Molecular systematics of the genus *Widdringtonia* Endl.

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Abstract

The systematics and phylogeny of the genus *Widdringtonia* Endl. is examined using psbA (chloroplast) and ITS (nuclear) sequence data. Support for the monophyly of the genus is demonstrated. It is found that *W. whytei* deserves species status and is sister to the other three species *W. nodiflora, W. cedarbergensis* and *W. schwarzii,* also *W. nodiflora* is found to be sister to a W. *cedarbergensis* and *W. schwarzii.* Almost no variation is found between *W. cedarbergensis* and *W. schwarzii.*

Introduction

Widdringtonia Endl.

The genus *Widdringtonia* (Cupressaceae) is a small genus of tropical to subtropical evergreen conifers (McIver 2001) that occur along the southern and eastern Mountain ranges of Southern Africa extending as far north as Malawi. Only three species taxa are commonly recognised within the genus: *W. cedarbergensis* Marsh, *W. nodiflora* (L.) Powrie, and W. *schwartzii* (Marloth) Mast.; however Pauw and Linder (1997) showed that *W. whytei* Rendle should be reinstated to species level status based on phenetic, phylogenetic, ecological and biological grounds. Marsh (1966) had previously nested *W. whytei* with *W. nodiflora* based on morphological evidence. Systematics of the genus are currently based only on morphological and ecological evidence.

Plants of *Widdringtonia* are monoecious trees or shrubs. Two types of leaves are found, needle-like in juveniles and scale-like in adults (Marsh 1966). *W. cedarbergensis* and *W. schwarzii* are very similar, differences between the two taxa can be found in growth form, seed size, seedling size and location. *W. nodiflora* and *W. whytei* are exceptionally similar in some features, but clear differences between the two taxa can be found in growth form and ecology (Pauw and Linder 1997).

W. cedarbergensis is a tree 5-7 m high up to 20 m with a pyramidal crown when young, spreading with age. Seed scars of *about* 4.5 × 6 mm are found and cotyledon leaves after germination are 35 mm long and 5 mm broad (Marsh 1966). The species is found in a small distribution range on the Cedarberg Mountains

near Clanwilliam in the Western Cape between altitudes of 915 and 1525 m on rocky ground (McIver 2001).

W. schwarzii is a tree normally 17-26 m high up to 30 m (White 1983) with a pyramidal crown usually not spreading with age. Seed scars of *about* 1.5 × 2.5 mm are found and cotyledon leaves after germination are 20-25 mm long and about 2 mm broad (Marsh 1966). The species is found in a small distribution range on the Baviaanskloof and Kouga Mountains in the Eastern Cape near Willowmore and are usually confined to south facing slopes in inaccessible steep sided gorges (personal observation).

W. nodiflora is a multi-stemmed shrub or narrow crowned tree (Pauw and Linder 1997). It coppices after fire and is common in fire prone heathlands. The species is found in scattered populations from Table Mountain near Cape Town to Mount Mulanje in southern Malawi occurring on humid mountain slopes (McIver 2001).

W. whytei is a tall, wide crowned forest tree (Pauw and Linder 1997). It has limited fire survival capability when compared with *W. nodiflora*. The species is found on Mt Mulanje in Malawi in sympatry with *W. nodiflora*.

Systematics and phylogeny

Morphological and molecular data were used to investigate the systematics and phylogeny of the Cupressaceae by Gadek et. al. (2000) using matK and rbcL sequence data. Included in this study were rbcL sequence data for *W. cedarbergensis* and matK data for *W. Schwarzii*. The relevant clades *Widdringtonia* is nested in are shown in Figures 1-4.

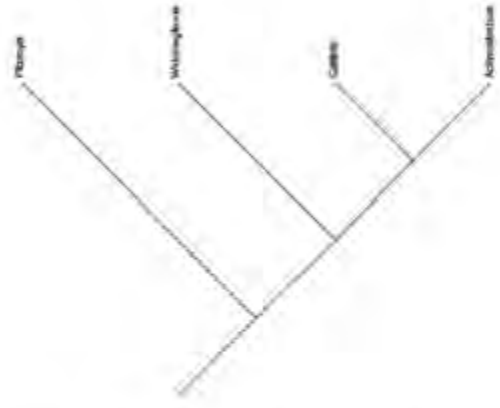

Figure 1. morphological relationship

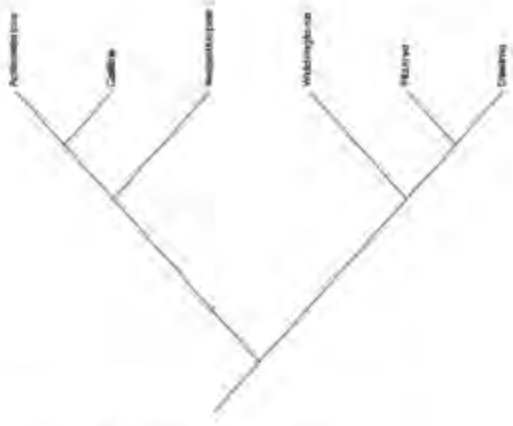

Figure 2. matK relationship

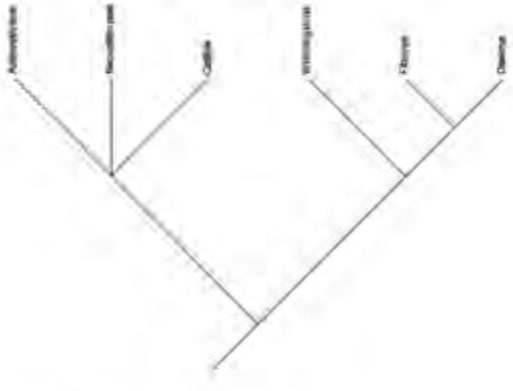

Figure 3. rbcL relationship

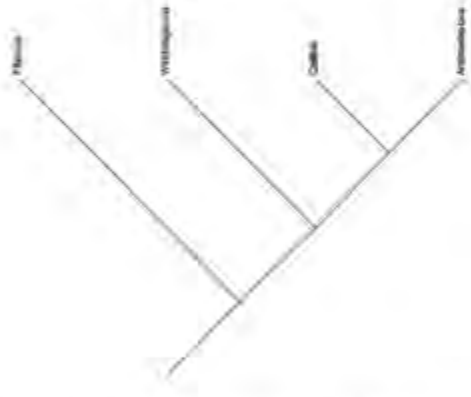

Figure 4. Strict consensus all the combined data. Diselma in a separate poorly supported clade (not shown)

Gadek et. al. (2000) recognised two major clades within the Cupressaceae, the callitroides in which *Widdringtonia* is nested and the cupressoids. All callitroides occur only in the Southern Hemisphere.

Biogeography

McIver (2001) used fossil evidence to suggest that *Widdringtonia* was present along the south-eastern coastline of North America and in Greenland around 95-80 Ma and in Europe around 95 and 40 Ma. There is only one fossil record of the genus from anywhere in the Southern Hemisphere thought to be of an undetermined Tertiary age and this record falls within the extant distribution of *Widdringtonia* (McIver 2001). McIver hypothesises that African taxa of *Widdringtonia* are descendants of populations that were present in Europe during the Tertiary (40Ma). Links shown with this possible origin are best seen with *W. nodiflora* for which seed cones and foliage are similar in size and morphology to fossils that appear in Europe (McIver 2001). McIver (2001) proposes that morphological evolution of cones within *Widdringtonia* involves an increase in seed cone size (based on fossil evidence). An extension of this hypothesis is that seed cone size increases are associated with adaptation to dryer climates (this holds true for extant taxa) (McIver 2001). Since McIver (2001) does not recognise *W. whytei* in her study it is presumed here that the linkage to *Widdringtonia* in Europe is also seen by her for *W. whytei* which is so morphologically similar to *W. nodiflora* (Pauw 1998).

Demographic status and conservation

W. cedarbergensis (Thomas and Bond 1997) and *W. whytei* (Pauw and Linder 1997) are specifically under threat of extinction. A multitude of studies has been carried out investigating the demographic status, ecology and conservation of *W.*

cedarbergensis. *Widdringtonia* demographic studies include *W. cedarbergensis* (Luckhoff 1971, Kruger and Haynes 1978, Manders 1986a, Manders 1986b, Manders 1987, Mustart and Bond 1994), *W. schwarzii* (Luckhoff 1963), *W. nodiflora* and *W. whytei* (Pauw and Linder 1997). Population level allozyme variation has been investigated of all species except *W. whytei* (Thomas and Bond 1997).

Thomas and Bond (1997) found patterns of allozyme variation varied between species, with *W. nodiflora* showing the greatest. Within species, allozyme variation occurs mainly within populations for *W. cedarbergensis*, between populations for *W. schwarzii* and between meta-populations of the more widespread *W. nodiflora*.

This study examines the systematics and phylogeny of *Widdringtonia* based on psbA (chloroplast DNA) and ITS (nuclear DNA) sequence data. The objectives are: 1. To test the monophyly of the genus, 2. to establish the phylogenetic relationships among species, 3. to examine differentiation between *W. cedarbergensis* and *W. schwarzii* using DNA evidence and 4. screen potential markers for phylogeographic studies of *W. cedarbergensis* and *W. schwarzii*.

Methodology

Sampling

Populations of *Widdringtonia* were visited and sampled for fresh leaf material. Four populations of *W. cedarbergensis*, and five of *W. schwarzii* were sampled (See Table 1 and Figure 5). Population sampling for *W. cedarbergensis* and *W. schwarzii* was aimed at covering most of the distributional range. Sampling of *W. nodiflora* consists only of Western Cape populations and only one *W. whytei* population from Mt. Mulanje was collected. For each population at least three individuals were collected (in most cases six).

Table 1. Species sampled, population, approximate location and sample size

Species	Population/Location	Sample size for region sequenced			Code
		PsbA	ITS	5S	
W. cedarbergensis	Die Hoek (33°43'40" S 19°08'40" E)	3	1	1	A
W. cedarbergensis	Pakhuis Pass (32°07'20" S 19°00'06" E)	5	1	1	P
W. cedarbergensis	Kleinkoupoort (32°15'44" S 19°05'52" E)	6	1	1	K
W. cedarbergensis	Welbedacht (32°24.529 S 19°10.803 E)	5			X
W. schwarzii	Saagkuilskloof (33°31.221 S 23° 39.059 E)	6	1	1	B
W. schwarzii	Sapreerivier, ((33°40.23.8 S 23°35.778 E)	6	1	1	S
W. schwarzii	Boschkloof, Baviaanskloof (Kougaberge)	1			E
W. schwarzii	Doringkloof, Baviaanskloof (Kougaberge)	1			C
W. schwarzii	Uitslag, Baviaanskloof (Baviaansberge)	1			U
W. nodiflora	Dutoitskloof (33°44'30" S 19°07'50" E)	3	1	1	D
W. nodiflora	Fernkloof, Hermanus	3			F
W. nodiflora	Table Mountain, near Grootkop	2			T
W. whytei	Mt Mulanje	3	1	1	M
Fitzroya cupressoides	Conifer Nursery (Sevenoaks, Kent, UK)	1			H
Actinostrobus sp.	'Australian garden', UCT	1			J

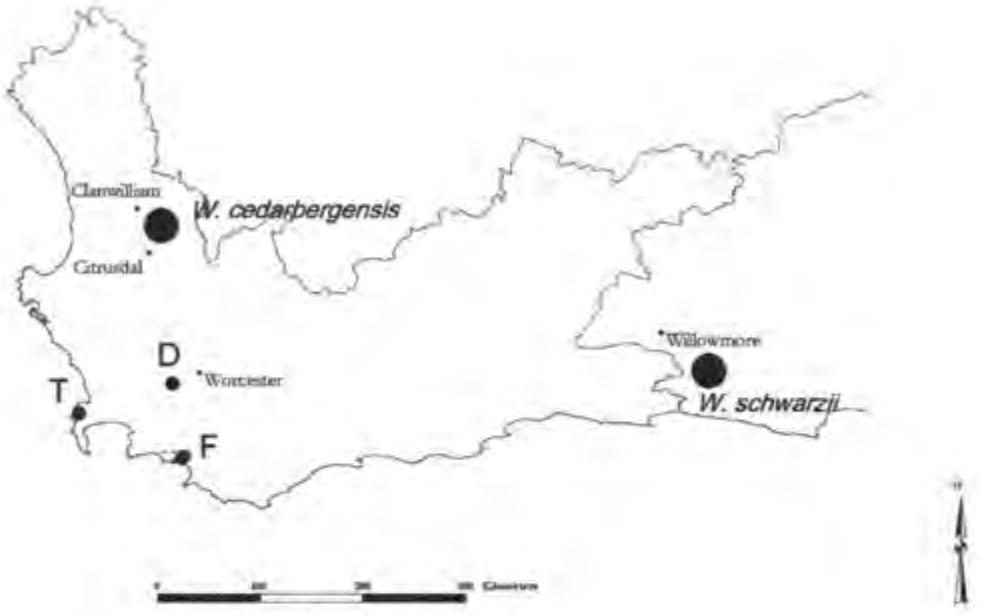

Figure 5. Sampling maps for W. cedarbergensis (4 populations), W. nodiflora (3 populations) and W. schwarzii (5 populations) in the south-western Cape.

Outgroups taxa were chosen on the basis of the proposed phylogeny of the Cupressaceae by Gadek et. al. (2000). For all separate data analyses, *Fitzroya* and *Actinostrobus* are either found as sister or basal clades with respect to *Widdringtonia*. While complete sampling of these taxa (given the assessment of monophyly in this study) would be preferential, simplistically these two genera are used as outgroups.

Data collection and analysis

Total DNA was extracted from fresh leaves dried in silica gel crystals using the CTAB (cetyldimethylammonium bromide) extraction method (Gawel & Jarret 1991). To prepare for the extraction, samples were first ground with PVP

(polyvinylpyrrolidone) to a fine powder using liquid nitrogen. Regions were amplified using the primers listed in Table 2 under conditions listed in Table 3.

Table 2. Primers used (PCR primers were used in sequencing)

Region	Code	Sequence	Reference
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'	
5S (F)	PIV	5' GGA GTT CTG ACG GGA TCC GG 3'	Cox et. al. 1992
5S (R)	PIII	5' GAG AGT AGT ACA TCG ATG GG 3'	

Table 3. PCR recipes and thermal conditions

Primer	Recipe	Thermal Condition
PsbAF TrnHR	30 ul reactions: 17.65 ul npH_20, 3 ul NH_4, 3 ul $MgCl_2$, 1.2 ul dNTPs, 1 ul of each primer, 0.15 ul Taq, 3 ul DNA.	97° 2min ; (97° 1min + 52° 1min + 72° 2min) 30cycles 72° 7min, 4° hold.
ITS5 ITS4	50 ul reactions: 29.7 ul npH_20, 5 ul NH_4, 2 ul dNTPs, 1.5 ul each primer, 0.3 ul Taq, 5 ul DNA	
PIV PIII	30 ul reactions: as for psbA	94° 2min ; (94° 1min+ 52° 1min + 72° 2min) 30cycles 72° 7min, 4° hold.

PCR templates were cleaned using the QIAquick[R] PCR Purification Kit. Sequencing reactions, using the same primers as for PCR, used ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (cycle sequence mix at 1/8 strength for psbA and 1/4 strength for ITS) products were resolved on an ABI3900 Prism, Automated Sequencer. Sequences were assembled and checked using the software DNAStar SeqMan and manually aligned and stored using DNAStar MegAlign software. Deleted segments were treated as missing data in the analysis. Unambiguous insertions and deletions ('indels') were scored as additional characters.

Exhaustive searches were conducted in PAUPv4.0 for each region separately. Strict consensus was used for multiples of equally parsimonious trees. Jackknife option in PAUP was used to indicate relative support for clades identified.

Results

The 5S database

The 5S locus was sequenced for three individuals of *W. cedarbergensis*, two of *W. schwarzii*, one of *W. nodiflora* and one of *W. whytei*. Taxa with more than one individual sequenced are from different populations (see Table 1). No variation is present in the 5S region among any of the samples with the exception of two base pair changes for *W. whytei*. With no informative characters, the 5S sequences were excluded from any further analyses.

The psbA database

The psbA locus was sequenced for all populations sampled including the two out-group taxa (see Table 1 for the number of individuals sampled for each population). Variation was found between taxa but none between populations or individuals. Because of the invariance of psbA for individuals within taxa further phylogenetic analysis was conducted using a single individual from each taxon. The final alignment comprises of 569 positions. In addition 18 indels were scored. Sequence data for *Fitzroya* and *Actinostrobus* showed a large variant section not present in *Widdringtonia*, given the difficulty of aligning this section, it was excluded from the analyses (bp 268-332) leaving a total of 507 characters.

One most parsimonious tree emerges from an exhaustive search of the psbA data with a tree length of 76 and a consistency index (excluding uninformative characters) of 0.8065 (see Figure 6). *Widdringtonia* is robustly resolved as monophyletic with a jackknife value of 100. *W. whytei* is sister to the remaining

taxa with jackknife support of 95, and *W.* nodiflora is sister to W. *cedarbergensis* and *W. schwarzii* with jackknife support of 98. There is no variation between *W. cedarbergensis* and *W. schwarzii.*

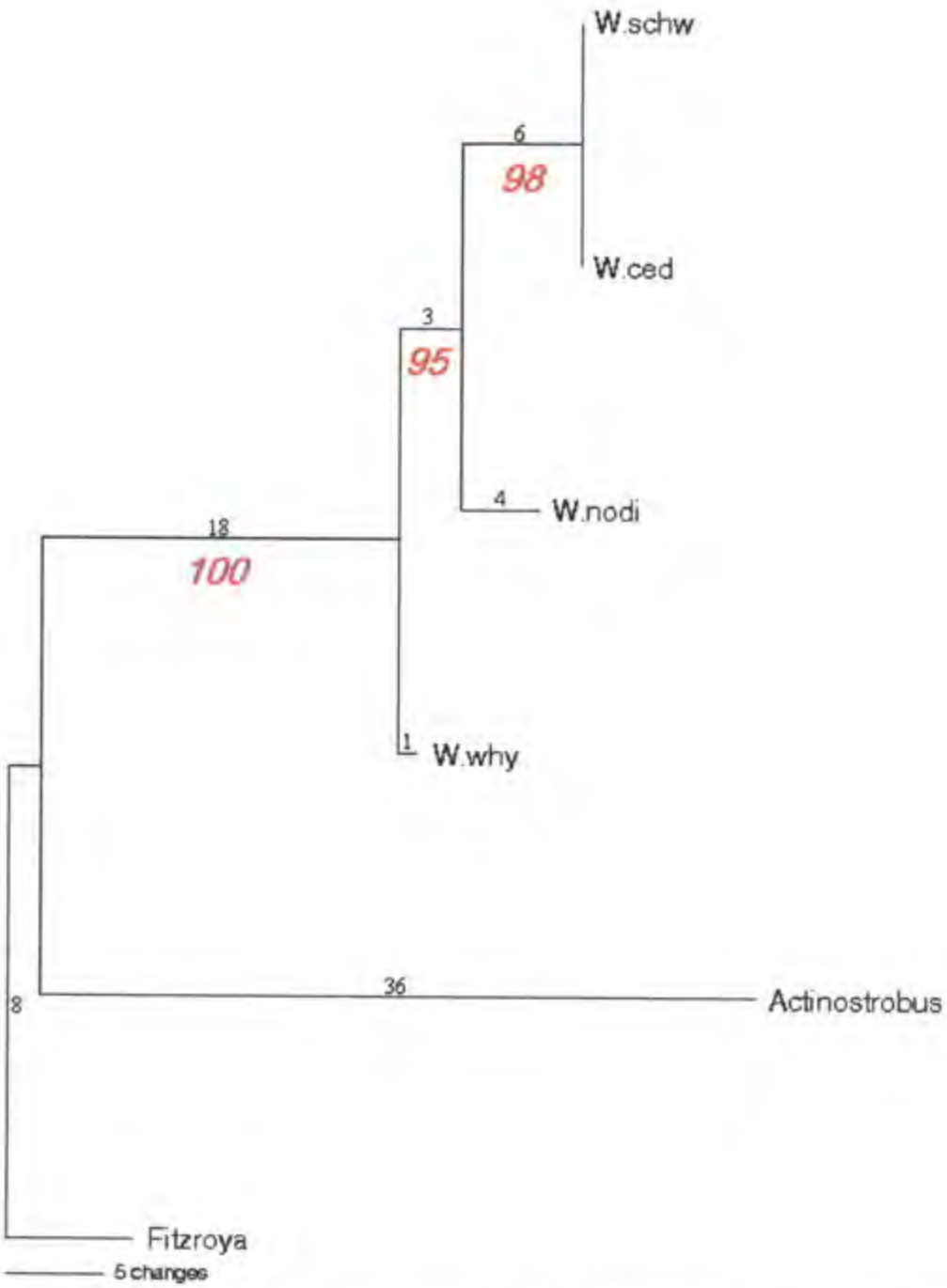

Figure 6. Phylogram of the psbA data. Length = 76, CI (ex.) = 0.8065, RI = 0.8125, RCI = 0.7484. Black text = tree length. Red text = jackknife support.

The ITS database

The ITS locus was sequenced as for 5S (see Table 1). Variation was found between species but none between populations or individuals. As with the psbA data, further phylogenetic analysis was conducted using a single individual from each taxon. The final alignment comprised of 840 positions. In addition 8 indels were coded for. There is no sequence data for *Fitzroya* and *Actinostrobus*. Based in the results from the psbA analysis, the tree is rooted using *W. whytei*.

One most parsimonious tree emerges from an exhaustive search of the ITS data With a tree length of 32 and a consistency index (excluding uninformative characters) of 1 is found (see Figure 7). A jackknife value of less than 50 is found for the *W. nodiflora*, *W. cedarbergensis* and *W. schwarzii* clade with *W. cedarbergensis* and *W. schwarzii* clade sharing six informative characters has a jackknife support of 100.

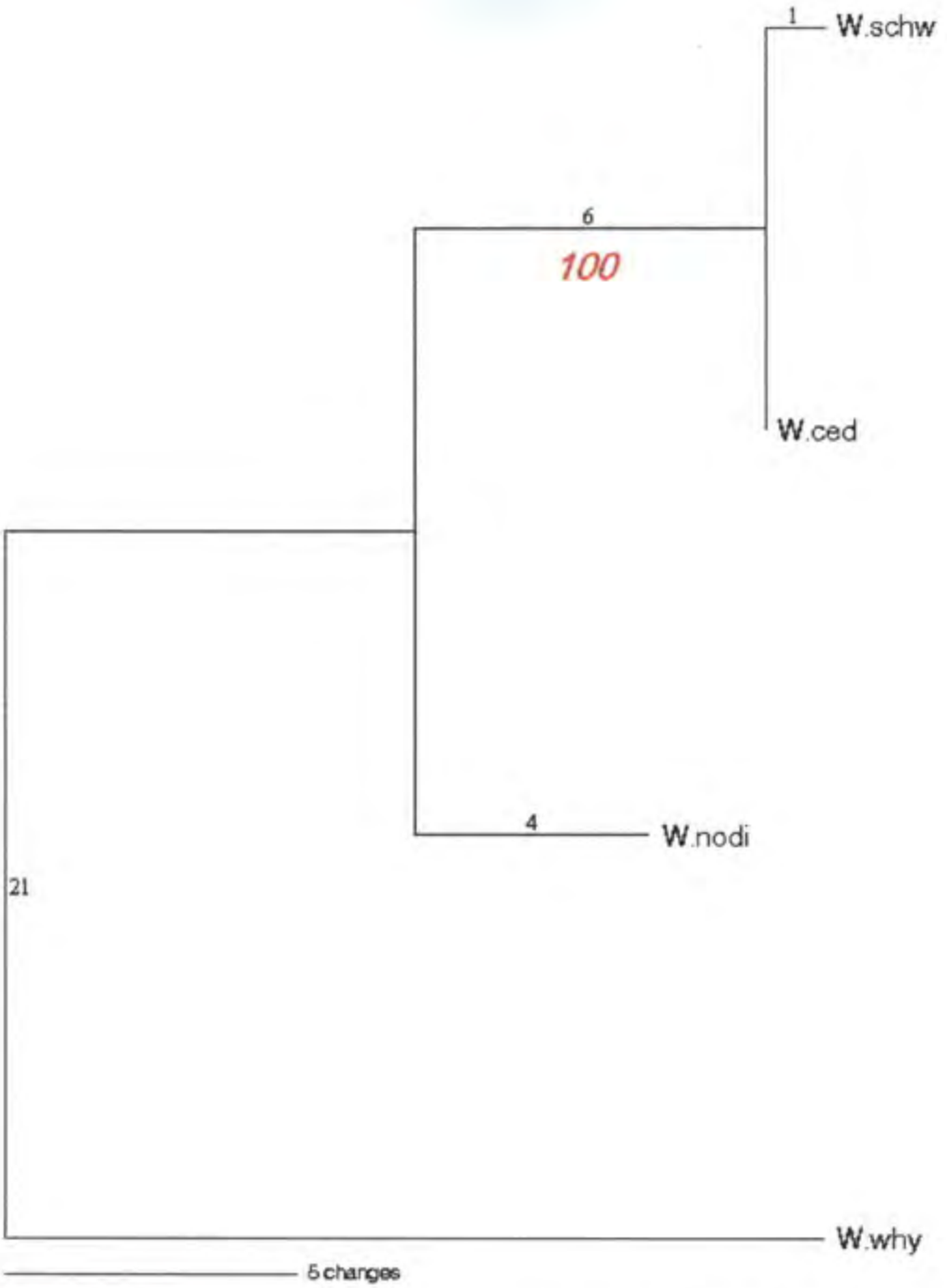

Figure 7. Phylogram of the ITS data. CI (ex.) = 1, RI = 1, RCI = 1. Black text = tree length. Red text = jackknife support.

Combined analysis

A combined analysis of all species level taxa resulted in a single most parsimonious tree with a length of 108, a consistency index (excluding uninformative characters) of 0.8378 (see Figure 8). Again the *Widdringtonia* clade nested as a robust monophyletic group with a jackknife of 100. *W. whytei* sister to the remaining species with a jackknife of 88, and *W. nodiflora* is sister to a W. *cedarbergensis* and *W. schwarzii* with a jackknife of 95.

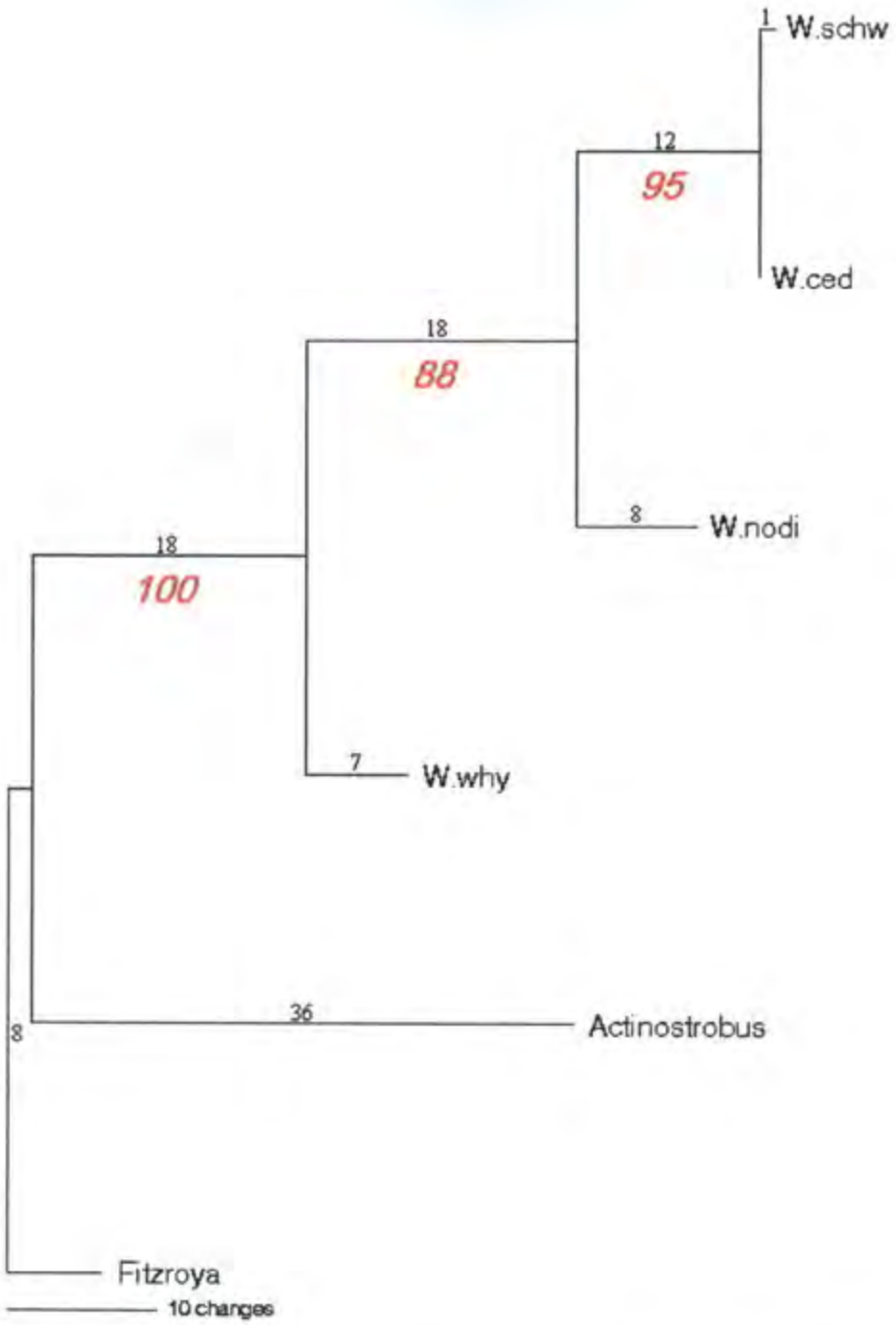

Figure 8. Phylogram of the combined data. Tree length = 108, CI (ex.) = 0.8378, RI = 0.8421, RCI = 0.7953. Black text = tree length. Red text = jackknife support.

Discussion

Phylogenetic relationships

Since no incongruence is found between the two regions, the combined data set is used to infer phylogenetic relationships.

The monophyly of the genus *Widdringtonia* is clearly supported given the robustness of the relationship. Evidence, however, is unfortunately only from the psbA region and with use of *Fitzroya* and *Actinostrobus* as outgroups. There is still a chance that monophyly could be rejected given inclusion of more outgroup taxa (especially taxa like Diselma and other Callitroids). Secondly incongruence is also a possibility between the chloroplast phylogeny and a nuclear phylogeny through events like hybridisation and introgression, should this be the case and incongruence has not been identified because the lack of nuclear data for the outgroup then monophyly might fall away. Given data in this study however it is clear that *Widdringtonia* is a monophyletic clade.

W. whytei Rendle

Results strongly support evidence found by Pauw and Linder (1997) that *W. whytei* is a separate taxon from *W. nodiflora*. Combined data show that *W. whytei* is sister to the other three species, as a result of the early divergence of the two. From a conservation perspective this also places high priority for the preservation of *W. whytei* based on the preservation of phylogenetic diversity (Humphries et. al. 1995, Crozier 1997, Hedderson 2002). As *W. whytei* is currently lumped with the wide

ranging *W. nodiflora* it does not receive adequate conservation and is at serious risk of extinction (Pauw 1998).

Variation between *W. cedarbergensis* and *W. schwarzii*

The very low levels of differentiation between *W. cedarbergensis* and *W. schwarzii* suggest that either the two are very recently diverged or a single taxon. Separating the two from a molecular perspective is a single base pair change. It is quite possible that this is simply intraspecific variation between two widely separated populations. Morphologically the two species are very much alike as discussed in the species descriptions; a difference in seed size and tree height could simply be an artefact of environmental variables. This is especially possible given the hypothesis proposed by McIver (2002) that larger seed cones are an adaptation to dryer climates. Whether *W. cedarbergensis* has the ability to produce smaller seed cones under more humid conditions or if *W. schwarzii* increases its seed cone size under drier conditions could answer some of these issues (i.e. whether differences in size are genetically fixed or the result of environmental conditions). Irrespective of whether they are considered one taxon or two, the phylogeny of *Widdringtonia* does enable a perspective on conservation priorities within the genus. Assessing priorities based on *phylogenetic diversity* using a branch length distance approach (Hedderson 2002) it seems that if choosing two taxa, *W. schwarzii* and *W. whytei* are the high priorities (highest inter node distance = 38), the third priority would probably be *W. nodiflora.*

As potential markers for phylogeographic studies of *W. cedarbergensis* and *W. schwarzii* psbA, ITS and 5S do not provide suitable variation at the population level. Possibly investigation into AFLP's (amplified fragment length polymorphisms) or locus specific micro-satellites could reveal the desired variation (Hedderson, Pers. Corr.). Rumours of a population of *W. schwarzii* north of the Baviaansberge needs to be investigated (possibly the Groot-winterhoekberge, Grootrivierberge) as this, if it exists, will provide another widely disjunct population with which to bring the difference between *W. cedarbergensis* and *W. schwarzii* into perspective.

Biogeography

The phylogeny of *Widdringtonia* does not completely contradict McIver's (2001) hypothesis of *W. nodiflora* and connections with European Tertiary *Widdringtonia* populations. Although lumping *W. whytei* with *W. nodiflora* and choosing to ignore the suggestion of species level taxon status proposed by Pauw and Linder (1997) does limit the ability to reconstruct the biogeographic history of *Widdringtonia*. Taking *W. whytei* into account it seems that the hypothesis of European connection is plausible (given the similar morphology to *W. nodiflora*). Possibly followed adaptation to fire *W. nodiflora* extended the range of *Widdringtonia*, spreading from the Cape and further north, linking up with *W. whytei* once again. *W. cedarbergensis* and *W. schwarzii* possibly arose from *W. nodiflora* with the onset of less humid Mediterranean conditions probably before *W. nodiflora* adapted to fire.

Conclusion

Widdringtonia is a monophyletic clade shown by molecular data of the psbA region in the chloroplast. *W. whytei* emerges from its apparent morphological similarity with *W. nodiflora* as truly separate taxa based on both psbA and nuclear ITS, supporting what Pauw and Linder (1997) found based on phenetic, phylogenetic, ecological and biological grounds. *W. cedarbergensis* and *W. schwarzii* are very similar, differing in only one base pair of the ITS region.

This fresh view into the systematics should help to bring perspective into the conservation of *Widdringtonia*, while not suggesting that *W. cedarbergensis* is of low conservation priority, just highlighting the high priority of *W. whytei*.

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