nitrogen using a mortar and pestle. Three to five samples for each growth form from each of the populations were used for DNA analysis. Total DNA concentrations were measured for each sample using an ND-1000 Spectrophotometer (NanoDrop Technologies, USA), to check for the presence of genomic DNA and then to dilute all samples to a concentration of 10 ng/µl.

ISSR primer selection, amplification and scoring

In a preliminary study, a total of twelve UBC primers (University of British Columbia ISSR primer set # 9; http://www.biotech.ubc.ca/naps) were tested on seven individuals representing an individual from each morphological population. Three primers were selected (Table 1) that gave the clearest and most reproducible banding pattern with a large number of scorable bands. ISSR PCR reactions were carried out in reaction mixtures of 30 µl, consisting of 17.6 µl sterile water, 3 µl 10X *Taq* polymerase reaction buffer (Bioline, London, UK), 3 µl of Mg^{2+} (50 mM), 1.2 µl dNTP mix (2.5 mM), 4 µl of primer (10 µM) and 0.2 µl SuperTherm *Taq* DNA polymerase (JMR Holdings, UK). ISSR PCR amplifications were carried out in a GeneAmp 2700 PCR System (Applied Biosystems, USA) with an initial denaturing step of 1-min 30-s at 94°C; 35 cycles each comprising 50°C (52°C for UBC primer 889) for 1-min 30-s, 72°C for 1 min, 2-min at 49 °C; and a final 3-min extension step at 72° C, after which the reactions were maintained at 4 °C until run on a gel.

Table 1: List of ISSR primers (University of British Columbia primer set # 9) screened for polymorphic bands.

Primer	Sequence	Primer	Sequence
813*	CTC TCT CTC TCT CTC TT	857	ACA CAC ACA CAC ACA CYG
814	CTC TCT CTC TCT CTC TA	859	TGT GTG TGT GTG TGT GRC
824	TCT CTC TCT CTC TCT CG	860	TGT GTG TGT GTG TGT GRA
840*	GAG AGA GAG AGA GAG AYT	873	GAC AGA CAG ACA GAC A
845	CTC TCT CTC TCT CTC TRG	889*	DBD ACA CAC ACA CAC AC
852	TCT CTC TCT CTC TCT CRA	890	VHV GTG TGT GTG TGT GT

***** *indicates primers used for scoring***

All samples were given a unique letter code generated randomly in Microsoft Excel for each primer and then re-sorted alphabetically. This was done to avoid possible scoring bias associated with any *a priori* knowledge of the samples. Eight microlitres of each PCR product was electophoresed for 90 minutes in 1.7% agarose gel, stained with1.4 µl ethidium bromide (0.5 µg/ml), and run at 80 volts in a 0.5X TBE buffer solution. The gels were then soaked for ten minutes in 0.5X TBE buffer solution with a high ethidium bromide concentration. Each gel contained three lanes with reference samples, one on either end and one in the middle. Gels were then visualized and photographed under UV light using a UVIDOC digital imaging system (UVITEC, Cambridge, U.K.). All gel photographs were saved as both hardcopy printouts and in digital format. The bands obtained from all the individuals of *A. linearis* were scored as present (1) or absent (0), with homology of bands ensured by comparison with the reference samples. Any samples that lacked clear banding in the gels for any primer were discarded from the analysis. Forty-four binary characters for 28 samples were then used as a combined ISSR dataset.

Once the gels were scored, the random unique codes were converted back into the individual identification codes. The raw data from the three primers were combined and used in a cluster analysis implementing an unweighted pair-group method (Jaccard coefficient) with arithmetic averages (UPGMA) in NTSYS-PC v2.10 (Rholf 2000). The robustness of the resulting pattern was evaluated by bootstrapping with 10,000 replicates in WINBOOT (Yap & Nelson 1996). In addition to the distance analysis, banding patterns were analysed with maximum parsimony (MP) using PAUP* 4.0 (Swofford 2002). Clade support was calculated with 2000 bootstrap replicates (Felsenstein 1985) and analysed using the following search parameters: a heuristic search with random sequence addition and 1000 replicates, TBR branch swapping, MULTREES in effect and MAXTREES set to 1000. ARLEQUIN (Excoffier *et al.* 2005) was used to perform an analysis of molecular variation (AMOVA; Excoffier *et al.* 1992) on the combined primer data. Groups were defined for the AMOVA by population.

DNA primer selection, amplification, sequencing and analysis

Four of the fastest evolving regions of the plastid genome (Shaw *et al.* 2005) and one region from the nuclear genome were amplified using PCR for seven samples representing all populations and morphological forms (Table 2). These samples were then examined for variability. Based on this survey, the primers trnL5′^{UAA}F and trnF^{GAA} (Taberlet *et al.* 1991) were selected to amplify all the samples because they showed haplotypic variability and have been extensively used in other plant studies (Shaw *et al.* 2005), although an alternative candidate could have been the trnT^{UGU}F - 5′trnL^{UAA}R region. PCRs were performed in volumes of 30 µl containing 2 µl of template DNA, 3 µl of 10X *Taq* polymerase reaction buffer (Bioline, London, UK), 3 µl $MgCl_2$ (50mM), 1 µl of each primer (10 µM), 1.2 µl of dNTPs (10 mM), 0.2 µl *Taq* polymerase and sterile H_2O up to 30 µl. PCR for all primers was performed on a GeneAmp 2700 PCR System (Applied Biosystems, USA) with an initial 2 min denaturing step at 94°C; 28 cycles, each comprising 94°C for 1 min, 50°C for 30 s, 72°C for 1 min; and a final 6-min extension step at 72°C. PCR products were sequenced using BigDye technology run on an ABI 3300 sequence analyser by Macrogen, Korea (http://dna.macrogen.com).

Table 2: The five primer pairs used to generate sequence data from seven *Aspalathus linearis* individuals from five localities representing three morphological forms.

Primer	Region	Source
trnL′^{UAA}F* - trnF^{GAA}*	Plastid	Taberlet *et al.* (1991)
trnT^{GGU} - trnD^{GUC}F*	Plastid	Demesure *et al.* (1995)
trnS^{GCU}* - trnG^{UCC}	Plastid	Shaw *et al.* (2005)
trnT^{UGU}F* - 5′trnL^{UAA}R	Plastid	Taberlet *et al.* (1991)
PIII-PIV*	Nuclear	Cox *et al.* (1992)

**** represents the primers used for sequencing. trnL′^{UAA}F - trnF^{GAA} was the only gene region sequenced in both directions.***

All sequences for each region were assembled and edited using CODON CODE ALIGNER 1.6 (www.codoncode.com/), then aligned in CLUSTAL W (www2.ebi.ac.uk/clustalw/) set to default parameters. The resulting data sets were analysed for each marker based on uncorrected *p* distances in PAUP* 4.0 (Swofford 2002). Haplotypes for the trnL′^{UAA}F - trnF^{GAA} plastid assemblages were identified using a parsimony analysis in PAUP* 4.0 (Swofford 2002). This involved a heuristic search with 1000 random addition sequence replicates, TBR branch swapping algorithms, MULTREES in effect, and a MAXTREES limit of 1000. The tree was rooted on an accession of *Aspalathus cordata* obtained from GenBank (AF518124; Crisp & Cook 2003).

Results

The means and standard deviations for seven variables ~~are~~ from 70 individuals from five localities are reported in Table 3. The PCA for the seven morphological variables is shown in Figure 2. The eigenvalues obtained by PCA indicate that two or three components provide a good summary of the data, where the first two components accounted for 72.4% of the variance, and the first three explained more than 83.4%. Subsequent components contribute less than 5% each. The populations were grouped into three distinct clusters. The first cluster, labelled the 'erect' form, is comprised of individuals from Witbank, Bo-Valletjie, Jeugkamp and the cultivated crop from Landskloof. The population at Bo-Valletjie grouped within the erect form, but it is a much larger, tree-like form, which had the greatest average leaf length (Table 3). The erect form was characterised by shorter average leaf length (with the exception of the Bo-Valletjie population), and a high, upright, branching angle with fewer number of stems at a height of 5 cm above the ground, a range of height to diameter ratios, and a variable height of first branching. The second cluster, labelled the 'prostrate' form, is comprised of individuals from Witbank and Langkloof. The third cluster, labelled the 'shrub' form, only contains individuals sampled from the wild population at Landskloof. Both the prostrate and shrub forms were characterised by longer leaves and a greater number of stems at 5 cm above the ground with a low, prostrate, branching angle (Table 3). The variables that separate the prostrate and shrub forms from the erect form are the average leaf length, the number of stems at 5 cm and branching angle (Figure 2.B). The shrub form was separated from the prostrate forms by a significantly greater height to diameter ratio with a significantly smaller average stem diameter at 5 cm than the prostrate forms (Figure 2.B; Table 3). The prostrate forms are very distinctive in the PCA ordination even though the two populations visited differed significantly in leaf length. The prostrate form and shrub form have many branches at the base, which in the prostrate form lie flat on the ground and in the shrub form lie relatively flat at first but then curve upwards.

Table 3: Mean (± std. dev.) values for seven morphological traits recorded in seven populations from the Wupperthal, Biedouw Valley and Suid Bokkeveld regions of the northern Cederberg. Dissimilar superscripts denote significant differences between populations at $p<0.05$. All quantitative data were analysed statistically using one-way ANOVA and post-hock Tukey HSD in Statistica 7.0 software.

Location	Bo-Valletjie	Witbank	Jeugkamp	Landskloof	Landskloof	Witbank	Langkloof
	32° 17' 38"E	32° 15' 33"E	32° 10' 46"E	31° 49' 51"E	31° 49' 36"E	32° 15' 33"E	32° 24' 07"E
GPS Coordinates	19° 11' 17"S	19° 10' 14"S	19° 09' 31"S	19° 04' 22"S	19° 04' 08"S	19° 10' 14"S	19° 14' 26"S
Growth Form	Erect	Erect	Erect	Erect (Cultivar)	Shrub	Prostrate	Prostrate
Sample Size	10	8	11	9	10	8	10
AVG leaf length	46.9 ± 6.9^{a}	22.4 ± 2.4^{c}	24.9 ± 1.8^{c}	24.1 ± 3.1^{c}	33.4 ± 5.3^{b}	25.9 ± 1.6^{c}	53.2 ± 8.6^{a}
Number of stems at 5 cm	2.7 ± 2.2^{c}	$4.8 \pm 1.2^{b,c}$	$3.5 \pm 2.5^{b,c}$	$7.7 \pm 2.0^{b,c}$	14.2 ± 7^{b}	26.6 ± 15.2^{a}	14.5 ± 8.2^{b}
Basal stem diameter (0 cm)	32.6 ± 15.3^{a}	$25 \pm 4^{a,c}$	37.5 ± 17.7^{a}	38.7 ± 16.2^{a}	$24.6 \pm 5.2^{a,d}$	$12.3 \pm 5.7^{b,c,d}$	5.2 ± 1.9^{b}
5cm Stem Diameter AVG	17.0 ± 5.5^{a}	11.7 ± 4.5^{a}	18.6 ± 6.5^{a}	17.9 ± 5.2^{a}	15.2 ± 3.4^{a}	5.6 ± 0.5^{b}	3.2 ± 0.8^{b}
Height to Diameter ratio	2.3 ± 0.7^{a}	1.2 ± 0.3^{b}	$1.9 \pm 0.7^{a,b}$	1.1 ± 0.2^{b}	0.7 ± 0.1^{b}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}
Branching angle	57 ± 11^{a}	48 ± 12^{a}	70 ± 8^{a}	65 ± 4.3^{a}	2 ± 6^{b}	3 ± 3.7^{b}	8 ± 5.4^{b}
Height 1st branching	127 ± 157^{a}	5 ± 0^{b}	$60 \pm 92^{a,b}$	1 ± 1^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}

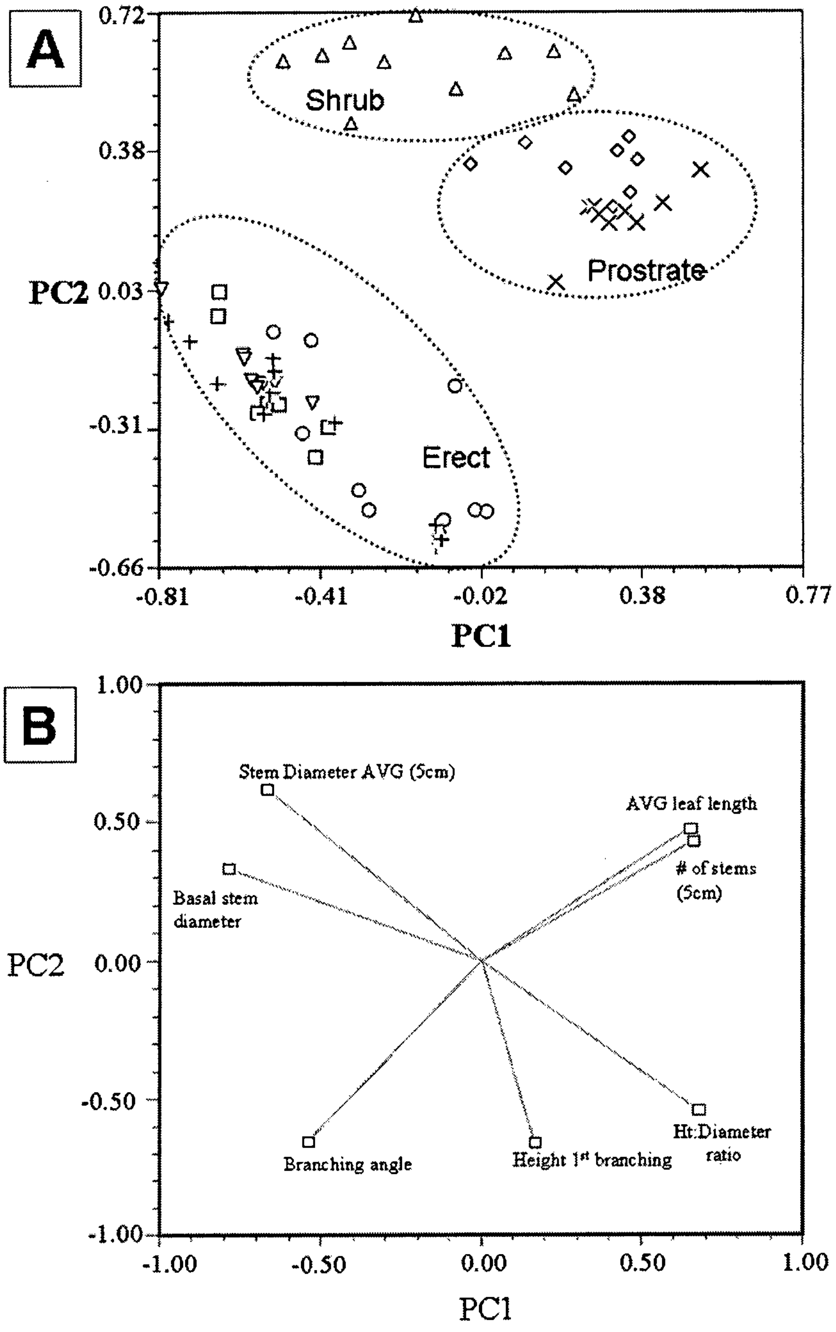


Figure 2: (A) Plot of the first two principle components (PC1 & PC2) obtained from a principle component analysis based on seven morphological variables listed in Table 3 from 70 individuals of *Aspalathus linearis* sampled from five localities in the Cederberg Fynbos region, namely Langkloof (×), Bo-Valletjie (○), Witbank (◇ & □), Jeugkamp (+), and Landskloof (cultivated - ∇, Δ-wild). Stippled lines are used to group individuals with similar morphological traits. (B) illustrates the correlation of the original variables with PC1 & PC2

Table 4: The ISSR haplotype and band summary for the combined dataset from three primers (813, 840 and 889) for seven populations of *Aspalathus linearis.*

Locality	Growth form	n	# haplotypes	# usable bands out of 44	# polymorphic bands	% of usable bands that are polymorphic
Bo-Valletjie	Erect	4	4	26	14	54
Jeugkamp	Erect	5	5	28	13	46
Landskloof	Shrub	2	2	28	6	21
Landskloof	Erect	2	2	28	12	43
Langkloof	Prostrate	5	5	44	26	59
Witbank	Erect	4	4	28	15	54
Witbank	Prostrate	3	3	44	21	48

Haplotype variation

All sequences were trimmed to exclude ambiguous sections at each end. Sequence lengths, number of distinct haplotypes and pairwise uncorrected *p* distances for each marker for seven individuals are reported in Table 5. Indel events were observed in trnL$'^{UAA}$F-trnF^{GAA}, trnT^{GGU}-trnD^{GUC}F and trnS^{GCU}-trnG^{UCC}. The uncorrected *p* distance values for the plastid primers were not found to be significantly different from one another (ANOVA, $F_{(3,83)} = 0.95$, $p > 0.4$). However the nuclear gene region PIII-PIV has a significantly greater uncorrected *p* distance value than the plastid regions (ANOVA, $F_{(4,105)} = 13.57$, $p<0.05$). The trnT^{GGU}-trnD^{GUC}F region had the highest number of haplotypes (7) and trnL$'^{UAA}$F-trnF^{GAA} had the next highest (4), and both trnDF^{GUC}-trnT^{UGU} and trnS^{GCU}-trnG^{UCC} had only two haplotypes.

Table 5: The genetic variability of four plastid (cDNA) and one nuclear markers from seven individuals of *Aspalathus linearis* sampled from five localities in the Cederberg Fynbos region. * indicates the primer used to sequence the gene region. PIII-PIV has a significantly greater D value than all the plastid markers.

Locus		Length	# of distinct haplotypes	# of B.P. difference	# of Indels	Uncorrected *p* distance (D) ($\times10^{-3}$)
trnL$'^{UAA}$F* - trnF^{GAA}*	(cDNA)	893	4	4	1	2.4±1.8
trnDF^{GUC}F* - trnT^{UGU}	(cDNA)	193	2	1	0	3.4±5
trnS^{GCU}* - trnG^{UCC}	(cDNA)	312	2	0	1	1.8±3
trnT^{GGU}F* - 5'trnL^{UAA}R	(cDNA)	779	7	7	3	3±2.2
PIII-PIV*	(nuclear)	342	5	7	0	10.3±6.3

ISSR Results

An example of an ISSR banding profile for the 889 primer is shown in Figure 3. Using the combined dataset from the three primers, each individual was found to have a unique ISSR fingerprint within each population, and each population was found to contain a high percentage of polymorphic bands (Table 4). Both UPGMA and parsimony did not identify any grouping corresponding to populations or forms and both the UPGMA dendrogram and the parsimony tree were reduced to polytomies when bootstrap results were included to form consensus trees (dendrogram and parsimony tree not shown). This implies that any structure identified was not robust due to the lack of a clear hierarchical structure. These conclusions were supported by the AMOVA (grouped by locality), which recovered 94% of variation within populations and only 6% amongst the populations (FST = 0.06; p=0.2).

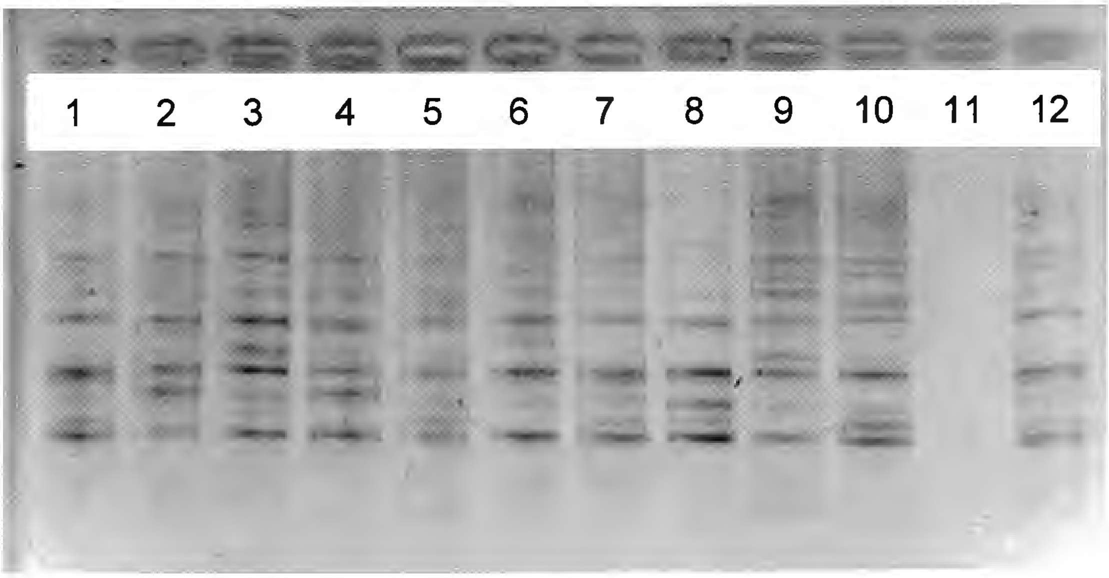


Figure 3: Example of an ISSR banding profile obtained on 1.7% agarose gel using the primer UBC 889 (University of British Columbia, kit #9) across 8 *Aspalathus linearis* individuals. Lanes 1, 7 and 12 are reference samples. Lane 11 did not amplify.

Four unique haplotypes were identified by the parsimony analysis, designated A-D (Figure 4), with three haplotypes directly connected to an ancestral haplotype (Figure 5). All of the prostrate forms and the erect form from Bo-Valletjie possess the ancestral haplotype. Note that all other haplotypes were derived from the ancestral haplotype. Both haplotypes C and D are separated from haplotype A by single transversion events. Haplotype B is separated from haplotype A by one transition, one transversion and one deletion event. The three shrub form individuals from Landskloof possess a unique derived haplotype (haplotype D). The erect individuals from Witbank, Jeugkamp and from the cultivated Landskloof population possess two other haplotypes derived from the ancestral one (haplotypes B and C). The haplotype frequency for each form from each locality is summarized in Table 6. An AMOVA was not applied to the haplotypic data because no haplotypes were shared between all populations and forms, and also because the haplotypes display a very simplistic network. The AMOVA includes a component that looks at the frequency of haplotypes within groups, and so that some haplotype sharing is required amongst groups.

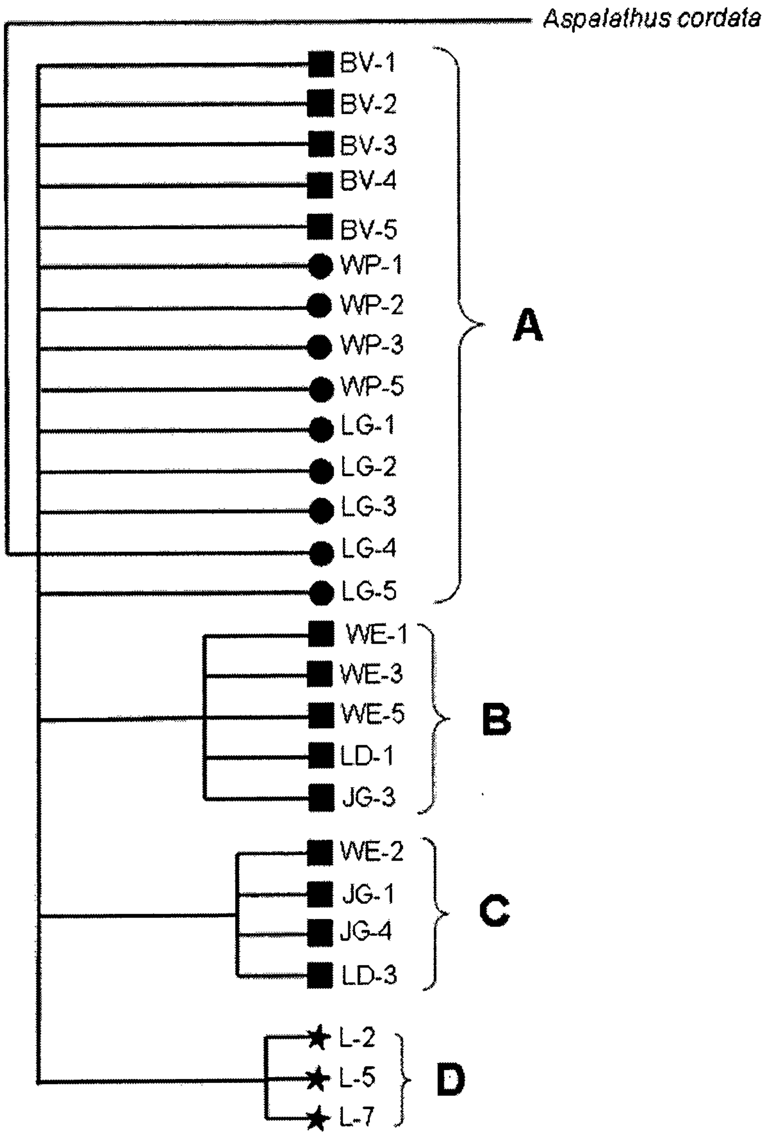


Figure 4: The parsimony tree of 28 trnL$^{'UAA}$F-trnF^{GAA} sequences of *Aspalathus linearis* sampled from five localities within the Cederberg region (BV – Bo-Valletjie; WP – Witbank; WE – Witbank; LG – Langkloof; LD – Landskloof cultivated population; L – Landskloof wild population), including the three forms identified in the morphological analysis, namely the prostrate (circles), erect (squares) and shrub (stars) forms. A single accession of *Aspalathus cordata* is used as an outgroup.

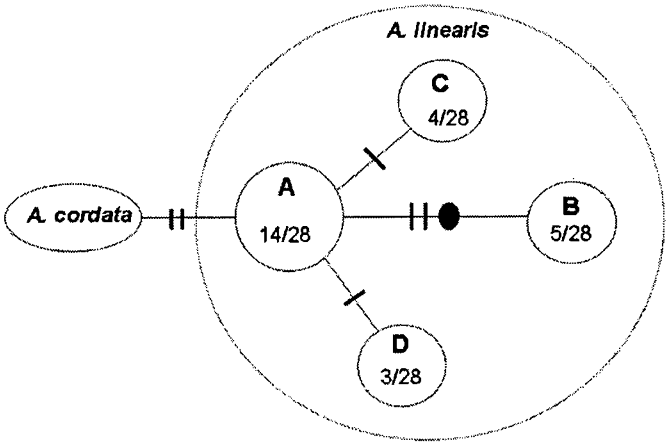


Figure 5: Haplotype network of 28 trnL$^{'UAA}$F – trnF^{GAA} sequences of *Aspalathus linearis* sampled across five localities in the Cederberg Fynbos region. An accession of *Aspalathus cordata* is used as an outgroup to root the network. A dash represents a base pair change and a solid circle represents a deletion event.

Table 6: Populations analysed in this study, the growth forms, regeneration strategy, numbers of individuals (N), haplotype frequencies and measures of genetic diversity (*H*). Regeneration strategy is obtained from Louw *et al.*(unpublished).

Population	Growth Form	Regeneration strategy	N	Haplotypes A	B	C	D
Witbank	Prostrate	Resprouter	4	4			
Langskloof	Prostrate	Resprouter	5	5			
Bo-Valletjie	Erect	Unknown	5	5			
Jeugkamp	Erect	Reseeder	3		1	2	
Witbank	Erect	Reseeder	4		3	1	
Landskloof	Erect (Cultivated)	Reseeder	2		1	1	
Landskloof	Shrub	Resprouter	3				4

Discussion:

Aspalathus linearis displays extraordinary variation in morphology, and the bulk of this variation is distributed amongst populations (Dahlgren 1968). Previous studies using a suite of different techniques, specifically i) allozymes (van der Bank *et al.* 1999), ii) phenolic compounds (van Heerden *et al.* 2003) and iii) isozymes (van der Bank *et al.* 1995), have demonstrated limited gene flow between populations of *A. linearis*. This is the first study to quantitatively assess the morphological variation of *A. linearis* focussing on a specific geographic region, and to evaluate genetic variables in relation to the separate morphological forms, specifically a single plastid non-coding region and ISSR. The main aim of this study is to evaluate and quantify the infraspecific diversity within and between populations of *Aspalathus linearis* in the Cederberg region. Knowledge regarding the infraspecific diversity of a species is important for conservation (Rojas 1992) and taxonomy. *Aspalathus linearis* is an economically important plant and both wild and cultivated populations are harvested to produce rooibos tea. It is a species that is being exploited with little scientific knowledge regarding its current biodiversity status and evolutionary history.

Are there distinct morphological groups?

Across the five sampled localities, the PCA revealed three easily distinguishable forms that have clear morphological character differences, despite some variation within the groups. However, an infraspecific classification of *A. linearis* should not be based on morphology alone as there is another important dimension to include in the classification, specifically that of regeneration strategy. Van Heerden *et al.* (2003) proposed an infraspecific classification system based on regeneration strategy where resprouting growth forms range in morphology from erect to prostrate and the reseeding forms are only erect. Louw *et al.* (unpublished) have identified the shrub and prostrate forms to be resprouters, and the erect forms from Landskloof, Jeugkamp and Witbank to be reseeders. Sprouters in general have multi-stemmed appearance at ground level as shoots coppice from a lignotuber after disturbance, whereas reseeders have a single main stem at ground level (Schutte *et al.* 1995). The regeneration strategy of the erect-form population at Bo-Valletjie remains unclear.

Are morphological groups genetically distinct?

Inferring genetic relationships between organisms based on a single genome is questionable as different parts of an organism's genome may have different evolutionary histories due to recombination, incomplete lineage sorting, introgression and paralogy (Takahata 1989; Doyle 1997). Therefore a multi-locus approach is usually recommended to avoid inferring evolutionary relationships based on a single gene region whose evolutionary history may not characterize the organismal evolutionary history. This study used a single plastid non-coding region, trnL$'^{UAA}$F - trnF^{GAA}, and a technique that makes use of unidentified sections of the entire, namely ISSR.

To assess plastid haplotypic variation in *A.linearis*, the region trnL$'^{UAA}$F - trnF^{GAA} was selected because: (i) it shows haplotypic variation as four distinct haplotypes were found, and (ii) it is the most widely used plastid region (Shaw *et al.* 2005), so these *A. linearis* sequences can easily be used in other studies. The trnL$'^{UAA}$F - trnF^{GAA} region revealed strong genetic differences between the different forms. Both the prostrate populations possessed only the ancestral haplotype A. However, this haplotype was not unique to the prostrate form, as it was also present in the erect population at Bo-Valletjie. The erect reseeders, however, all possessed haplotypes B and C, and the shrub form only possessed haplotype D. Two to five samples of the plastid trnL$'^{UAA}$F-trnF^{GAA} region is too small a sample size to infer population level dynamics within a species. Any patterns observed in the data may very well be an artefact of the sample size. However, if the apparent fixation of haplotype A in the prostrate forms and the Bo-Valletjie population is a true reflection of the haplotype distribution and diversity between these populations and the remaining erect-form populations, then this would suggest that there may have been long-term isolation between the two. The same could be said for the fixation of haplotype D in the shrub form. The fixation of the various haplotypes is likely due to genetic drift, which occurs in small populations that do not undergo outcrossing. Genetic drift is the change in allele frequencies between generations due to random sampling of gametes. The rate of

?

genetic drift in large populations is very low (Nei *et al.* 1975), which suggests that populations with fixated haplotypes may have undergone one or more bottleneck events.

The ISSR technique was unable to gain significant support for any relationships between the sampled individuals and so could not be used to support or contradict the plastid sequence data. There are three possible explanations as to why the technique failed to find support: (i) *Aspalathus linearis* forms a panmictic species across all of the localities sampled, (ii) populations may be separated but have not been separated for long enough for genetic differentiation to occur, but we may possible expect a discrepancy between nuclear and plastid genome because the nuclear effective population size is twice as large as the plastid genome, and (iii) the ISSR method is flawed. I believe that the results of the ISSR technique are due to a flaw in the method, specifically that there were not enough informative bands identified for each individual. The solution to this problem is to increase the total number of polymorphic bands, which can be done by increasing the number of primers used. This study identified an average of 9 polymorphic bands per primer, but only used three primers. Previous studies have found similar levels of band polymorphism, but these have generally used many more primers. For example, Li & Ge (2001) found an average of nine polymorphic bands when using three primers; Barth *et al.* (2002) recovered an average of 3.4 polymorphic bands using 11 primers and Wolfe & Randle (2001) an average of 20.5 polymorphic bands with 11 primers.

Does interbreeding occur between the different forms?

Both prostrate and erect forms were found at Witbank, growing in sympatry and randomly distributed over a very small area (< 1000 m^2). The fact that they do not share any haplotypes and that no hybrids (i.e. morphological intermediates) have been observed suggests that these two forms are reproductively isolated from one another. Similarly, at Landskloof no haplotypes are shared between the wild resprouting shrub form and the cultivated reseeding erect form in Landskloof. Irwin (2002) suggests that genealogies based on nonrecombining genetic units, such as plastid markers, may show geographically separated clades even when there are no barriers to gene flow due to chance alone. However, the fact that both erect and prostrate forms at Witbank do not share any haplotypes suggests that the observed differences between geographically distant populations are probably not due to stochastic causes alone. Seventeen species of Hymenoptera have been observed visiting the flowers of *A. linearis,* most of which were found to be generalist pollinators within the genus *Aspalathus* (Gess & Gess 1994). This suggests that any isolation between the prostrating and erect forms at Witbank is not due to divergence in pollinators exploited. Alternative mechanisms that may be preventing gene flow between the regeneration strategies are differences in flowering times or genetic incompatibility. The evidence that resprouters and reseeders are genetically isolated supports the findings of van der Bank *et al.* (1999) who found that resprouting populations found over 300 km apart, in Franschoek and Gifberg, were more genetically similar than to any of the five reseeding populations found within 50 km of the Gifberg population.

The broad correspondence between the haplotypes and the regeneration strategies suggests that there has been long-term isolation between resprouters and reseeders. If gene flow was occurring between resprouting and reseeding populations, we would expect to see some sharing of haplotypes between these forms. The regeneration strategy of the Bo-Valletjie population is currently uncertain. If the Bo-Valletjie population is found to be resprouting, then it would lend additional supports to this hypothesis. However, even if the Bo-Valletjie population is found to consist of reseeders, it does not discredit the hypothesis of isolation, as haplotype A may have become fixed in this population through a combination of incomplete lineage sorting and genetic drift.

Conclusions

This is the first study to quantitatively define the morphological variation observed in *A. linearis* and has established the feasibility of using DNA plastid sequences as a method to extend the research of hypotheses regarding the effects of genetic swamping by the cultivated erect reseeder "Rocklands" type, as well as the effects of selection and fire-survival strategies on genetic diversity. I suggest that the wild erect reseeding forms are at far greater risk to genetic swamping by the cultivated *A. linearis* populations than the prostrate or erect resprouters as the seeders and sprouters appear to be reproductively isolated.

Future work is required to confirm the morphological and haplotypic results of this study can be confirmed in a number of ways: i) increasing the number of individuals sampled per population, ii) increasing the number of plastid regions and ISSR primers, iii) including a nuclear locus or multiple loci, and iv) increasing the number of populations sampled.

This paper shows that the plastid marker trnL$^{'UAA}$F - trnF^{GAA} displays variability which likely displays genetic structuring and so is an ideal non-coding spacer region to study population level dynamics within *Aspalathus linearis*. This study identifies other plastid regions and a nuclear region as potentially useful markers for a multi-locus molecular approach to studying taxonomic and ecological questions within the species. Bond & Midgley (2003) state that to bridge the gap between the ecology and evolution of the resprouting and reseeding strategies, the genetic differences between related species with contrasting sprouter-seeder life-histories is required. I propose that *Aspalathus linearis* may be an ideal target species for such a study as it contains both sprouting and seeding populations within the same species. Also the results of this study suggest that plastid nucleotide sequences display a suitable degree of divergence and structuring to answer such evolutionary questions.

Further sampling across more populations of sprouters and seeders may show that individuals using the two strategies belong to monophyletic sister clades, in which case a revision of the species may be in order, where the resprouters and reseeders are split into separate species. Given the result of this study and the study by van der Bank (1999) the splitting of *Aspalathus linearis* would be supported by both the biological species concept (BSC; Mayr 1942) and genealogical species concept (Baum & Shaw 1995) as sprouting and seeding populations that occur in sympatry do not cross pollinate to form hybrids and the sprouters and seeders may represent separate monophyletic genealogies.

Acknowledgements

G.A. Verboom, J. Bishop and R. La Grange provided valuable comments and criticisms of earlier drafts. I would especially like to thank Tony Verboom for the countless hours he devoted to helping me with this project and answering my seemingly infinite number of questions and problems. Also I would like to thank Timm Hoffman and Rhoda Louw, who started me along the path of this project with the collecting trip and valuable insights into the rooibos world.

References:

ÁLVAREZ I. & WENDEL J.F. (2003) Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution*, 29:417–434.

BARTH S., MELCHINGER A.E. & LÜBBERSTEDT T.H. (2002) Genetic diversity in *Arabidopsis thaliana* L. Heynh. investigated by cleaved amplified polymorphic sequence (CAPS) and inter-simple sequence repeat (ISSR) markers. *Molecular Ecology*, 11:495-505.

BAUM D. & SHAW K.L. (1995) Genealogical perspectives on the species problem. Pages 289-303 *in* P.C. Hochand and A.C. Stephenson, editors. Experimental and molecular approaches to plant biosystematics. Missouri Botanical Garden, St. Louis, MO.

BOND W.J. & MIDGLEY J.J. (2003) The evolutionary ecology of sprouting in woody plants. *International Journal of Plant Sciences*, 164:S103-S114.

CLEGG M.T., GAUT B.S., LEARN G.H. JR. & MORTON B.R. (1994) Rates and patterns of chloroplast DNA evolution. *Proceedings of the National Academy of Sciences, USA*, 91:6795-6901.

COWLING R.M. (1987) Fire and its role in coexistence and speciation in Gondwanan shrublands. *South African Journal of Science*, 83:106-112.

COX A.V., BENNET M.D. & DYER T.A. (1992) Use of the polymerase chain reaction to detect spacer size heterogeneity in plant 5S-rRNA gene clusters and to locate such clusters in wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*, 83: 684-690.

CRISP M.D. & COOK L.G. (2003) Phylogeny and embryo sac evolution in the endemic Australasian Papilionoid tribes Mirbelieae and Bossiaeeae. Pages 253-268 *in* B. B. Klitgaard and A. Bruneau, editors. Advances in Legume Systematics, part 10, Higher Level Systematics. Royal Botanic Gardens, Kew.

DAHLGREN R. (1968). Revision of the genus *Aspalathus* II: The species with ericoid and pinoid leaves. 7 subgenus *Nortieria.* With remarks on Rooibos cultivation. *Botany Notiser* 121:165-208.

DAHLGREN R. (1988). Revision of the genus Aspalathus. *Flora of Southern Africa*, 16(3,6):1-430.

DEMESURE B., SODZI N. & PETIT R.J. (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology*, 4:129-131.

DOYLE J.J. (1997) Trees within trees: genes and species, molecules and morphology. *Systematic Biology*, 46(3):537-553.

ELLSTRAND N.C., PRENTICE H.C. & HANCOCK J.F. (1999) Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology and Systematics*, 30:539-563.

EXCOFFIER L., SMOUSE P.E., QUATTRO J.M. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131:479-491.

EXCOFFIER L., LAVAL G. & SCHNEIDER S. (2005) Arlequin ver. 3.0: An integrated software package for populatin genetics data analysis. *Evolutionary Bioinformatics Online*, 1:47-50.

FELSENSTEIN, J. (1985) Phylogenies and the comparative method. *The American Naturalist*, 125:1-15.

GAWEL N.J. & JARRET R.L. (1991) A modified CTAB DNA extraction procedure for *Musa* and *Ipomea*. *Plant Molecular Biology Reporter*, 9:262-266.

GESS S.K. & GESS F.W. (1994) Potential pollinators of the Cape Group of Crotalarieae (*sensu* Polhill) (Fabales: Papilionaceae), with implications for seed production in cultivated rooibos tea. *African Entomology*, 2(2):97-106.

GODWIN I.D., AITKEN E.A.B. & SMITH L.W. (1997) Application of inter simple sequence repeats (ISSR) markers to plant genetics. *Electrophoresis*, 18:1524-1528.

GOLDBLATT P. & MANNING J.C. (2002) Plant Diversity of the Cape Region of Southern Africa. *Annals of the Missouri Botanical Garden*, 89(2):281-302.

HENDERSON A. (2006) Traditional morphometrics in plants systematics and its role in palm systematics. *Botanical Journal of the Linnean Society*, 151:103-111.

IRWIN D. E. (2002) Phylogeographic breaks without geographic barriers to gene flow. *Evolution*, 56:2383-2394.

JAMES F.C. & MCCULLOCH C.E. (1990) Multivariate analysis in Ecology and Systematics: Panacea or Pandora's Box? *Annual Review of Ecology and Systematics*, 21:129-166.

JOLIFFE I.T. (1986) *Principle Component Analysis*. Springer. New York.

LE MAITRE D.C. & MIDGLEY J.J. (1992) Plant reproductive ecology. Pages 135-174 *in* R.M. Cowling, editor. The ecology of Fynbos - nutrients, fire and diversity. Oxford University Press, Cape Town.

LI A. & GE S. (2001) Genetic variation and clonal diversity of *Psammochloa villosa* (Poaceae) detected ISSR markers. *Annals of Botany*, 87:585-590.

NEI M., MARUYAMA T. & CHAKRABORTY R. (1975) The Bottleneck Effect and Genetic Variability in Populations. *Evolution*, 29(1):1-10.

MAYR E. (1942) *Systematics and Origins of Species*. Columbia University Press, New York.

RHOLF F.J. (2000) *NTSYS-PC. Numerical Taxonomy and Multivariate Analysis System*, Version 2.01q. Exeter Software, Setauket, New York.

ROJAS M. (1992) The Species Problem and Conservation: What are we protecting? *Conservation Biology*, 6(2):170-178.

ROTANDI A., MAGLI M., RICCIOLINI C. & BALDONI L. (2003) Morphological and molecular analyses for the characterization of a group of Italian olive cultivars. *Euphysica*, 132:129-137.

SCHUTTE A.L., VLOK J.H.J. & VAN WYK, B.-E. (1995) Fire-survival strategy — a character of taxonomic, ecological and evolutionary importance in fynbos legumes. *Plant Systematics and Evolution*, 195:243-259.

SHAW J., LICKEY E.B., BECK J.T., FARMER S.B., LIU W., MILLER J., SIRIPUN K.C., WINDER C.T., SCHILLING E.E. & SMALL R.L. (2005) The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*, 92:142-166.

SICA M., GAMBA G., MONTIERI S., GAUDIO L. & ACETO S. (2005) ISSR markers show differentiation among Italian populations of *Asparagus acutifolius*. *BMC Genetics*, 6:17-23.

SWOFFORD D.L. (2002) PAUP*: Phylogenetic Analysis Using Parsimony (* and other methods), version 4b10. Sinauer, Sunderland MA.

TABERLET P., GIELLY L. PAUTOU G. & BOUVET J. (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, 17:1105-1109.

TAKAHATA N. (1989) Gene geneaology in three related populations: consistency probability between gene and population trees. *Genetics*, 122:957-966.

TSUMURA Y., OHBA K. & STRAUSS S.H. (1996) Diversity and inheritance of Inter-simple sequence repeat polymorphisms in Douglas fir (*Pseudotsuga menziesii*) and Sugi (*Cryptomeria japonica*) *Theoretical and Applied Genetics*, 92:40-45.

VAN DER BANK M., VAN WYK B.-E. & VAN DER BANK H. (1995) Biochemical genetic variation in four wild populations of *Aspalathus linearis* (Rooibos Tea). *Biochemical Systematics and Ecology*, 23(3):257-262.

VAN DER BANK M., VAN DER BANK F.H. & VAN WYK B.-E. (1999) Evolution of sprouting versus seeding in *Aspalathus linearis*. *Plant Systematics and Evolution*, 219:27-38.

VAN HEERDEN F.R., VAN WYK B.-E., VILJOEN A.M. & STEENKAMP P.A. (2003) Phenolic variation in wild populations of *Aspalathus linearis* (rooibos tea). *Biochemical Systematics and Ecology*, 31:885-895.

VESK P.A., WARTON D.I. & WESTOBY M. (2004) Sprouting by semi-arid plants: testing dichotomy and predictive traits. *OIKOS*, 107:72-89.

WOLFE A.D. & RANDLE C.P. (2001) Relationships within and among species of the holoparasitic genus *Hyobanche* (Orobanchaceae) inferred from ISSR banding patterns and nucleotide sequences. *Systematic Botany*, 26(1):120-130.

WOLFE A.D. & LISTON A. (1998) Contributions of PCR-based methods to plant systematics and evolutionary biology. Pages 43 - 86 *in* D. E. Soltis, P. S. Soltis, and J. J. Doyle, editors. Molecular systematics of plants II: DNA sequencing. Kluwer, London.

YANG W., DE OLIVEIRA A.C., GODWIN I., SCHERTZ K. & BENNETZEN J.L. (1996) Comparison of DNA marker technologies in characterizing plant genome diversity: variability in Chinese sorghums. *Crop Science*, 36:1669-1676.

YAP I.V. & NELSON R.J. (1996) WINBOOT: *A program for performing bootrap analysis of binary data to determine confidence limits of UPGMA-based dendrograms*, 1st edn. International Rice Research Institute, Manila, Philippines.

YASODHA R., KATHIRVEL M., SUMATHI R., GURUMURTHI K., ARCHAK S. & NAGARAJU J. (2004) Genetic analyses of Casuarinas using ISSR and FISSR markers. *Genetica*, 122:161-172.

ZIĘTKIEWICZ E., RAFALSKI A. & LABUDA D. (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase reaction amplification. *Genomics*, 20:176-183.

References:

ACHYUTHAN H. (2003) Petrologic analysis and geochemistry of the Late Neogen-Early Quaternary hardpan calcretes of Western Rajasthan, India. *Quaternary International*, **106-107**:3-10.

ALONSO-ZARZA A.M. & ARENAS C. (2004) Cenozoic calcretes from the Teruel Graben, Spain: microstructure, stable isotope geochemistry and environmental significance. *Sedimental Geology*, **167**:91-108.

ANDREWS J.E., SINGHVI A.K., KAILATH A.J., KUHN R., DENNIS P.F., TANDON S.K. & DHIR R.P. (1998) Do stable isotope data from calcrete record late Pleistocene monsoonal climate variation in the Thar Desert of India. *Quaternary Research*, **50**:240-251.

BIRKELAND P.W. (1984) *Soils and Geomorphology*. Oxford University Press, New York, pp372.

BRUYN L.A.L. DE & CONACHER A.J. (1990) The role of termites and ants in soil modification - a review. *Australian Journal of Soil Research*, **28**(1):55-93.

CAPO R.C. & CHADWICK O.A. (1999) Sources of strontium and calcium in desert soil and calcrete. *Earth and Planetary Science Letters*, **170**:61-72.

CERLING T.E. (1984) The stable isotopic composition of modern solid carbonate and its relationship to climate. *Earth and Planetary Science Letters*, **71**:229-240.

CERLING T.E. & QUADE J. (1993) Stable carbon and oxygen isotopes in soil carbonates. In: Swart P.K., Lohmann K.C., McKenzie J., Savin S. (Eds.), Climate changes in continental isotopic records, *American Geophysical Union, Geophysical Monographs*, **78**:217-231.

COATON W.G.H. (1981) Fossilised nests of Hodotermitidae (Isoptera) from the Clanwilliam district, Cape Province. *Journal of the Entomological Society of Southern Africa*, **44**:79-81.

DANSGAARD, W. (1964) Stable isotopes in precipitation. *Tellus*, 16:436-468.

DEAN W.R.J. (1992) Effects of animal activity on the absorption rate of soils in the southern Karoo, South Africa. *Journal of the Grassland Society South Africa*, **9**:178-180.

DUBBIN W. (2001) Soils – past and present. *Geology Today*, **17**:225-228.

EDWARDS G. & WALKER D.A. (1983) *C_3, C_4: mechanisms, and cellular and environmental regulation, or photosynthesis*. Blackwell Scientific, Oxford.

FRIEDMAN I. & O'NEIL J.R. (1977) Compilation of stable isotope fractionation factors of geochemical interest. In: Data of Geochemistry, ed. M Fleischer, *US Geological Survey Professional Paper 440-K.* 12pp.

HARRIS C., OOM B.M. & DIAMOND R.E. (1999) A preliminary investigation of the oxygen and hydrogen isotope hydrology of the greater Cape Town area and an assessment of the potential for using stable isotopes as tracers. *Water SA*, **25**(1):15-24.

HOLMGREN K., LEE-THORP J.A., COOPER G.R.J., LUNDBLAD K., PARTRIDGE T.C., SCOTT L., SITHALDEEN R., TALMA A.S. & TYSON P.D. (2003) Persistent millennial-scale climatic variability over the past 25,000 years in Southern Africa. *Quaternary Science Reviews*, **22**:2311-2326.

JONES J.A. (1990) Termites, soil fertility and carbon cycling in dry tropical Africa: a hypothesis. *Journal of Tropical Ecology*, **6**:291-305.

KHADKIKAR A.S., CHAMYAL L.S., RAMESH R. (2000) The character and genesis of calcrete in Late Quaternary alluvial deposits, Gujurat, western India, and its bearing on the interpretation of ancient climates. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **162**:239-261.

KOCH P.L. (1998) Isotopic reconstruction of past continental environments. *Annual Review of Earth Planetary Sciences*, **26**:573-613.

LIU B., PHILLIPS F.M. & CAMPBELL A.R. (1996) Stable carbon and oxygen isotopes of pedogenic carbonates, Ajo Mountains, southern Arizona: implications for paleoenvironmental change. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **124**:233–246.

LOVEGROVE B.G. & SIEGFRIED W.R. (1986) Distribution and formation of Mima-like earth mounds in the western Cape Province of South Africa. *South African Journal of Science*, **85**:108-112.

MIDGLEY G.F. & MUSIL C.F. (1990) Substrate effects of zoogenic soil mounds on vegetation composition in the Worcester-Robertson valley, Cape Province, South Africa. *South African Journal of Science*, **56**:158-166.

MIDGLEY J.J., HARRIS C., HESSE H. & SWIFT A. (2002) *Heuweltjie* age and vegetation change based on $\delta^{13}C$ and 14C analyses. *South African Journal of Science*, **98**:202-204.

MOONEY H.A., TROUGHTON, J.H. & BERRY, J.A. (1977) Carbon isotope ratio measurements of succulent plants in Southern Africa. *Oecologia*, **30**(4):295-305.

MOORE J.M. & PICKER M.D. (1991) *Heuweltjies* (earth mounds) in the Clanwilliam district, Cape Province, South Africa: 4000 year old termite nests. *Oecologia (Berlin)*, **86**:424-432.

MUELLER G. & GASTNER, M. (1971) The "Karbonate-Bombe," a simple device for the determination of the carbonate content in sediments, soils, and other materials. *Neues Jahrb. Mineral.*, **10**:466–469.

OKELLO-OLOYA T., SPAIN A.V. & JOHN R.D. (1985) Selected chemical characteristics of the mounds of two species of Amitermes (Isoptera, Termitinae) and their adjacent surface soils from northeastern Australia. *Revue d'écologie et de biologie du sol*, **22**(3):291-311.

PARKINGTON J., CARTWRIGHT C., COWLING R.M., BAXTER A.& MEADOWS M. (2000) Palaeovegetation at the last glacial maximum in the western Cape, South Africa: wood charcoal and pollen evidence from Elands Bay Cave .*South African Journal of Science*, **96**:543–546.

PICKER M.D., HOFFMAN M.T. & LEVERTON B. (2006) Density of *Microhodothermes viator* (Hodotermitidae) mounds in southern Africa in relation to rainfall and vegetative productivity gradients. *Journal of Zoology*, Published article online 27 Jul 2006 [doi:10.1111/j.1469-7998.2006.00189.x].

QUADE J., CHIVAS A.R. & MCCULLOCH M.T. (1995) Strontium and carbon isotope tracers and the origins of soil carbonate in South Australia and Victoria. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **113**:103–117.

ROWE P.J. & MAHER B.A. (2000) 'Cold' stage formation of calcrete nodules in the Chinese Loess Plateau: evidence from U-series dating and stable isotope analysis. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **157**:109-125.

SCHMID S., WORDEN R.H. & FISHER Q.J. (2006) Variations of stable isotopes with depth in regolith calcite cements in the Broken Hill Region, Australia: Palaeoclimate evolution signal? *Journal of Geochemical Exploration*, **89**:355-358.

SRIVASTAVA P. (2001) Paleoclimatic implications of pedogenic carbonates in Holocene soils of the Gangetic Plains, India. *Paleogeography, Paleoclimatology, Paleoecology* **172**:207-222.

TALMA A.S. & VOGEL J.C. (1992) Late Quaternary paleotemperatures derived from a speleothem from Cango Caves, Cape Province, South Africa. *Quaternary Research*, **37**:203-213.

VESTE M., HERPPICH W.B. & VON WILLERT D.J. (2001) Variability of CAM in leaf-deciduous succulents from the Succulent Karoo (South africa). *Basic and Applied Ecology*, **2**(3):283-288.

Appendix I:

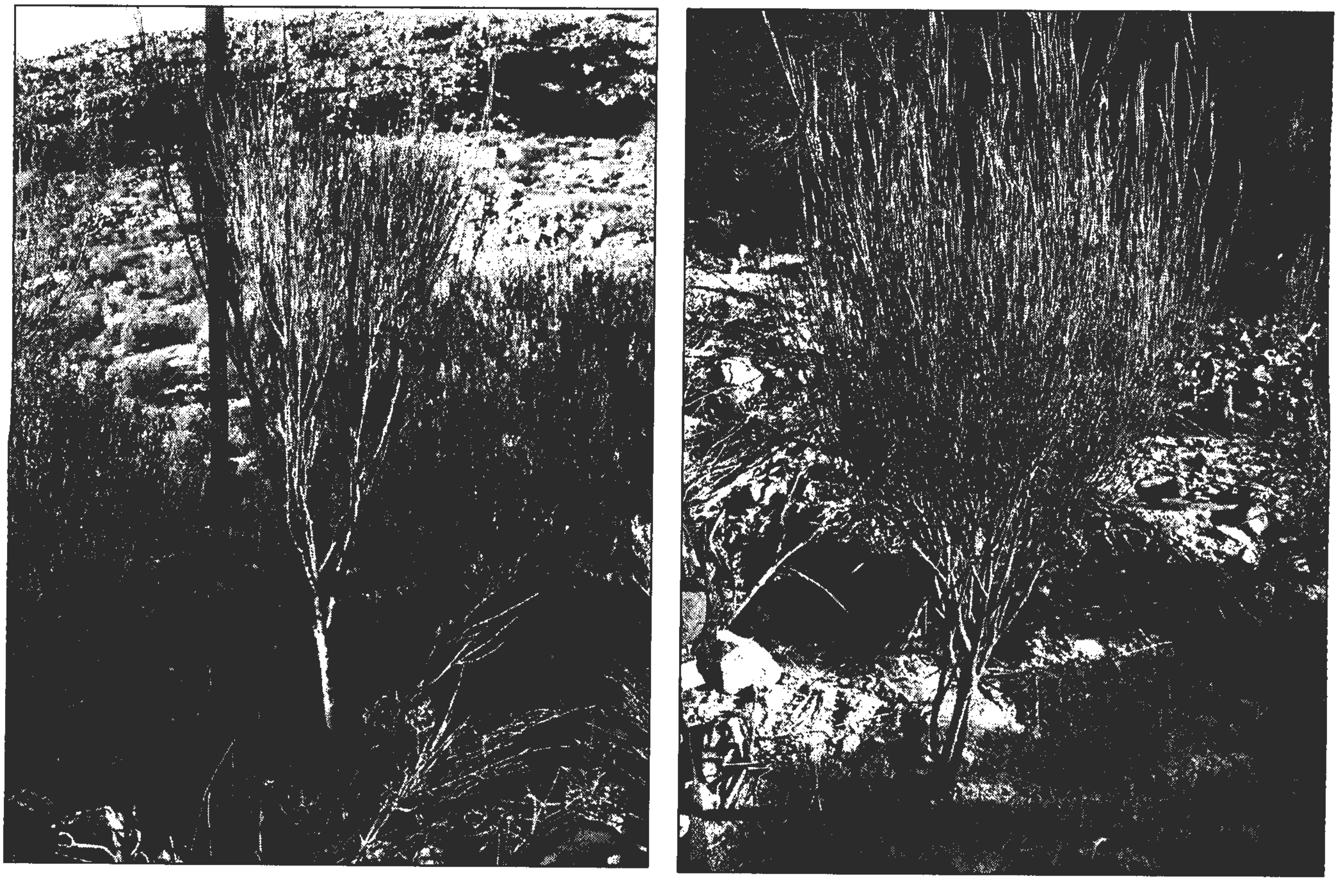


Figure 6: Two examples of 'erect' form individuals of *Aspalathus linearis*

Figure 7: An example of a 'prostrate' form of *Aspalathus linearis*