Genetic and morphological means of differentiating within and between populations of *Widdringtonia whytei* and *Widdringtonia nodiflora* on Mount Mulanje, Malawi

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Abstract

There has been confusion in the past as to the taxonomy of *Widdringtonia* on Mount Mulanje. At present it is accepted that there are two species present. The aim of this study was to assess the level of genetic variation present across six populations of *Widdringtonia whytei* and *Widdringtonia nodiflora* on the mountain, and determine whether there are two genetically separate species present. Analysis of genetic variation included PsbA (chloroplast DNA), ITS (nuclear DNA), microsatellite and ISSR gene region markers. Out of the markers that amplified successfully, it was found that there is little genetic variation present with no differentiation between or within taxonomic groups for the gene regions analyzed. Morphometric analysis of cones and leaves grouped *W. whytei* and *W. nodiflora* separately, with a midgroup more closely associated with definite *W. whytei*. This compared favourably with observations of individuals from the different groups in the field. It is suggested that based on the low level of genetic variation across the mountain, *W. whytei* and *W. nodiflora* have diverged very recently from a *W. nodiflora* ancestor.

Keywords:

Widdringtonia whytei, *Widdringtonia nodiflora*, Mount Mulanje, genetic variation, morphology, phylogeny, divergence.

Introduction

Widdringtonia whytei is endemic to Mount Mulanje in Malawi, and in 1984 was declared the national tree (Chapman 1995) as it is a unique feature of the mountain and has great economic value. The species is characteristically found in afromontane forest habitat as an emergent species above the forest canopy, which constrasts with the general habitat and growth form of other species within the genus. This feature also gives a unique character to the forests of Mount Mulanje.

The genus *Widdringtonia* consists of four currently recognized species, all of which are endemic to Southern Africa. Although the phylogeny of the genus *Widdringtonia* (De Roo and Hedderson unpublished) and the family Cupressaceae (Gadek et al 2000) suggests a Southern Hemisphere origin for the genus, fossil evidence suggests that the genus was present in North America around 95mya (McIver 2001). Fossilized wood on Kerguelen Island in the South Indian Ocean is evidence of its presence there during the Pleistocene (Philippe et al 1998). *Widdringtonia whytei* is found in the northernmost area of present distribution of the genus, along with the most widespread currently recognised species, *Widdringtonia nodiflora*. This species is found throughout most of the distribution of the genus, occurring in scattered populations from Mount Mulanje in the north along the mountain ranges of the east coast of Southern Africa as far as the Cape Peninsula at the Southwestern tip of Africa. *W. cedarbergensis* is endemic to a small area in the northern Western Cape Province and *W. schwarzii* is found in the Eastern Cape of South Africa (Coates Palgrave 1977).

Until recently, *W. whytei* had been lumped within *W. nodiflora* (Marsh, 1966), as both species are found on Mount Mulanje, and without careful observation the two could appear to be forms of the same species. Pauw and Linder (1997) show that *W. nodiflora* and *W. whytei* on Mount Mulanje are differentiated physically, biologically and ecologically. These species differ in their growth form, with *W. nodiflora* usually coppicing from its base after fire and having multiple trunks, compared with a single non-coppicing trunk in *W. whytei*. Because of this feature, *W. whytei* is restricted to forest habitat while *W. nodiflora* is usually found in fire-prone scrub and grassland habitat.

Since being described in 1891 and its economic potential realized, *W. whytei* has been exterminated from much of its original habitat on Mount Mulanje, chiefly from logging operations because of its valuable wood. The species is also prone to destruction from too frequent fires started by illegal hunters or by farmers around the mountain clearing land for farming (Bayliss et al 2007). European cypress aphid *Cinuru cupessi* Buckton was introduced with the planting of Mexican Cypress *Cupressus lusitanica* Mill and now feeds on Mulanje Cedar. *Pinus patula* (Mexican pine) and *Rubus ellipticus* (Himalayan Raspberry) (Chapman 1995) compete for habitat following disturbance as they grow too fast and smother young cedar seedlings (pers. obs.).

Attempts have been made to replant Mulanje Cedar where it was previously logged, as well as establishing plantations on Zomba plateau (Chapman 1995). However, it has been suggested that these initiatives used some seed of what is *W. nodiflora* and not *W. whytei*, the former being less economically useful, and of limited benefit in conserving the latter red-data species.

As the Mulanje cedar is a localized endemic, it is at present classified as "Endangered" under criteria Ala-d, B1 and B2a-e of the IUCN's Red List Categories (IUCN 2010). Bayliss et al (2007) found that the total area of occupancy of the species declined by 616.7 ha during the 15 years since the previous survey, and of the cedar trees recorded on the mountain, 32.27% were dead. The main cause of these deaths is an issue of debate.

Seed for propagation has been collected from populations across the mountain, and so supplementing wild populations with propagated plants may have a negative impact on the genetic integrity of these populations if populations are genetically structured. Certain genotypes may be better able to survive in local conditions, and as there is much environmental heterogeneity across the mountain, this may be resulting in outbreeding depression in which planted trees of inferior genetics breed with wild trees and compromise their reproductive ability, thus resulting in decreased recruitment in future. For economic purposes of *W. whytei* in plantations, it is important to determine whether trees with favourable morphology for timber production are determined by genetics or environmental conditions.

De Roo (2002) used genetic markers (ITS and PsbA) to assess levels of interspecific variation and produce a phylogeny for the genus. This initially suggested that *W. whytei* and *W. nodiflora* are well differentiated genetically, although this study only made use of specimens of *W. nodiflora* from the Western Cape and a single specimen of *W. whytei.* Later work by De Roo and Hedderson (unpublished) using *W. nodiflora* specimens from further north show that *W. whytei* and *W. nodiflora* are in fact much closer genetically. *W. nodiflora* was found to be basal to all other species in the genus. However, *W nodiflora* also forms distinct genetic clusters in different parts of its range and so is likely to consist of more than one species as was the case prior to being lumped into *W. nodiflora* by Marsh (1966). As only a single individual of *W.whytei* was included in the de Roo phylogeny, it is not known what level of genetic variation exists within this species, or if the one sample used was collected from true *W. whytei* or *W. nodiflora.*

In order to determine levels and nature of genetic variation within *Widdringtonia* on Mount Mulanje, four main types of markers could be utilised to look for variation on different scales. The first two (Chloroplast and Nuclear DNA markers) are those used by De Roo (2002) which were found to work successfully for *Widdringtonia.* While these were useful on a larger scale (ie. inter-specific variation), different markers are needed to investigate finer-scale genetic variation such as that within or between populations. Microsatellite markers have been utilised within related genera including *Austrocedrus* (Arana et al 2008) and *Cupressus* (Sebastiani et al 2005; Xu et al 2008). Inter-simple sequence repeats (ISSRs) are another means of detecting finer-scale variation within species, such as population structuring in *Cupressus chenggiana* (Hao et al 2006).

The aim of this study is to determine the levels of genetic diversity that are present within *Widdringtonia whytei*, both on an intra- and inter-population level. I also examine genetic variation between different tree growth forms on Mount Mulanje to investigate the idea that there are two species present as opposed to only one. If there is variation present specifically between groups, this may help to delineate the boundary between *W. whytei* and *W. nodiflora.*

The hypothesis is that there is genetic variation present, which should correspond with morphological and biological differentiation as noted by Pauw and Linder (1997).

Therefore, as part of this study, morphometric analysis of individuals within each supposed taxonomic group will help provide a base by which to judge the results for genetic variation within and between *W. whytei* and *W. nodiflora*. Alternatively there may not be genetic variation present among or between these taxonomic groups, and morphological variation may be environmental as opposed to genetic to a greater or lesser degree.

Methods

Taxonomic grouping of individuals on Mount Mulanje

Widdringtonia specimens were identified according to current taxonomic classification as either *W. whytei* or *W. nodiflora* by Hassan Patel (pers. comm.). For the purpose of classification for data analysis, *Widdringtonia* on Mount Mulanje is divided into three different recognisable groups. True *W. whytei* has a tall, straight trunk, with branching starting from at least 10 metres or so from the base. Any Widdringtonia which did not match this description was classified as *W. nodiflora*, ie. trees branching from low down. However, this included both tall trees possessing small, egg-shaped cones like the true W. *whytei* as well as smaller trees sometimes actively resprouting from their base, with large round cones (pers. obs). *W. nodiflora* was therefore divided into two groups determined primarily by cone shape where cones were present, as well as resprouting where there was evidence of this having taken place. True *W. nodiflora* has large round cones and may resprout while the taller *W. nodiflora* with small cones is referred to as the forestry *W. nodiflora*, as this followed their classification as *W. nodiflora* by forestry workers interested in their growth and wood productivity. This classification was done as such due to the unsure taxonomy of the forestry group between *W. nodiflora* or *W. whytei*.

Description of study sites

Mount Mulanje is an Igneous massif of granite in Southern Malawi. The mountain rises above the Chiradzulu plain to 3002m above sea level at its highest point, making it the highest mountain in the region (Bayliss 2007). Although the region experiences summer rainfall, rainfall on parts of the mountain is higher and less seasonal. Along with the Mulanje Cedar, one of the most prominent botanical features, 70 out of 1330 plant species on the mountain are endemic (Strugnell 2002).

The distribution of *W. whytei* consists of a number of populations of varying sizes within afromontane forest patches scattered across the plateau of Mount Mulanje. These forest patches are mostly confined to protected habitats such as valleys where fires do not frequently reach. Forests are surrounded by grassland in more exposed areas on the mountain. Six of these forests (Figure 1) were chosen from which to collect data.

Sombani forest consists of a healthy population of medium/large trees within intact broadleaf forest canopy, with little sign of disturbance other than very old and very wide stumps of Cedars probably felled many decades ago. A recent fire burnt the edge of the forest but did not penetrate far into it. Some ancient, gnarled trees were present on the far edge of the forest but most were dead at the time of this survey. The scrub just outside of the forest also contained the only three trees of *W. nodiflora* that were resprouting from the base, following an earlier fire evident from the recently burnt trunks of these trees.

A large part of Nathaka forest was recently burnt, although there were a number of *W. whytei* trees that survived as their bark was only singed and their foliage is high up. There were also a number of tall forestry *W. nodiflora* trees. These trees were all close to the edge of the original forest and their identification as such was based on their branching starting from low down on the trunk.

Chinzama forest is the smallest of the forest patches surveyed, and likely amongst the oldest Cedars that were encountered. The forest canopy was less well developed with many of the cedars showing signs of stress and senescence. This forest also contained some true *W. nodiflora* trees although these were determined on cone shape alone, as they only had a single stem from the base.

Mvunje forest was the most recently and heavily disturbed site that was surveyed. Until recently there had been a number of healthy, ancient, tall cedar trees present. Most of these trees had been ring-barked or felled illegally within a short time prior to surveying, as some leaves on canopy branches were still green. These branches also contained a large number of cones which held much seed. There was however relatively good natural recruitment of seedlings, although quite a lot of seedlings had also been planted to supplement recovery of the cedar population.

Two sites were surveyed within Lichenya forest. This was the most extensive area of continuous forest habitat encountered. The first site was higher up the course of the Lichenya River on its eastern side, and contained many medium sized cedars within a well developed canopy of broadleaf trees. Similar habitat was found at the second site, further down the river course on the western side of the valley. Lichenya forest is likely wetter and older than the other sites surveyed apart from Chinzama forest due to the abundance of epiphytic orchids, mosses and ferns present along trunks and branches of broadleaf trees and cedars, along with a well developed understorey of plectranthus and ferns amongst other plant groups.

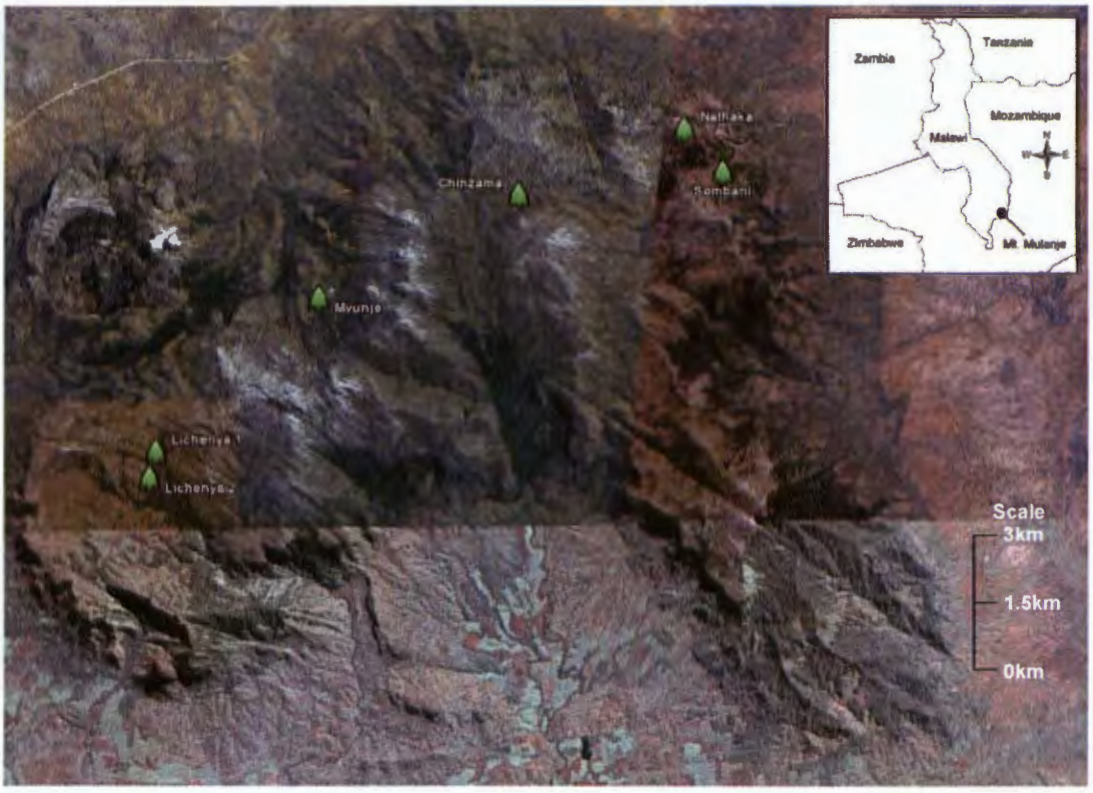

Figure 1. Location of study sites on Mount Mulanje, Malawi (Google™ Earth 2010).

Collection of field data

Samples were collected from the mountain over the week 7th to 14th June 2010. Leaf material was collected from five true *W. whytei* and forestry *W. nodiflora* individuals at each site, except where less than five individuals were present in the population. True *W. nodiflora* was only collected from the two sites where it was found to occur. In total 103

leaf samples were collected, 60 true *W. whytei*, 17 supposed *W. nodiflora*, and 5 definite *W. nodiflora*. 21 juvenile specimens were also collected which could not be identified to species due to their size. These samples were labelled according to site where collected and putative taxonomic group, and dried using silica gel crystals to be used for DNA extraction. Cones containing seed were collected from a few individuals within each of the three groups for morphometric analysis to compare with the results found by Pauw and Linder (1997).

Table 1. *Widdringtonia* populations from which samples were collected

Population	Location	Latitude	Longitude
S	Sombani	S 15° 53' 31.0"	E 35° 42' 38.0"
N	Nathaka	S 15° 52' 49.6"	E 35° 42' 0.3"
C	Chinzama	S 15° 53' 53.0"	E 35° 39' 15.9"
M	Mvunje	S 15° 55' 31.2"	E 35° 35' 56.3"
L1	Lichenya 1	S 15° 58' 0.36"	E 35° 33' 12.5"
L2	Lichenya 2	S 15° 58' 26.10"	E 35° 33' 5.28"

Data analysis

DNA was extracted from 2 mg of silica-dried leaf material using the CTAB method of Gawel and Jarret (1991) with the addition of polyvinylpyrrolidone-40 (PVP) during grinding of leaf material. PVP addition was necessary in order to dissolve the resin present in the plant material which otherwise prevented successful extraction of DNA.

The ITS region of the 18S-26S rRNA cistron (nuclear DNA) and psbA (chloroplast DNA) sequence data were shown to be useful in working with the four recognized species of *Widdringtonia* (De Roo 2002). These two markers were used to investigate variation at least between species and help to designate the supposed *W. nodiflora* group to either true *W. nodiflora* or *W. whytei*.

Table 2. Primers used for amplification of nuclear (PsbA) and Chloroplast DNA (ITS)

Region	Code	Sequence	Ref
PsbA (F)	PsbAF	5' GTT AGT CAT GAA CGT AAT GCT C 3'	Sang et al 1997
PsbA (R)	TrnHR	5' CGC GCA TGG TGG ATT CAC AAA 3'	
ITS (F)	ITS5	5' GGA AGT AAA AGT CGT AAC AAG G 3'	Baldwin 1992
ITS (R)	ITS4	5' TCC TCC GCT TAT TGA TAT GC 3'	

Microsatellite markers could provide a useful means of detecting genetic variation on a very local scale between individuals within populations or taxa. However, these markers are usually species or genus specific and none have been developed at present for *Widdringtonia*. Developing microsatellite markers was not possible given the time constraints of this study. Nine microsatellite loci have been developed by Arana et al (2008) for the species *Austrocedrus chilensis*, which is in the same subfamily as *Widdringtonia* (Gadek et al 2000). This could be close enough genetically to allow these microsatellites to be utilized for *Widdringtonia*, and these were evaluated for amplification and utility in *Widdringtonia*.

Table 3. Primers used for microsatellite amplification

Primer	Sequence
Achi1 F	5' GCATATGTTAATTTGTGTATGTCATTG 3'
EU168263 R	5' CACTTGTACAAAATAGGTTCATGC TT 3'
Achi2 F	5' CATGCCAATGGAATCTCAAA 3'
EU168264 R	5' AAGGATCCAAACATGCAAGA 3'
Achi3 F	5' CCCATCTAATCATCCACTACTTACAT 3'
EU168265 R	5' TGTTATATTTCATGTTGTTTGTAGTCC 3'
Achi4 F	5' AACCATCCAATGACACATCCT 3'
EU168266 R	5' TCTATGGCAGCAAGCTCAAA 3'
Achi5 F	5' CCTAACCATGGAATATGAAATGAA 3'
EU168267 R	5' TCTCAATGTCTTAGTCAGATTGTTTC 3'
Achi6 F	5' TCTTAATGTGCTTGAGTTCATGTC 3'
EU168271 R	5' CGGTTATGTCTCCATTGATATTTCT 3'
Achi7 F	5' GGTAGCGCTAACATACATGC 3'
EU168268 R	5' TCACAAAGAAATACAACATCTAAA 3'
Achi8 F	5' AAGGTGGACTTTAAATGTGCAATAG 3'
EU168269 R	5' GACATTGGCAACACCATTGA 3'
Achi9 F	5' CCTCTTGATTTGGGATTTGG 3'
EU168270 R	5' TCAATGGGTTAACTAGCAATTGTG 3'

Following PCR, samples were electrophoresed for 5min at 100V using 3µl of PCR product on a 1% agarose gel stained with goldview (Guangzhou Geneshun Biotech Ltd.) in 0.5xTBE buffer. This was to determine whether amplification had been successful.

ITS and PsbA-trnH amplicons were sequenced and sequence data was combined with the data set obtained for *Widdringtonia* by De Roo and Hedderson (unpublished) to determine the position of *W. whytei* and *W. nodiflora* from Mulanje in relation to other members of the genus.

ISSR primers are multilocus markers which determine genetic variation between individuals in terms of presence or absence of alleles as shown by bands on agarose gel. This method thus does not require sequencing of genetic data as the bands on the gel are scored as present or absent. These markers are capable of detecting fine-scale variation between individuals even within a population.

Table 4. Primers used to amplify inter-simple sequence repeats (ISSR)

Primer	Sequence
813	$(CT)_8T$
814	$(CT)_8A$
824	$(TC)_8G$
845	$(CT)_8RG$
852	$(CT)_8RA$
859	**$(TG)_8RC$**
860	**$(TG)_8RA$**
889	**$DBD(AC)_7$**
890	**$VHV(GT)_7$**

Single letter abbreviations for degenerate primer positions: Y = (C, T), R = (A, G), B = (non A), D = (non C), V = (non T), H = (non G)

Primers in bold were successful in amplifying ISSRs

PCRs were performed using nine of these primers for which stock was available. The standard protocol as set out by Wolfe (pers comm.) was followed initially to test which primers showed signs of working successfully on the DNA. Primers that did not amplify were excluded from further analysis. The PCR process was optimized using those

primers that showed amplification in the initial PCR and expanded to a large group of 30 individuals from all three taxonomic groups and across all populations surveyed.

Successful ISSR amplification samples were electrophoresed for 1 hour at 70V using 5µl of PCR product on a 2% agarose gel stained with ethidium bromide in 0.5xTBE buffer. This allowed for bands to be determined and compared between samples.

Table 5. PCR recipes and thermal conditions

Primer	Recipe	Thermal conditions
PsbA TrnHR	30µl reactions: 16.4µl npH_20, 3µl NH_4 buffer, 4.2µl $MgCl_2$, 1.2µl dNTPs, 1µl of each primer, 0.2µl Taq, 3µl DNA.	94° 3min ; (94° 1min + 52° 45sec + 72° 1min) 30 cycles ; 72° 3min, 4° hold
ITS5 ITS4	34µl reactions: as for PsbA but with 4µl BSA.	As for PsbA
Micro-satellite primers	10µl reactions: 7.17µl npH_20, 1µl NH_4 buffer, 0.2µl $MgCl_2$, 0.4µl dNTPs, 0.33µl of each primer, 0.0667µl Taq, 0.5µl DNA.	94° 5min ; (94° 1min + 50° 1min + 72° 1min) 30 cycles ; 72° 8min, 4° hold
859 860 889 890	25µl reactions: 16.4µl npH_20, 2.5µl NH_4 buffer, 3µl $MgCl_2$, 0.5µl dNTPs, 2µl primer, 0.1µl Taq, 0.5µl DNA.	94° 1:30min ; (94° 40sec + 45° 45sec + 72° 1:30min) 35 cycles ; 94° 45sec, 44° 45sec, 72° 5min, 6° hold

Comparison of morphology between *W. whytei* and *W. nodiflora*

Morphometric analysis was conducted on cones and seeds from individuals within each supposed taxonomic group with the aim of finding a way to differentiate between them and determine whether the forestry *W. nodiflora* is closer in morphology to true *W. nodiflora* or true *W. whytei* or is separated from both groups. This should confirm the finding of morphometric differences between tree forms by Pauw and Linder (1997). Cones and seed from *W. nodiflora* from the Western Cape in South Africa were included in this analysis as a benchmark for true morphological *W. nodiflora*.

Measurements conducted included cone length (mm) and width (mm), as well as seed length (mm) and width (mm). Cone measurements included 16 true *W. nodiflora*, 9 of the midgroup, 37 true *W. whytei*, and 20 *W. nodiflora* from the Western Cape. Seed measurements included 23 true *W. nodiflora*, 14 of the midgroup, 51 true *W. whytei* and 20 *W. nodiflora* from the Western Cape. These measurements were plotted on graphs of

length along the X axis and width along the Y axis to illustrate the general range of dimensions for each group and how well they cluster relative to each other. The significance of the difference in dimensions between different groups was determined using a t-test for independent samples by groups using Statistica 8.0.

Results

ITS and PsbA

A total of six samples, which included two definite *W. whytei*, two forestry *W. nodiflora* and three definite *W. nodiflora*, were sequenced for each of these gene regions. No genetic variation was found between any of these individuals for the psbA-trnH region, and very limited genetic variation was found for ITS region. Any genetic variation that was found was in one or two individuals and never specific to one group.

Blasting the samples for the psbA-trnH region against Genbank showed that the sequence data resembles species within Asteraceae far more closely than any Gymnosperms, thus indicating genetic contamination of samples with Asteraceous DNA. ITS sequence data did however conform closest to samples of *Widdringtonia* in Genbank and so these PCRs were not genetically contaminated. The ITS sequence data aligned well with the sequence data obtained for this gene region by De Roo and Hedderson (unpublished). This allowed the samples to be incorporated into a phylogenetic tree of the genus *Widdringtonia*.

The phylogeny of *Widdringtonia* contains two main clades as shown in Figures 2 and 3. The first clade contains *W. whytei* and *W. nodiflora* from the northern part of the species distribution. The second clade contains *W. nodiflora* from the Cape as well as *W. cederbergensis* and *W. schwarzii*. Only one individual sequence for ITS from a true *W. nodiflora* on Mount Mulanje did not cluster with the previously sequenced sample of *W. whytei* and the other six samples that I sequenced, but was placed within the overall clade with individuals of *W. nodiflora* from populations in Zimbabwe and northern South Africa. Within my samples from Mount Mulanje, the individuals with longest branch lengths are within true *W. whytei* and true *W. nodiflora* samples (0.01), while forestry *W. nodiflora* has shortest branch lengths of 0.0009.

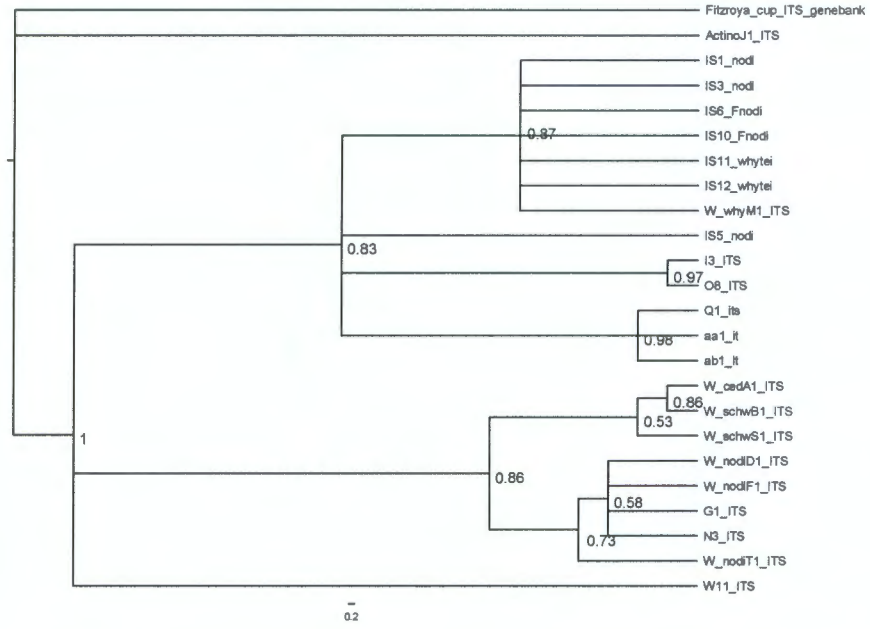

Figure 2. Phylogenetic tree for the genus *Widdringtonia*, showing posterior probabilities of nodes. IS1-IS12 indicate the seven samples collected and sequenced in this survery.

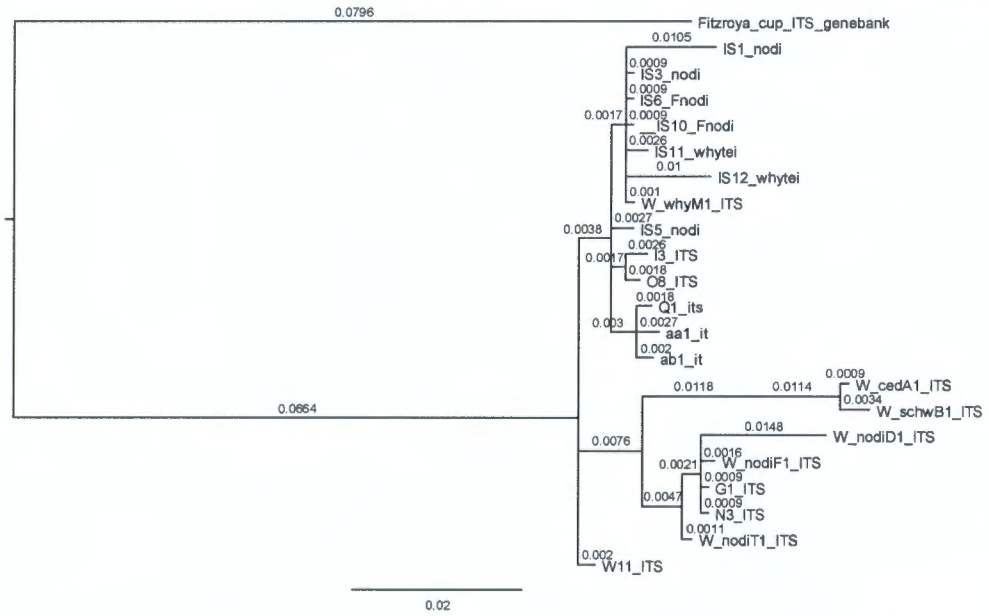

Figure 3. Phylogenetic tree for the genus *Widdringtonia*, illustrating branch lengths. IS1-IS12 indicate the seven samples collected and sequenced in this survey.

Nine microsatellite primers developed for *Austrocedrus* (Arana et al 2007) were tested for amplification but none appeared to work for *Widdringtonia* using the PCR conditions utilised by Arana et al (2007). Changing the PCR conditions and concentrations of contents of the PCR mix did not lead to successful PCR amplification.

Of the nine ISSR primers tested, only four appeared to amplify successfully. For these primers that worked, bands were not well differentiated, but resolution did not improve with optimization of the PCR conditions. However, bands were distinct enough to determine that there is no variation present for these DNA regions across all samples amplified.

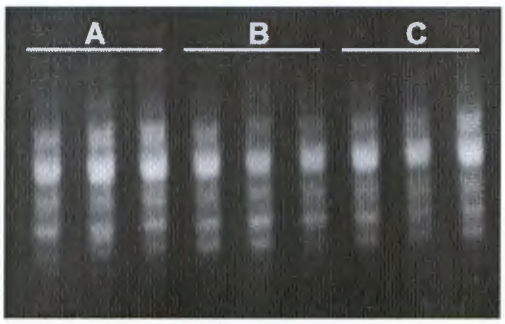

Figure 4. ISSR primer 859

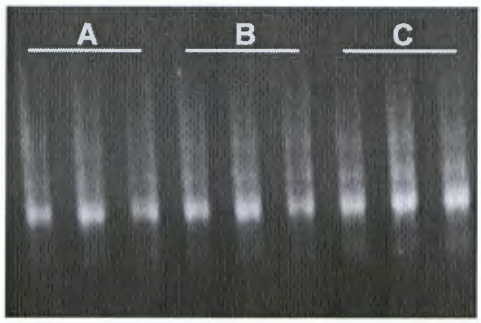

Figure 5. ISSR primer 860

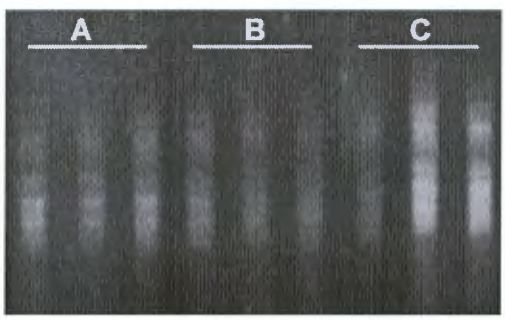

Figure 6. ISSR primer 889

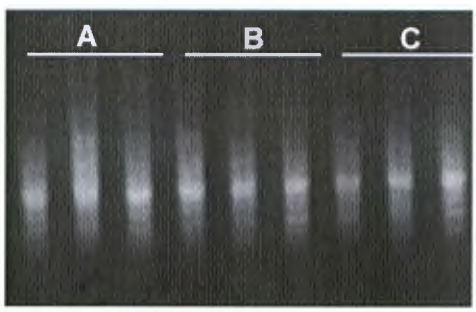

Figure 7. ISSR primer 890

Figure 4 – 7. Key: **A** = true *Widdringtonia nodiflora*, **B** = forestry *Widdringtonia nodiflora*, **C** = true *Widdringtonia whytei*.

Morphological comparison between *W. whytei* and *W. nodiflora*

Morphometric analysis revealed that certain features of cones and seeds differ significantly between samples of definite *W. nodiflora* and *W. whytei* on Mount Mulanje. Cones of true *W. whytei* do not differ significantly from the forestry *W. nodiflora* group. There is less variation in size within the forestry *W. nodiflora* group than true *W. whytei* but this may be due to the different sampling sizes. Different sample size was the reason for not performing a multivariate test on the data, such as an analysis of variance (ANOVA). The *W. whytei* and *W. nodiflora* groups differ significantly from definite *W. nodiflora* in terms of both length and width of cones (Figure 8), although width (37.33% , $P<0.001$) more than length (16.48% , $P<0.05$). The significant difference between groups is both in terms of ratio of length to width of cones as well as overall size of cones. *W. nodiflora* cones from the Western Cape are larger in size but not significantly different in shape ie. Ratio of length to width.

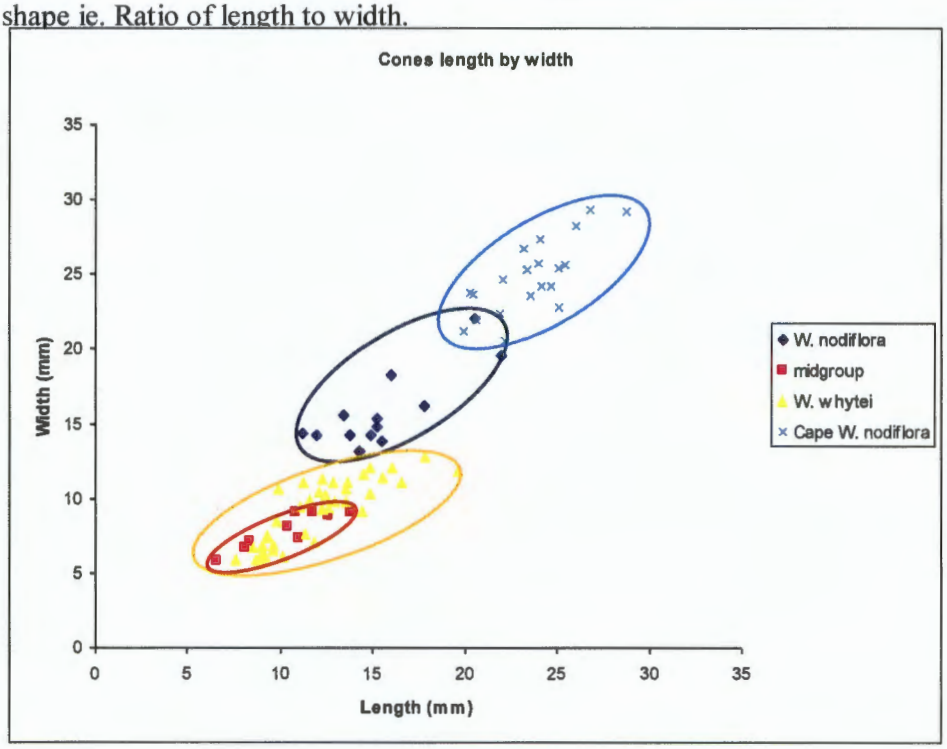

Figure 8. Graph showing length and width of cones from different groups as defined by colours, and area of occupancy outlined by circles.

Seeds of true *W. whytei* and forestry *W. nodiflora* do not differ significantly. There is a significant difference between *W. whytei* and true *W. nodiflora* (Figure 9) in terms of length (17.26%, P<0.001) and width (28.78%, P<0.001), although there is some overlap between these groups. The difference is more in terms of size than ratio of length to width. Western Cape *W. nodiflora* seeds are similar in size to true *W. nodiflora* on Mount Mulanje.

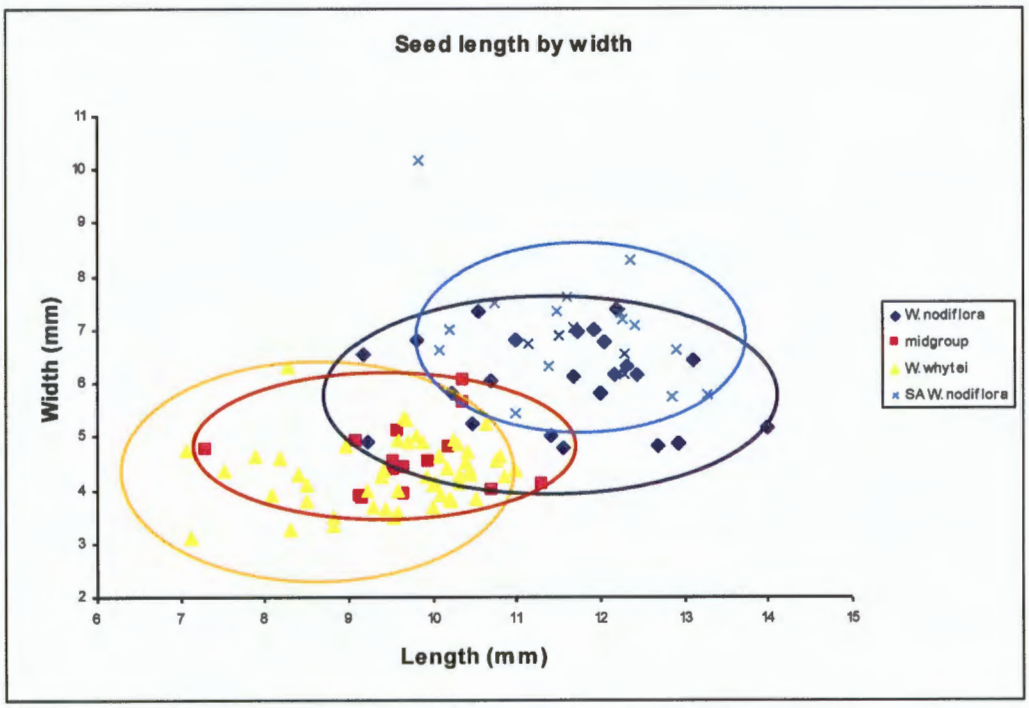

Figure 9. Graph showing length and width of seeds from different groups as defined by colours, and area of occupancy outlined by circles.

Table 6. Results of T-tests of significant difference between groups based on seed and cone dimensions. Difference between means is shown as percentage.

	Seed dimensions				Cone dimensions			
	length		width		length		width	
Group	% diff in mean	P value	% diff in mean	P value	% diff in mean	P value	% diff in mean	P value
N-W	17.26	< 0.001	28.78	< 0.001	16.48	< 0.05	37.33	< 0.001
N-M	15.85	< 0.001	24.30	< 0.001	27.85	< 0.01	45.83	< 0.001
W-M	1.68	NS	5.93	NS	13.60	NS	13.56	NS

Discussion

Genetic variation

Although it was hoped that analysis of genetic data would reveal enough variation to be able to assess levels of within- and between-population genetic variation, no significant level of variation was detected within the regions assayed. However, this is still an important finding, if this is indeed a true reflection of the genetics of *W. whytei* and *W. nodiflora*.

The reliability of this study was however compromised by the fact that the psbA-trnH region was found to have been contaminated by Asteraceous DNA after the sequences failed to align to previously exracted DNA of *Widdringtonia*. It was unfortunate that this was only detected late in the study as there was not sufficient time to try and rerun the PCR and aquire sequence data from it. It may also have been beneficial to have amplified another chloroplast DNA region.

The ITS sequence data was incorporated into a phylogenetic tree of the genus, which inferred that all but one of the samples which I collected align closely with the sample of *W. whytei* from De Roo and Hedderson (unpublished). Whether that individual was a true *W. whytei* or a *W. nodiflora* cannot be determined from my data alone. However, there is evidence of divergence in some true *W. nodiflora* and *W. whytei* individuals from their longer branch lengths, while others have short branches from the base of this clade.

Although the ITS sequence data was not contaminated with non-*Widdringtonia* DNA, it is not known whether the ISSR data resulted from amplification of *Widdringtonia* DNA or from contaminant DNA of some non-*Widdringtonia* taxon. Judging by the fact that the ITS sequences of *Widdringtonia* showed no overall variation between taxonomic groupings, it is assumed that the ISSR results also show *Widdringtonia* alleles.

It is unusual that ISSRs do not show some variation within *W. whytei* or between this and true *W. nodiflora*, where there is some level of morphological differentiation. Even within small samples from a few populations of one species there is usually some differentiation between individuals. A study of *Liparia parva* and *Liparia splendens* by Letten (2005) showed variation in band patterns between a few close populations within

each species. Perhaps this difference in patterns of variation is due to differing dispersal ability, as *Liparia* is animal-pollinated while *Widdringtonia* is wind-pollinated.

A study of genetic variation in *Cupressus chenggiana* using ISSRs found a higher proportion of variance among populations than has been found in other conifer groups (Hao et al 2006), suggested to be due to the relative isolation among each population. The populations studied in *C. chenggiana* are a lot further apart than any populations of *W. whytei* on Mount Mulanje. The furthest populations surveyed (Sombani and Lichenya 2) are still less than 20km apart.

It is unfortunate that microsatellite markers did not amplify for *W. whytei* as this could have shown finer-scale genetic variation within the species, possibly between populations or within populations.

Morphometric variation between taxonomic groups.

The cones and seed of forestry *W. nodiflora*, are nested well within the range of cone dimensions of definite *W. whytei*. The definite taxa are significantly different in terms of length and width of cones and seeds (Figures 8 and 9), while there is no corresponding level of genetic differentiation that clusters individuals of these groups separately.

When surveying the six populations on the mountain, a number of differing features were evident. At Sombani forest, the first tree observed at the edge of the forest was identified by a local forester as *W. nodiflora*, although it seemed very similar in all respects to a tree growing next to it, which was identified as *W. whytei*. The only difference evident was that the first tree was branching from lower down than the second. However, this feature would not have been obvious without it having been pointed out. Deeper into the forest all *Widdringtonia* individuals present were identified as *W. whytei*, with long straight trunks only branching high up in the forest canopy. Three true *W. nodiflora* individuals were encountered next to Sombani forest in forest-edge scrub, which had recently been burnt and were actively resprouting multiple shoots from the base. There were mature cones already present on these plants, which were very similar in shape to cones on *W. nodiflora* growing in South Africa (Figure 8). The resprouting growth was the same form as that of plants in South Africa recovering following fire. The pre-fire size of these plants could be determined at a few metres in height from the burnt trunks that were still

standing or had fallen but had not yet disintegrated. Some other trees close by that had not been burnt were of a similar size and had multiple stems.

From the morphometric results obtained as well as field observations explained, it seems that the forestry *W. nodiflora* is in fact more closely grouped with *W. whytei* than true *W. nodiflora*. Whether this is as a result of genetics or of environmental conditions affecting growth form cannot be concluded at this stage. The fact that all trees of this group were observed outside or on the edge of forest patches, as well as the lack of genetic differentiation between these groups suggests that these two growth forms are purely a result of growing in different conditions. It appears obvious that a tree will be forced to grow tall and straight if within a forest habitat as it competes for light. Where competition is not such an issue and light is accessible closer to the ground it is not necessary to grow as tall before producing branches and leaves.

Not one true *W. nodiflora* of substantial size was encountered. This species may not be able to survive within closed canopy forest if it cannot grow tall enough to reach the light. Alternatively, could it be possible that cones decrease in size as trees grow bigger so that trees beyond a certain size will not possess the large cones typical of *W. nodiflora*. Large trees growing in a forest will not burn and so will not show evidence of resprouting from their base. This would support an hypothesis that there is one species on the mountain, and any variation present is likely a result of environmental conditions.

Morphological variation as a result of genetic or environmental variation.

Plantations of Mulanje Cedar were established on Zomba Plateau in 1907, but a proportion of these turned out to be of atypical form (narrow-crowned) unlike the majority which were of typical (wide-crowned) Mulanje Cedar form (Venkatesh 1987). It appears that the atypical form would presently be classified as *W. nodiflora* and the typical form as *W. whytei*. Chapola (1990) refers to differing wood properties, with the wood of the atypical form being less dense and of a shorter fibre length than that of the typical form. However, there is no reference to the conditions under which the trees were growing, or if other features such as cone shape and size were taken into account.

It would be interesting to know whether the *W. nodiflora* individuals in this plantation were true *W. nodiflora* as I have classified it in my study, or if they conform to the

forestry *W. nodiflora* group. If the latter is true, the difference in growth form and wood quality could perhaps be due to environmental conditions rather than genetics, especially if the supposed *W. nodiflora* trees were more common near the edge of the plantation. Chapman (1995) also makes reference to a number of different situations where Mulanje Cedar was planted. In some areas such as Chikangawa on the Viphya plateau, with a sheltered aspect and frequent mists the cedars grew very well, while in other places most trees turned out to be of the atypical form. It was thought that this was due to seed having mistakingly been collected from *W. nodiflora* trees instead of *W. whytei* trees, which is quite likely as *W. nodiflora* branches lower and thus cones are usually much easier to get to. It is also possible that these trees may merely have been grown under conditions where there was less competition for light, or unfavourable growing conditions caused trees to be stunted and produce lower quality wood.

However, there could just be genetically distinct growth forms within *W. whytei* as in many North temperate conifers such as Scots Pine and Norway Spruce (Venkatesh 1987). These are not necessarily distinct enough to be worthy of classification into different species, but perhaps recognised as varieties or subspecies within the species.

Because allelic variation was not found does not mean that it does not exist within *Widdringtonia* on Mount Mulanje. It could be that there are only a limited number of alleles that have become fixed for a particular morphological character in each species. It may require searching through many different genetic markers before these particular alleles are pinpointed. It is most likely though that much of the genetics is invariable between *W. whytei* and *W. nodiflora* on Mount Mulanje.

Determining historical processes from available data

The historical landscape was at times more forested than it is today and so *W. whytei* could have occurred more extensively in the past than at present. During the Last Glacial Maximum, montane forest was widespread in the Lake Malawi catchment (Debusk 1998) and so this would likely have increased the area of potential occurrence of the Mulanje Cedar. Prior to that conditions were extremely dry and that may have led to a population bottleneck, where the population of *Widdringtonia* on Mount Mulanje was effectively reduced to a minute population in which very little genetic variation was

conserved. However, while this could account for the lack of variation within *W. whytei*, it would not account for the lack of genetic differentiation between *W. whytei* and *W. nodiflora*. This rather suggests a recent divergence between these species, which also agrees with the phylogeny of the genus in which all groups in this study are clustered within the same clade, without enough genetic variation present to differentiate between currently accepted taxonomic groups.

The phylogeny of the genus *Widdringtonia* shows *W. nodiflora* (defined in a broad sense) to be basal to all other species in the genus (De Roo unpublished). Perhaps during the colder, drier conditions since the development of the cold Circum-Antarctic current (Cowling and Richardson 1995), more open grassy habitats allowed for the expansion of the range of *W. nodiflora*. A more recent reduction of this habitat at lower altitudes separated the different populations, leading to subsequent genetic divergence between different regions where it is found today. More recent expansion of forest would also have provided a niche where this species would have been at a disadvantage in competition for light as it devoted more resources to wood production at its base rather than in its trunk. A trade-off whereby more resources are devoted to wood production in the trunk rather than at the base would allow a tree to compete better in light-limited and fire-protected conditions. However, more exposed conditions where forest could not reach would have allowed *W. nodiflora* to persist close to *W. whytei* as they differentiated in response to different habitat niches.

However, while certain key morphological traits appear to have become fixed for each taxon, likely due to high selection pressure in the different habitats, this has taken place too recently for neutral genetic variation to have accumulated and become fixed for different taxa so as to provide genetic means of differentiating between them. This is a similar phenomenon as is observed between *W. cederbergensis* and *W. schwarzii* where there is very limited genetic and morphological variation evident between these taxa (De Roo 2002). While these two species were geographically separated relatively recently, so have *W. whytei* and *W. nodiflora* recently been separated ecologically. If the same factors continue to keep these species separated, they should continue to diverge until further genetic variation builds up between these taxa. As for the forestry *W. nodiflora*, it appears that this is merely an alternative growth form of *W. whytei*, either due to environmental or

genetic factors. Further study of the conditions under which this growth form is found in nature as well as plantations would provide a better means of determining its true taxonomic status. Investigating alternative genetic markers could provide better means of examining genetic variation within and between *W. whytei* and *W. nodiflora.*

In conclusion, it appears that there are in fact two species present on Mount Mulanje, *Widdringtonia whytei* and *Widdringtonia nodiflora. W. whytei* includes two growth forms which both possess small, egg shaped cones. *W. nodiflora* includes relatively smaller trees found outside the forest canopy with larger rounded cones and able to resprout after a fire. There is very little genetic variation present within these species across the mountain, and apparently no genetic structure is present across populations. Investigation of other genetic markers is necessary to be able to confirm this finding.

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