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The evolution of the Afrotemperate-endemic genus *Macowania* (Asteraceae) in the Drakensberg region of South Africa

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Abstract

The cosmopolitan Asteraceae tribe Gnaphalieae, or paper daisies or everlastings, form a significant component of both the dry and cool temperate floras of southern Africa. Within this tribe exists a small Afrotemperate genus, *Macowania,* endemic to the grassland biome of South Africa and occurring almost exclusively within the Drakensberg region, apart from two disjunct species in North Africa. The age, relationships and geographic origin of *Macowania* is investigated in order to provide insight into the factors affecting speciation, especially uplift events, on this small Afrotemperate genus. A well-supported phylogenetic hypothesis based on both nuclear and chloroplast genes suggests that *Macowania* is sister to a clade corresponding to the *Relhania* clade s.s., and that these are in turn sister to a clade containing the genera *Athrixia* and *Pentatrichia. Macowania* is monophyletic only with the inclusion of the enigmatic monotypic genus *Arrowsmithia*, resulting in the future synonymy of *Macowania* with *Arrowsmithia*. The anomalous species *M. pinifolia*, previously part of the genus *Athrixia*, is placed in a polytomy with the *Relhania* s.s. clade and the remaining species of *Macowania* and *Arrowsmithia*. DNA sequence data could not be obtained for several *Macowania* species, including the taxa from North Africa. The placement of these species within *Macowania* is confirmed by means of a parsimony analysis of morphological characters against a molecular backbone constraint tree. One species, *M. tenuifolia*, is well-supported in two different placements within *Macowania* by chloroplast and nuclear DNA sequence data. The best position of this species is inferred by incongruence decomposition analysis and morphological affinities. Bayesian relaxed clock methods and ancestral area reconstruction using maximum likelihood and squared change parsimony estimate the age and ancestral area of the genus, and determine the timing and route of colonisation of the Drakensberg. Diversification within *Macowania* is consistent in timing with the uplift events during the Miocene and Pliocene that resulted in significant vertical movement in eastern South Africa, suggesting that colonisation of the high-elevation Drakensberg grassland by *Macowania* was promoted by uplift. The topographic heterogeneity and increased river action resulting from the uplift may also have promoted evolution into new habitats and potentially mediated the movement of the ancestor of *Macowania* into the Drakensberg region via riparian habitats.

Introduction

The grassland biome of South Africa describes an herbaceous vegetation type of relatively short and simple structure that is dominated by graminoids. Small and medium-sized shrubs also occur, but are usually confined to specific habitats such as smaller escarpments or *koppies* (Mucina & Rutherford, 2006). The grassland biome is distributed on the high central plateau, the inland areas of the eastern seaboard, the mountainous areas of KwaZulu-Natal and the central parts of the Eastern Cape (Mucina & Rutherford, 2006). The grasslands associated with the Drakensberg region experience high rainfall and low temperatures (Mucina & Rutherford, 2006), being associated with some of the highest elevations in South Africa.

The Drakensberg Alpine Centre (DAC) is one of five centres of plat endemism located within the boundary of the grassland biome, (Mucina & Rutherford, 2006; Carbutt & Edwards, 2006) and is regarded as the only true alpine centre in southern Africa. Extending over some 40,000km ^{2}, the centre supports high flowering plant diversity and relatively high floristic endemism (16%) (Carbutt & Edwards, 2004), accommodating many rare endemics with highly specific habitat preferences (Carbutt & Edwards, 2006). Endemic grasses include a range of danthonioid and pooid species, while non-graminoid endemics include representatives of, particularly, Asteraceae, Hypoxidaceae, Orchidaceae, Hyacinthaceae, Scrophulariaceae, Ranunculaceae, Lamiaceae and Aizoaceae (Mucina & Rutherford, 2006). Together with the Cape Floristic Region (CFR), the DAC has been identified as the southern, or Gondwanan, source of the temperate flora of South Africa (Hilliard & Burtt, 1987; Linder, 1990; Galley *et al,* 2006). While the origins of the floristic diversity within the grassland biome remains poorly understood, current understanding suggests that the sequence of speciation events leading to the endemic flora is quite recent, commencing around 5 Ma (Galley *et al,* 2006). Many of the Afrotemperate species have been found to have emanated from the Cape, with many diverse Cape clades (Linder, 2003) also having members in the Afrotemperate region (Cowling, 1983).

In the early Miocene (23.03-5.33 Ma), major continental uplift in the eastern part of South Africa—the Post-African 1 cycle—caused hot mantle material, from approximately 3000km to 1000km under southern Africa's continental crust, to flow onto the surface and rejuvenate the landscape (Partridge & Maud, 1987). The event caused uplift of between 150-300m and in the process the habitats and substrates available for plant colonisation would have been significantly altered (Partridge & Maud, 1987; Cowling *et al,* 2009). This was followed by a second, more pronounced uplift event in the Pliocene (5.3-2.6 Ma)—the Post-African II cycle—which caused approximately 600-900m of uplift resulting in further habitat modification, giving rise to a diversity of escarpment-edge habitats that presently support endemic plant assemblages (Partridge & Maud, 1987). Apart from its impact on substrata availability, the significant vertical movement that took place in the eastern region this time would have created the cold, high altitude zone that is currently occupied by alpine grassland vegetation (Mucina & Rutherford, 2006). Overall, the two post-African uplift events would have had a profound effect on the diversification of the southern African flora through their effect on topography, climate and soil

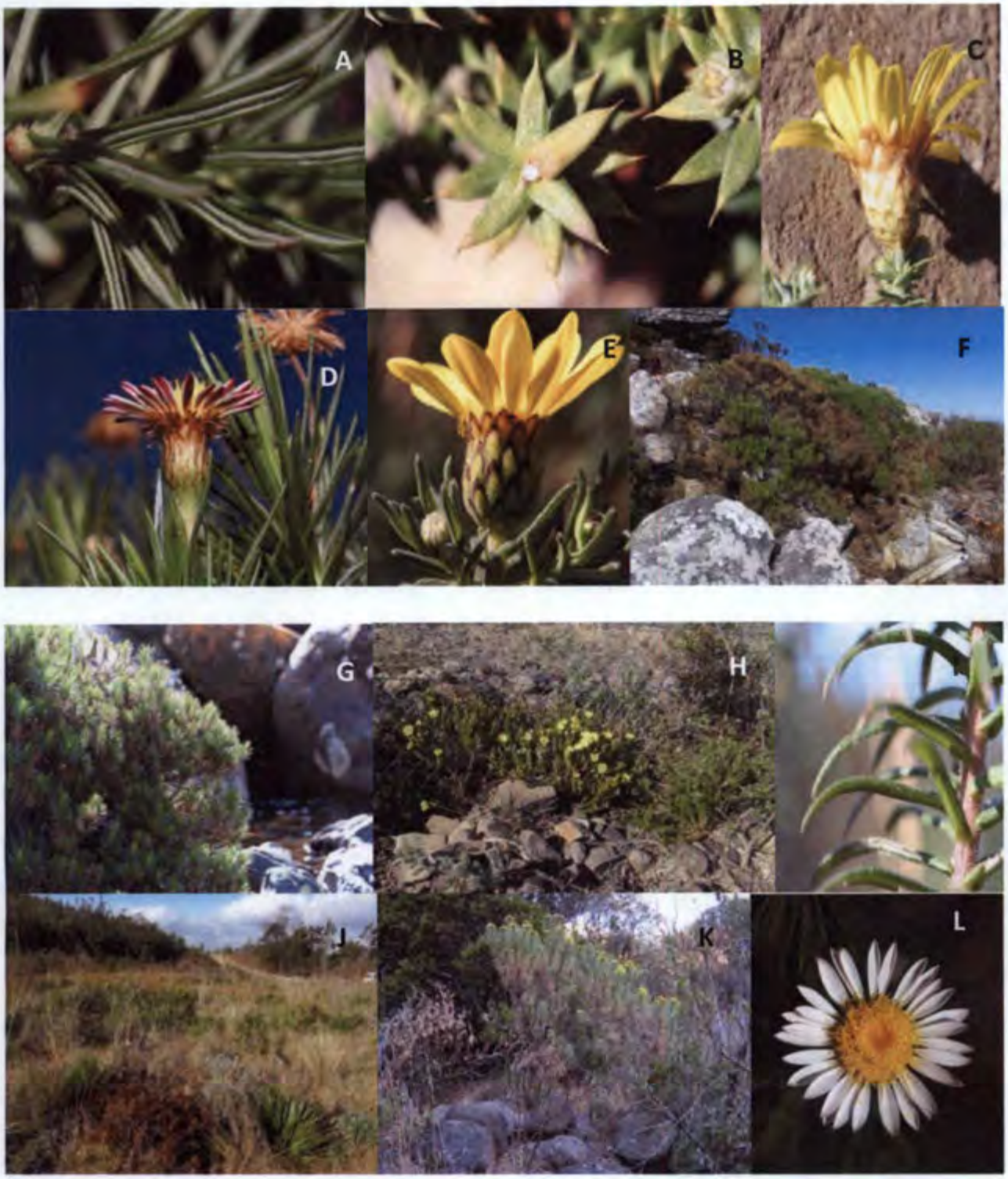

Fig 1: Habit and habitat of *Macowania and Arrowsmithia* species. A) Leaves of *M. revoluta* showing characteristic prominent midrib abaxially. (B) Pungent leaves of *Arrowsmithia* styphelioides that lack the prominent midrib (C) Capitulum of *A. styphelioides.* (D) *M. pinifolia* with mauve underside of ray florets. (E) Dark margined involucre of *M. revoluta* (F) Rocky habitat of *M. tenuifolia* (G) Riparian habitat of *M. pinifolia* (H) Rocky habitat of *A. styphelioides.* (I) Characteristic alternate leaves of *M. revoluta.* (J) 'Damp' open wetland habitat of *M. revoluta.* (K) Riparian habitat of *M. corymbosa* (L) Inflorescence of *M. pinifolia.* Photos: N.G. Bergh and C. McKune

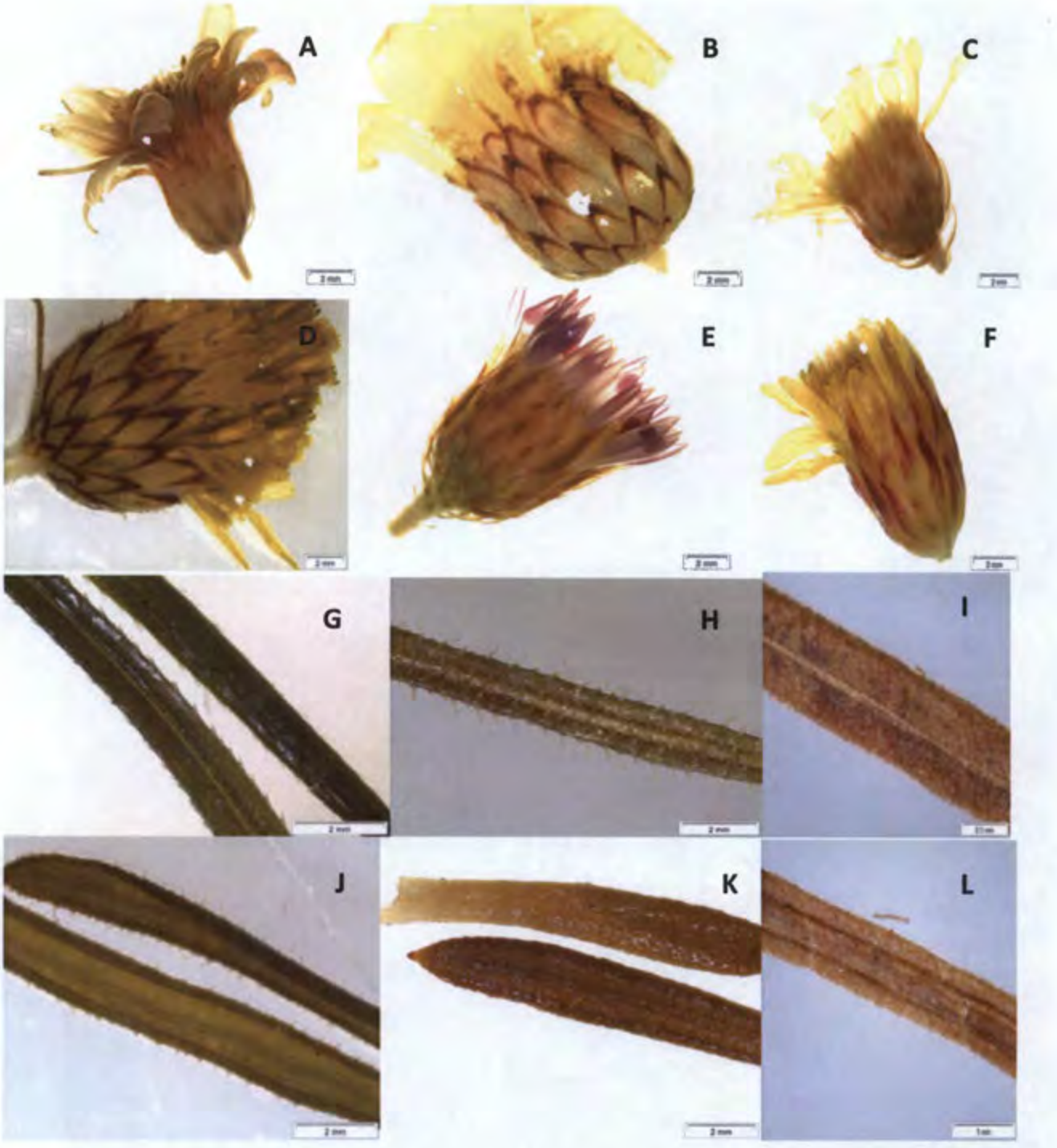

Fig. 2. Morphological features of *Macowania* and Arrowsmithia species: capitulum and leaf characters. (A) *M. pulvinaris* capitulum (B) *M. revoluta* capitulum showing bracts with dark edges. (C) *M. deflexa* capitulum (D) *M. corymbosa* capitulum; note similarity to *M. revoluta* (E) *M. pinifolia* capitulum showing mauve underside of ray florets (F) *M. tenuifolia* capitulum (G) *M. pinifolia* abaxial (right) and adaxial (left) leaf surfaces (H) *M. corymbosa* abaxial leaf surface. (I) *M. ericifolia* adaxial leaf surface. (J) *M. revoluta* abaxial leaf surface (K) *M. glandulosa* abaxial (bottom) and adaxial (top) leaf surfaces (L) *M. ericifolia* abaxial leaf surface Photos: J. Bentley and N.G. Bergh. All scale bars represent 2mm, except L (1mm) and I (0.5mm).

heterogeneity. Galley *et al* (2006) suggest the uplifted Drakensberg zone has played a significant role as stepping stone in the northward spread of the Cape flora.

Molecular phylogenetic inference provides a tool with which to investigate the historical construction of species-rich biomes (Pennington *et al,* 2006; Verboom *et al,* 2009). Investigating the diversification process within a group of species can provide insight into the historical processes and events that have shaped the species composition and landscape of a region. Modern molecular phylogenetic frameworks based on nucleotide substitution data and likelihood statistics, as well as relaxed molecular clocks allow for highly resolved phylogenies and temporal placement of speciation events. Galley *et al* (2006) provide a well-supported hypothesis based on molecular evidence that suggests a south to north migration of several Cape clades—clades which are suggested to have undergone their early diversification in the Cape Floristic Region (CFR), and of which half of the species are still to be found in the region (Linder, 2003)—accounting for the presence of a Cape element within the Afrotemperate Drakensberg region.

The cosmopolitan Asteraceae tribe Gnaphalieae, comprising the paper daisies or everlastings, forms a significant component of both the dry and cool temperate floras of southern Africa (Bergh & Linder, 2009). Members of Gnaphalieae are herbs or shrubs characterised by entire leaves and papery involucral bracts (Fig. 1 a, b, c, d, f, l; Fig. 2 a-l). The *Relhania* clade *sensu sticto* is an early diverging lineage of the Gnaphalieae which probably evolved in the winter rainfall region of southern Africa and diversified from the Miocene, and contains species from *Relhania* and *Oedera,* amongst others (Bergh & Linder, 2009). Related to the *Relhania* clade s.s. is a small genus, *Macowania,* comprising twelve endemic high-altitude Afrotemperate species, two of which are disjunctly distributed in North Africa. *Macowania* species are all woody shrubs or subshrubs with highly distinctive linear, revolute leaves characterised by a prominent midrib on the abaxial leaf surface (Fig. 1 a, b, g-k; Fig. 2 g-l). Related to both of these clades is the *Relhania* clade *sensu amplo* Bergh & Linder (2009), containing species from *Athrixia, Pentatrichia* and others. The heads in *Macowania* are surrounded by a typical gnaphalioid involucre of dry, brown bracts, which in two species, *M. revoluta and M. corymbosa,* are dark-margined (Fig. 1 f; Fig. 2 b, d), leading Hilliard (1976) to consider these as sister species. Most species have yellow flowers, although the two North African species have pale-yellow to white flowers. One species, *M. pinifolia,* is anomalous in the genus as it lacks the typical leaf character and has ray florets which are white above and mauve below (Fig. 1 d, l; Fig. 2 e, g). This species was considered by Hilliard (1985) to be better placed in a related genus, *Athrixia,* although its placement in this genus is also somewhat incongruous. *Athrixia* is one of the genera considered to be closely related to *Macowania,* but it is readily distinguished on the basis of leaf, involucral and achene characters as well as by the possession of purple ray flowers. Another taxon thought to be close to *Macowania* is the monotypic *Arrowsmithia styphelioides* which is narrowly endemic to the Eastern Cape mountains, near Hogsback. While *A. styphelioides* does not share the leaf characters of *Macowania* (Fig. 1 b) it does share similarities in floral structure (Fig. 1 c).

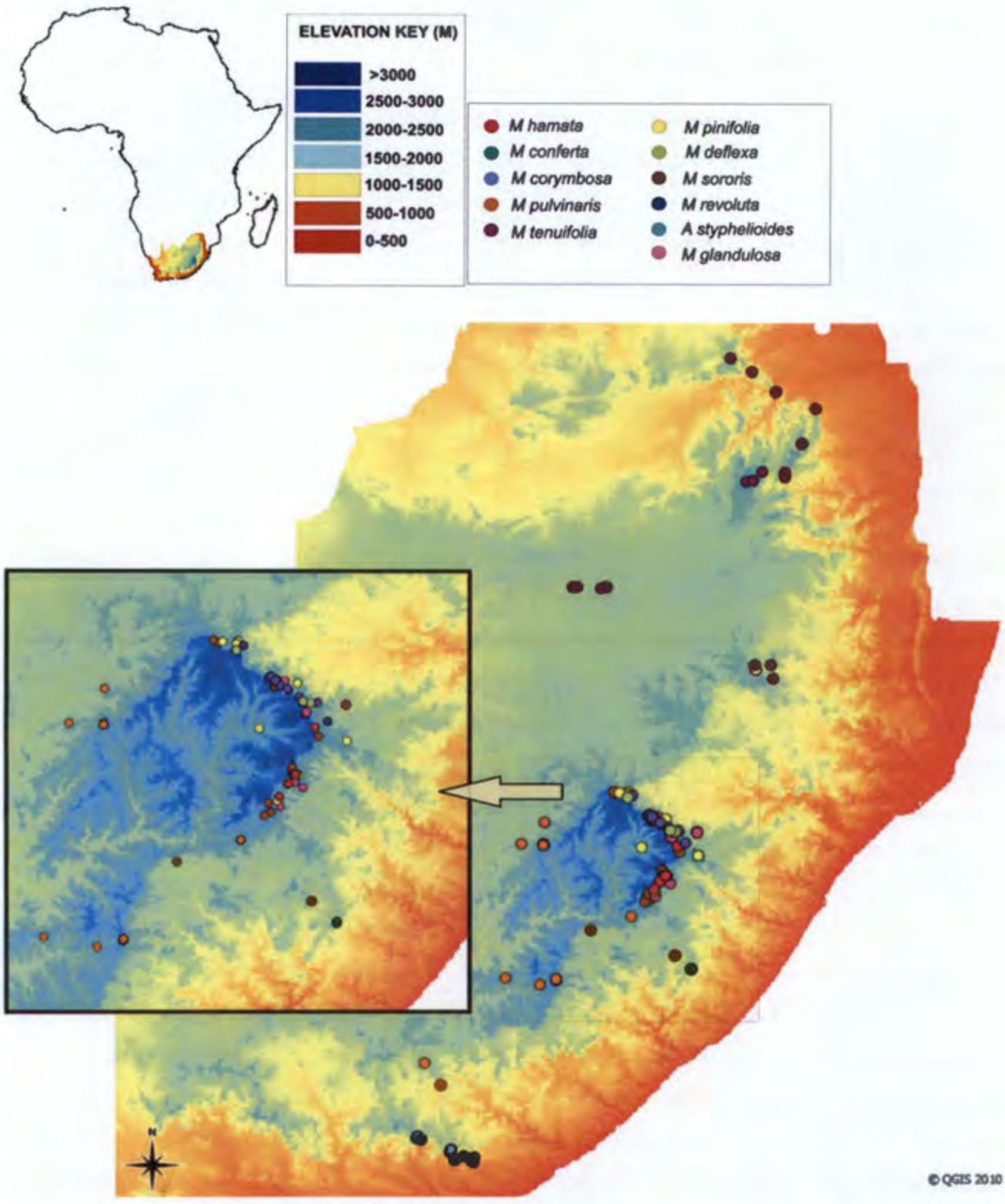

Fig. 3: Map showing the distributions of *Macowania* species in South Africa. Note that locality information was not available at the time of writing for the two North African species, *M. abyssinica* and *M. ericifolia*. The distribution ranges of *Macowania* species were quantified using field collection locality data as well as information from herbarium specimens in BOL, PRE and NBG and PRECIS data. The accuracy with which each specimen could be localized depended on the quality of the original locality data, and a measure of accuracy was assigned to each record. Digital 1:50,000 maps of South Africa (Surveys and Mapping, Mowbray, Cape Town) were used to determine the locality coordinates. Due to the unavailability of

digital maps of Lesotho, a 1:250,000 map of Lesotho was used to estimate coordinates for the distribution of the genus within Lesotho. The distributions were then depicted spatially using the programme Quantum GIS v1.5.0. (QGIS development team, 2010). The map was obtained from the CGIAR-CSI SRTM 90m Database (Jarvis *et al*, 2008).

Macowania species generally occur within two habitat types including riparian habitats (Fig. 1 g, j, k) and non-riparian habitats (Fig. 1 f, h) and mostly, although not exclusively, at high altitudes (Fig. 3). The distribution of the genus extends from the Eastern Cape Drakensberg in the Hogsback vicinity, on several isolated peaks including Ngeli Mountain and throughout the KwaZulu-Natal Drakensberg, with one species occurring within the Mpumulanga Drakensberg and Gauteng (Fig. 3), and a further two species extending into North Africa.

The primary aim of this study is to generate a phylogeny of the basal gnaphaloid lineages with an emphasis on the genus *Macowania* and its immediate relatives. The monophyly of *Macowania* will be investigated and its relationships to other members of the *Relhania* clade evaluated. Several *Athrixia* species are included in the analysis in order to determine the correct generic assignment of *M. pinifolia*. The phylogeny presented is largely molecular but morphology is used to determine the placements of species for which DNA sequences could not be obtained.

A second objective of this study is to use the presence of high-altitude endemics within *Macowania* and *Relhania* s.s. and *Relhania* s.a. to test the hypothesis that the high-altitude Drakensberg flora is recent in origin, possibly being associated with Pliocene uplift. In order to assign the origin of taxa within a certain habitat or biome, the endemic taxa should be younger than the biome to which they are endemic. Dating analyses have the power to provide insights into the timing of evolutionary events and, in conjunction with historical reconstructions of altitude and biome preference, can be used to test the hypothesis that the occupation of alpine grassland environments coincided with Miocene-Pliocene uplift events.

Materials and Methods

Sequence data

Two DNA extraction methods were utilised in this study. In most cases, total genomic DNA was extracted using the ionic detergent CTAB (hexadecyltrimethylammonium bromide) buffer method (Doyle & Doyle, 1987) with DNA being suspended and diluted in 100µl of sterile, distilled water. A second method, for the direct PCR amplification of DNA (Bellstedt *et al*, 2010), was also applied with the aim of obtaining higher DNA yields from old (≥ten yr) herbarium specimens. Total DNAs of 28 species of *Macowania, Athrixia, Pentatrichia, Comborhiza, Arrowsmithia* and *Relhania* (Table 1) were extracted from 2-15mg dried leaf material depending on the extraction method used. Most material was collected in the field, but some species were sampled from

herbarium specimens, especially the Bolus (BOL) and Pretoria (PRE) herbaria. Vouchers of new collections were deposited in the Compton Herbarium (NBG).

Table 1: Table displaying names, accessions, province distribution and data sampled of the taxa used in this project. DNA samples extracted and sequenced in this study are indicated with *. The remaining samples were supplied by N.G. Bergh (unpublished data). The provinces column describes the species' distributions. Voucher codes represent collectors' names and collection number. Province codes are as follows: EC=Eastern Cape, NS=north of South Africa, KZN=KwaZulu-Natal, L=Lesotho, FS=Free State, G=Gauteng, LIM=Limpopo, NC=Northern Cape, WC=Western Cape, M=Mpumalanga, NW=North West, S=Swaziland, N=Namibia.

Species Name	Data sampled	Voucher	Province
Ingroup taxa			
*Arrowsmithia styphelioides**	trnTL, ITS, ETS, morph	NGB2129	EC
Macowania abyssinica	morph (literature only)	n/a	NS
M. conferta	morph (literature only)	n/a	EC, KZN
*M. corymbosa**	psbAF, trnTL, ITS, ETS, morph	NGB2177	KZN
*M. corymbosa**	psbAF, trnTL, ITS, ETS, morph	JB002	KZN
*M. deflexa**	psbAF, trnTL, ITS, ETS, morph	NGB2173	KZN
*M. deflexa**	psbAF, trnTL, ITS, ETS, morph	NGB2178	KZN
M. ericifolia	morph	S1443	NS
*M. glandulosa**	psbAF, trnTL, ITS, ETS, morph	NGB2181	KZN
*M. hamata**	psbAF, trnTL, ITS, ETS, morph	NGB2166	KZN
*M. pinifolia**	psbAF, trnTL, ITS, ETS, morph	JB003	KZN, L
*M. pinifolia**	psbAF, ITS, ETS, morph	JB004	KZN, L
*M. pulvinaris**	trnTL, ITS, ETS, morph	NGB2140	KZN, L, FS, EC
*M. revoluta**	ITS, ETS, morph	JB001	EC
*M. revoluta**	psbAF, trnTL, ITS, ETS, morph	JB005	EC
*M. sororis**	psbAF, trnTL, ITS, ETS, morph	NGB2161	KZN, L
*M. tenuifolia**	psbAF, trnTL, ITS, ETS, morph	NGB2211	KZN, M, LIM, G
Outgroup Taxa			
Alatoseta tenuis	ITS, ETS, morph	JM3187	WC
Amphiglossa corrudifolia	psbAF, ITS, ETS, morph	K1291	NC, WC

Anisothrix kuntzei	trnTL, ITS, ETS, morph	NGB2089	WC
*Athrixia angustissima**	ITS, ETS, morph	NGB1449	FS, KZN, L, NC, EC
*A. arachnoidea**	psbAF, trnTL, ITS, ETS, morph	NGB218	M, KZN
*A. capensis**	ITS, ETS, morph	NGB2198	WC, EC
*A. crinita**	ITS, morph	NGB2209	WC
*A. elata**	psbAF, trnTL, ITS, ETS, morph	NGB2203	LIM, NW, G, M, S, FS, L
*A. fontinalis**	morph (literature only)	NGB2137	NS
A. gerrardii	ITS, ETS, morph	NGB1523	M, KZN, EC
*A. heterophylla**	ITS, ETS, morph	NGB2174	WC
*A. phylicoides**	psbAF, ITS, ETS, morph	NGB2180	WC, EC
*A. rosmarinifolia**	ITS, ETS, morph	WW10436	NS
*A. rosmarinifolia**	ITS, ETS, morph	K1705	NS
*Comborhiza virgata**	psbAF, trnTL, ITS, ETS, morph	NGB2174	KZN
Galeomma oculus-cati	psbAF, trnTL, ITS, ETS, morph	NGB1703A	NC
Ifloga spicata	psbAF, trnTL, ITS, ETS, morph	L17590	NS
Leysera gnaphaloides	ETS, morph	NGB1441	N, NC, WC, EC
Metalasia densa	psbAF, ITS, ETS, morph	NGB1266	M, S, FS, KZN, L, NC, WC, EC
Oedera genistifolia	trnTL, ITS, ETS, morph	NGB1572	NC, WC, EC
O. squarosa	trnTL, ITS, ETS, morph	NGB1065	NC, WC, EC
*Pentatrichia alata**	psbAF, trnTL, ITS, ETS, morph	NGB2209	LIM, M
P. petrosa	trnTL, ITS, ETS, morph	EK2143	N, NC
Philyrophillum brandbergense	trnTL, ITS, ETS, morph	EK2144	N
*Relhania acerosa**	trnTL, ITS, ETS, morph	NGB2137	KZN, L
*R. dieterlenii**	psbAF, trnTL, ITS, ETS, morph	NGB2148	L
R. rotundifolia	trnTL, ITS, ETS, morph	TO sn	WC
Rhynchopsidium sessiliflorum	trnTL, ITS, ETS, morph	NGB2062	WC, EC
Rosenia glandulosa	ETS, ITS, morph	NGB1729	WC
Printzia polifolia	ETS, ITS, morph	NGB1558	WC, EC

Two nuclear regions and two chloroplast regions that have proven phylogenetic utility in the Gnaphalieae were analysed (Bergh & Linder, 2009) (Table 2). The external transcribed spacer (ETS) of nuclear ribosomal DNA was amplified using the primers 18S-ETS (Baldwin & Markos, 1998) and AST-1 (Markos & Baldwin, 2001) while the

associated internal transcribed spacer (ITS) was amplified using ITS4 and ITS5 (White *et al,* 1990), resulting in a product consisting of the ITS1 and ITS2 introns and the intervening 5.8S ribosomal gene. The two plastid regions included the trnT-trnL region using the primers 'trna' and 'trnb' of Taberlet *et al* (1999) and secondly, the psbA-trnH^{gug} spacer using trnH-R and psbA-F (Sang *et al,* 1997).

Table 2: Primer sequences used

ITS 4	5'	TCC TCC GCT TAT TGA TAT GC	3'
ITS 5	5'	GGA AGT AAA AGT CGT AAC AAG G	3'
AST-1	5'	CGT AAA GGT GCA TGA GTG GTG T	3'
18S-ETS	5'	CGC GCA TGG TGG ATT CAC AAA TC	3'
trnH-R	5'	CGC GCA TGG TGG ATT CAC AAA TC	3'
psbA-F	5'	GTT ATG CAT GAA CGT AAT GCT C	3'
trna	5'	CAT TAC AAA TGC GAT GCT CT	3'
trnb	5'	TCT ACC GAT TTC GCC ATA TC	3'

The polymerase chain reaction (PCR) amplifies DNA generating numerous copies of the target DNA sequence through repeated heating and cooling cycles. Samples were ground using a mixer mill (MM 400, Retsch, Haan, Germany). PCR reactions were done in 50µl volumes, each consisting of 21.6 µl of nuclease-free H_2O, 5 µl of 10xbuffer (Kapa Biosystems Inc., MA, USA) 3 µl of 25µM $MgCl_2$, 2 µl of dNTP 10µM dilution, 1 µl of DMSO, 2.5µl of each primer (10µl) and 0.4µl of Taq DNA polymerase (Kapa Biosystems Inc., MA, USA). Six microlitres of DNA extract, or a dilution thereof (10^{-3}), was then added to the reaction mixture. Amplification was carried out on a thermal cycler (Applied Biosystems (2720) CA, USA) using the following thermal profiles: initial denaturation of two minutes at 94°C, 35 cycles consisting of 94°C for 45 sec (denaturation), 52°C for 45 sec (annealing), followed by 72°C for two min (extension) and a final extension step at 72°C for eight min. The DNA extracts obtained from four difficult herbarium specimens (*Athrixia capensis, A. crinita, A. heterophylla* and *Macowania ericifolia)* were purified using a Qiagen PCR clean-up column (Qiagen, GmBH, Hilden, Germany) prior to PCR. Successfully amplified products were then sent to Macrogen (Macrogen Inc, Korea) for sequencing in both directions, using the primers used for the original PCR. In addition to the sequences generated during this study, several outgroup sequences were provided by N. Bergh (unpublished data).

Chromatograms of the forward and reverse sequences were examined, corrected where necessary and assembled using ChromasPro software (v. 1.5; C McCarthy Technelysium Pty. Ltd, Helensvale, Australia). The consensus sequences were aligned manually using BioEdit Sequence Alignment Editor (v. 7.0.0; T Hall 1997-

2007; Hall, 1999). Sections of the sequence that proved difficult to align were coded as missing data, or excluded in the phylogenetic analyses.

Although herbarium material was acquired for *M. ericifolia* the DNA extraction as well as clean-up column did not yield usable sequence, probably due to the age of the material (>100 yr). Material of M. *abyssinica* was not available (East African Herbarium did not respond to the loan request), and an attempt to scale Ngeli Mountain, the only known locality for *M. conferta*, was unsuccessful due to adverse weather. In addition, the chloroplast regions proved difficult to amplify in general. Thus the dataset contains a large amount of missing data, especially for the chloroplast regions. For this reason, there is little overlap in the set of species which have sequences of both chloroplast regions.

Morphology

A morphological matrix was produced based on that used for a cladistic study by Anderberg & Bremer (1991) on the *Relhania* generic group. A new morphological matrix was assembled having reworked the questions from Anderberg & Bremer's (1991) study to make them more applicable to the genus *Macowania* (Appendix 1). Characters were based on habit, leaf morphology and anatomy, capitulum arrangement, flower colour and sex of the florets in a capitulum. Characters and character states were identified through the use of herbarium material (specimens from the PRE and NBG herbaria) and field observations, as well as from taxonomic literature (Hilliard & Burtt, 1976; Hilliard & Burtt, 1985; Anderberg & Bremer, 1991; Anderberg, 1991; Bremer, 1976a; Anderberg, 1988; Bremer, 1978 and Bremer, 1976b).

Phylogenetic analysis

Phylogenetic analyses were conducted separately on the chloroplast and nuclear DNA sequence data. The two chloroplast and the two nuclear regions were concatenated because they each represent a single genetic locus (chloroplast genome, and ribosomal DNA locus) and so should represent a single gene tree. There was no evidence of conflict amongst loci within each of these partitions. The resulting chloroplast and nuclear gene trees were compared in order to determine the existence of well-supported incongruence (incongruence supported by bootstrap percentages of 75% or greater, and PP values of 0.95 or greater). Prior to the analysis, unalignable stretches of sequence were excluded from the analysis to avoid biases resulting from unsatisfactory sequence alignment (76 positions in trnT-trnL, and 24 positions in psbA-trnH). For parsimony analysis, uninformative characters were also excluded. In all cases, trees were rooted on *Printzia polifolia,* except for the chloroplast trees (there were no chloroplast sequence data available for this species) which were rooted on *Galeomma oculus-cati*. Conflict was found between the nuclear and plastid topologies with regards to one taxon, *M.*

tenuifolia. A 'conflict decomposition' analysis was implemented (Pirie *et al*, 2008) whereby *M. tenuifolia* was duplicated to represent separate plastid and nuclear accessions. One accession copy was represented by the corresponding plastid sequences only, with the nuclear partition coded as missing data while the other accession copy represented nuclear sequence only, with the plastid sequences coded as missing data. For the purpose of the Bayesian and BEAST analyses, only the nuclear accession of *M. tenuifolia* was included as it was more consistent with morphology (see discussion). Plastid data was coded as missing.

Unweighted parsimony tree searches were conducted in PAUP v.4.0 (Swofford, 2002). Each search involved an initial heuristic tree search using 10,000 random-addition replicates with nearest neighbour interchange (NNI) branch-swapping, and saving one tree per round of branch swapping. The shortest trees from the preliminary search were then subjected to tree bisection and reconnection (TBR) branch swapping, saving one tree per replicate. Node support was determined using 1000 non-parametric bootstrap (Felsenstein, 1985) replicates with the maximum number of trees saved per replicate being set at 500 trees.

Nucleotide substitution parameters and topology were examined via Bayesian inference using MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001) with MrModeltest v.2.2 (Nylander, 2004) being used to identify the appropriate molecular models for each gene region. MrModeltest v2.2 suggested the GTR model for ITS, GTR+G for ETS and the F81 model for the chloroplast data. Since Bayesian analysis tends to be more robust to model overspecification (Huelsenbeck & Rannala, 2004), slightly more complex models were implemented for each region namely, GTR+G for the ITS region, GTR+I+G for ETS, and GTR for the chloroplast regions. The addition of more parameters allows for a truer reflection of biology by allowing for biased substitution rates and among-site rate variation (Huelsenbeck & Crandall, 1997). These same models of evolution were utilised in the BEAST analysis, and the maximum clade credibility trees from the analyses returned similar tree topologies. Separate models were applied to each gene region in order to allow the model parameters to be estimated separately for each gene region. Rates of substitution were allowed to vary under a Dirichlet prior. Two independent Monte Carlo Markov chains (MCMC) were run for 10,000,000 generations, with sampling every 1,000 generations. The chain heating parameter was set at 0.2. Convergence of the MCMC chains as well as the 'burn-in' duration were determined by examining the average standard deviation of the split frequencies and likelihood score traces obtained from two simultaneous random runs using different trees. This was done using Tracer v1.3 (Drummond & Rambaut, 2007). After discarding burn-in samples, trees were summarised either by means of a maximum clade credibility tree, generated using Logcombiner v.1.5.4 (Drummond & Rambaut, 2007) and TreeAnnotator v.1.5.4 (Drummond & Rambaut, 2007), or by means of 75% majority rule consensus in PAUP. Trees were then visualised in FigTree v.1.3.1 (Rambaut, 2006).

Due to the lack of DNA sequence data for three *Macowania* species (*M. ericifolia, M. abyssinica* and *M. conferta*) for which no fresh leaf material could be obtained, a separate morphological analysis was run in order to infer the placements of these taxa and so test the monophyly of *Macowania*. The topology generated by the Bayesian

analysis using all DNA data, apart from the deleted plastid data for *M. tenuifolia,* was used as a backbone constraint tree for a parsimony analysis using only morphological data. The positions of these three taxa were thus determined only by the morphological data, while the relative positions of the remaining taxa were fixed. This constrained parsimony analysis was performed in PAUP v. 4.0 (Swafford, 2002) implementing the same heuristic search process described above for the nucleotide data but using the backbone constraint commands. A bootstrap analysis was also performed using only morphological data matrix applying the same heuristic search method as described above.

Molecular age estimation

Divergence times were estimated using all nucleotide data (excluding the plastid data for *M. tenuifolia),* under a lognormal relaxed Bayesian clock as implemented in the program BEAST v1.5.4 (Drummond & Rambaut, 2007). Being Bayesian in nature, BEAST allows for the specification of prior knowledge (e.g. calibration priors) along with the information provided by the molecular sequence data (Drummond & Rambaut, 2007). Importantly, too, BEAST allows for rates of molecular evolution to vary across the tree through the implementation of a relaxed phylogenetic 'clock,' with topology and branch lengths being simultaneously estimated from the data. An important realisation has come with the fact that substitution rate variation among lineages differs and that significant departures from clock-like evolution warrant the need for relaxed clocks that allow the rate to vary across the tree (Drummond *et al,* 2006).

The BEAST .xml file used in this study was configured using the program BEAUti v1.5.4 (Drummond & Rambaut, 2007). A mixed model approach was followed, in which substitution model parameters were estimated separately for each data partition. The same models were used as were used in the MrBayes analysis, and the rates of evolution on the tree branches were set using a relaxed clock with rates drawn from a lognormal distribution. Several short, preliminary BEAST runs were performed in order to examine MCMC chain performance, after which two final BEAST runs were initiated with 30,000,000 iterations each. The output was tested for convergence using Tracer v1.3 and the first ten to twenty percent of samples were discarded as burn-in, depending on the outcome of the tree. The post burn-in samples were then combined using Logcombiner v.1.5.4 and the maximum clade credibility tree, with median node heights, determined using TreeAnnotator v1.5.4, with the posterior probability limit set to 0.5.

The root node (treemodel.Rootheight in BEAST) was calibrated based on a previous molecular dating study by Kim *et al* (2005) based on the crown age of a large subfamily of Asteroideae. This study estimated the node to range from 35 to 39 Ma based on a slow and fast rate calibration of *ndhF* from several angiosperm families. It was also estimated to vary from 26 to 29 Ma based on a non-parametric rate smoothing (NPRS) dating exercise calibrated to an outgroup fossil (Kim *et al,* 2005). A normal distribution prior was specified for this node. The age

prior on the crown node of the *Relhania* group of genera (including the *Relhania* clade s.s. and *Relhania* clade s.a.) was based on an age estimate by Bergh & Linder (2009: node K) and specified as a normal distribution. A calibration prior was also applied to the crown node of rest of the Gnaphalieae which constitutes the Gnaphalieae excluding the *Relhania* clades (Bergh & Linder 2009: node C). This prior was also specified as a normal distribution. The use of secondary calibration points, although useful in many instances whereby limited fossil evidence is available, is subject to error and that the potential problems that may arise should be understood and accounted for. This can be achieved through using various precautionary measures such as confidence intervals and the specification of accurate priors (Forest, 2009).

Ancestral area reconstruction

The BEAST maximum clade credibility tree was used for the reconstructions of ancestral biome preferences and altitudinal ranges. Two distributional traits were reconstructed independently, namely minimum altitude range and biome.

Since the appearance of a high-altitude flora of the Drakensberg would be signalled by the appearance of species restricted to high altitudes, I opted to reconstruct historical shifts in species' *minimum* altitude limits. Minimum altitude range also provides an indication of a species tolerance to environmental conditions and thus insight into species-specific habitats. The minimum altitudinal limits of extant species were determined using collection locality information on herbarium specimens at BOL, NBG and PRE, and in the annotated checklist provided by Germishuizen & Meyer (2003). Squared change parsimony was used to reconstruct the continuous altitude data using the Ancestral State Reconstruction Package in Mesquite v.2.73 (Maddison & Maddison, 2006). Squared change parsimony aims to minimise the number of squared changes along the branches of phylogenetic tree in inferring ancestral states, and produces a more or less normal distribution of changes along the branches (Maddison, 1991).

Biome information was based on georeferenced locality data for each species referenced into the biome distributions of Mucina & Rutherford (2006). Biomes were divided into seven regions as follows: fynbos, succulent karoo, Albany thicket and savanna, while the grassland biome was divided into three sub-regions including the Drakensberg grassland bioregion, mesic highveld grassland biome and the sub-escarpment grassland bioregion (Mucina & Rutherford, 2006). The Drakensberg grassland bioregion occurs from the Lesotho highlands and immediate surrounds in KwaZulu-Natal stretching along the high-lying area of the escarpment in the Eastern Cape (Mucina & Rutherford, 2006). The mesic highveld grassland bioregion constitutes the largest grassland bioregion and is found mostly within the regions of the highveld that experience high precipitation (Mucina & Rutherford, 2006). The sub-escarpment grassland bioregion occurs at relatively low altitudes on the foothills of the Drakensberg and eastern escarpment (Mucina & Rutherford, 2006). The Drakensberg grassland bioregion has the

highest mean altitude, followed by the mesic Highveld grassland bioregion and then the sub-escarpment grassland bioregion (Mucina & Rutherford, 2006). The Drakensberg grassland bioregion also has lower temperatures and a higher incidence of frost compared with the other grassland bioregions (Mucina & Rutherford, 2006). A dispersal-extinction-cladogenesis (DEC) likelihood model for estimating geographic range evolution and likelihoods of ancestral states was implemented using the programme LaGrange v.2 (Ree & Smith, 2008). The DEC model specifies instantaneous rates between ranges along phylogenetic branches and uses this to estimate the likelihood of ancestral states at cladogenesis events (Ree & Smith, 2008). No distributional or dispersal constraints were applied.

Results

Sequence characteristics and phylogenetic analyses

The sequence alignments for the ITS and ETS regions were 650 and 449 base pairs long, respectively. The two regions contained 235 and 205 parsimony-informative characters (i.e. variable characters that are not autapomorphies) as estimated by PAUP v.4.0. The aligned plastid regions trnT-trnL and psbA-trnF were 592 and 465 base pairs long, with 54 and 85 parsimony-informative characters, respectively. The nuclear regions were therefore the most phylogenetically informative, averaging 40.5% parsimony-informative characters as compared to only 13.5 % for the chloroplast regions.

Due both to large amounts of missing data and to low levels of phylogenetic information, the chloroplast tree showed poor resolution amongst major clades and did not recover *Macowania* as monophyletic (Fig. 4). Nevertheless, the plastid data returned several sister-relationships that were well-supported by both parsimony bootstrap percentage (BS) and Bayesian PP values. Examples include the grouping of the two accessions of *M. corymbosa* and the sister-relationship of *M. revoluta* and *M. tenuifolia*. In contrast to the chloroplast tree, the topology obtained from the two nuclear regions was well-resolved, with many relationships receiving good support (Fig. 5). Although the monophyly of *Macowania* was not supported, strong support values were obtained for relationships within *Macowania*. *Arrowsmithia styphelioides* was included in *Macowania* with high support at several nodes (PP of 1 and BS of 100 % for a clade of *Macowania* and *Arrowsmithia*, excluding *M. pinifolia*, and PP of 1 and BS of 89% for *A. styphelioides* as sister to *M. revoluta*). The core clade of *Macowania* is therefore represented by those species already within *Macowania*, with the inclusion of *Arrowsmithia*, and excluding *M. pinifolia* as it is in a polytomy with *Macowania* and *Relhania* s.s.

In general, the chloroplast and nuclear topologies are in agreement. However, conflict was observed between the chloroplast and nuclear data in respect of the placement of *M. tenuifolia*, (Fig. 5). The nuclear data place *M. tenuifolia* as sister to *M. pulvinaris*, although with little support. However, this pair is strongly-supported as being

included in a clade with *M. sororis* and *M. deflexa* (PP = 1, BS = 91%), and this clade in turn is sister to *M. glandulosa*, again with good support (PP = 1, BS = 91%). In contrast, the chloroplast data place *M. tenuifolia* as sister to *M. revoluta* with good support (PP = 0.99, BS = 86%).

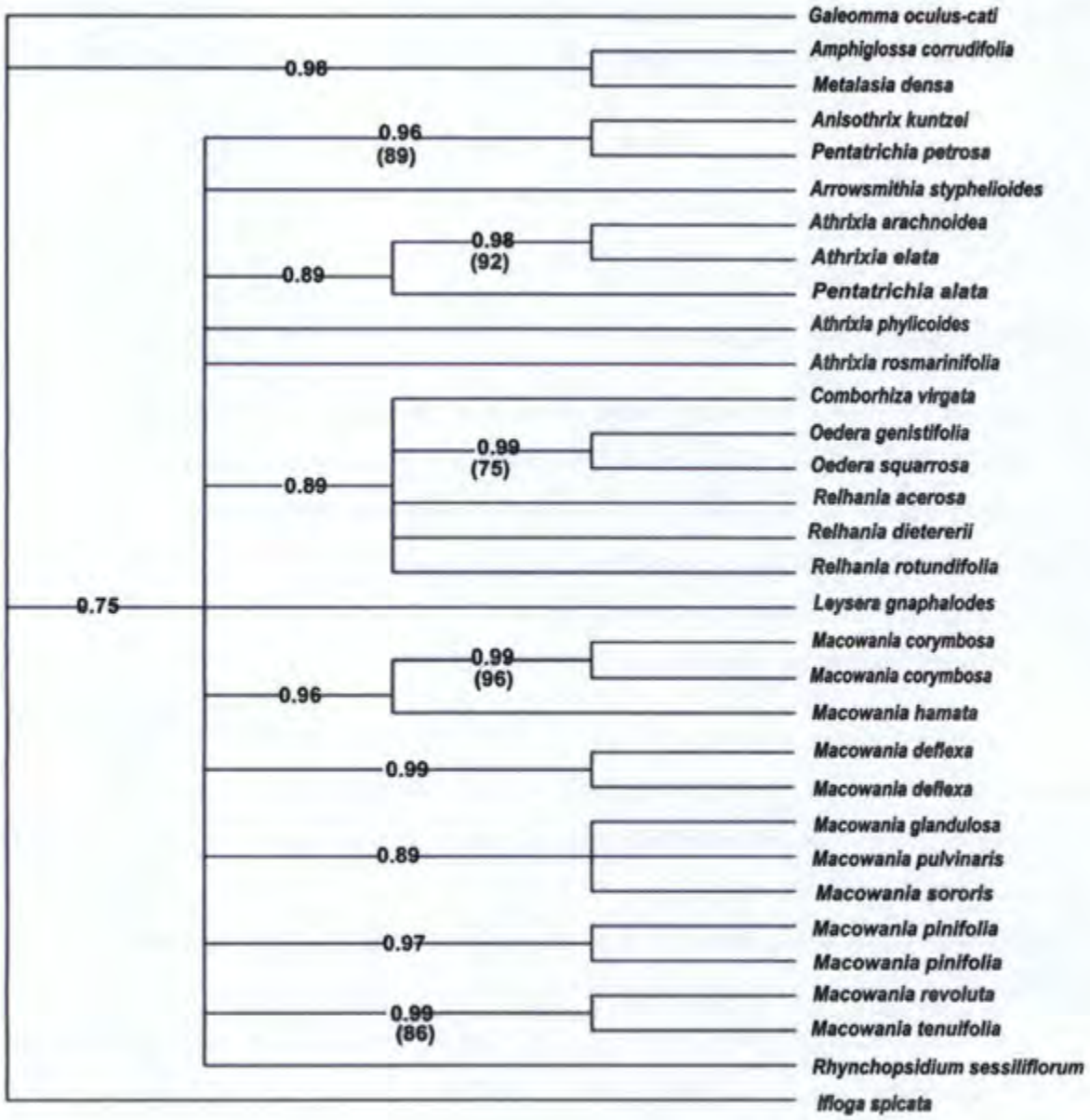

Fig. 4: Plastid topology. The plastid topology represents a 75% majority-rule consensus tree generated in PAUP from all the trees produced by Bayesian analysis in MrBayes (excluding the burn-in). Values correspond to those arrived at in the Bayesian analysis ≥0.75, followed by bracketed bootstrap percentages ≥75% in brackets.

In the 'conflict decomposition' tree (Fig. 6) there is support for both positions of *M. tenuifolia*. It is placed sister to *M. revoluta* (*M. tenuifolia*, chloroplast data, PP=1, bs=97%) and, while the sister relationship of *M. tenuifolia* and *M. pulvinaris* in the nuclear tree is not supported, there is substantial support for these two taxa as members of a clade that also contains *M. sororis* and *M. deflexa* (*M. tenuifolia*, nuclear data, PP=1, bs=92%).

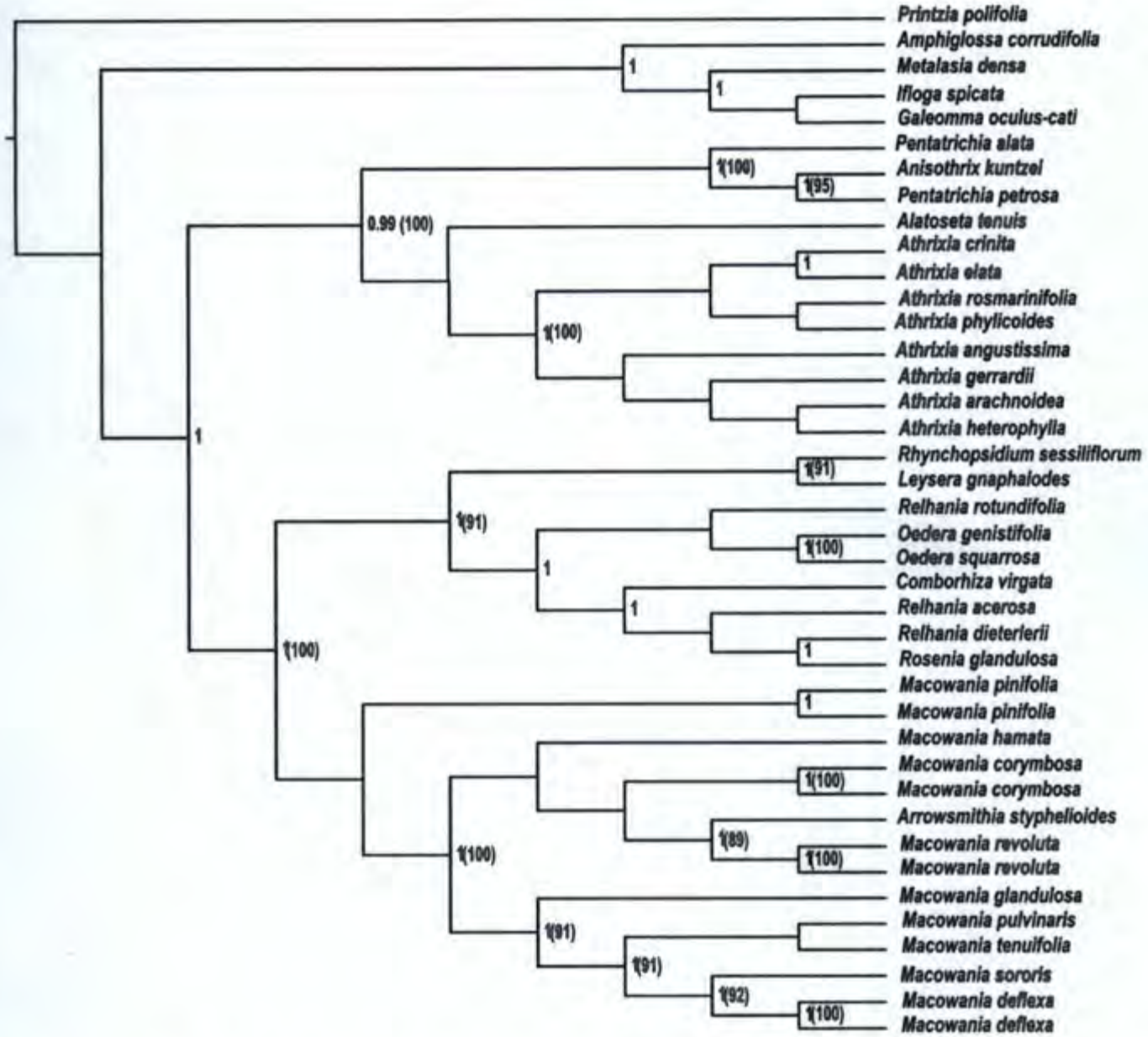

Fig. 5: Nuclear topology. The Nuclear topology is represented by the MrBayes maximum clade credibility tree. Values at the nodes represent Bayesian posterior probabilities followed by bootstrap proportions (in brackets). Only PP values ≥0.95 and bootstrap percentages ≥75% are shown.

The morphological data support the placement of *M. conferta, M. abyssinica* and *M. ericifolia* within the core clade of *Macowania* (excluding *M. pinifolia*) with high bootstrap support (Fig. 7). Due to the fact that this analysis was performed with a backbone constraint based on the data from the BEAST analysis, any bootstrap percentages reflect the frequency with which any of the three unconstrained *Macowania* species were placed within a particular clade on the basis of morphology. A bootstrap value of 100% indicates that none of these species ever fell within the *Relhania* clade s.a., but slightly lower bootstrap proportions within the *Relhania* clade s.s. suggests that the three taxa were sometimes grouped within this clade. Within the *Macowania* clade it appears as if one

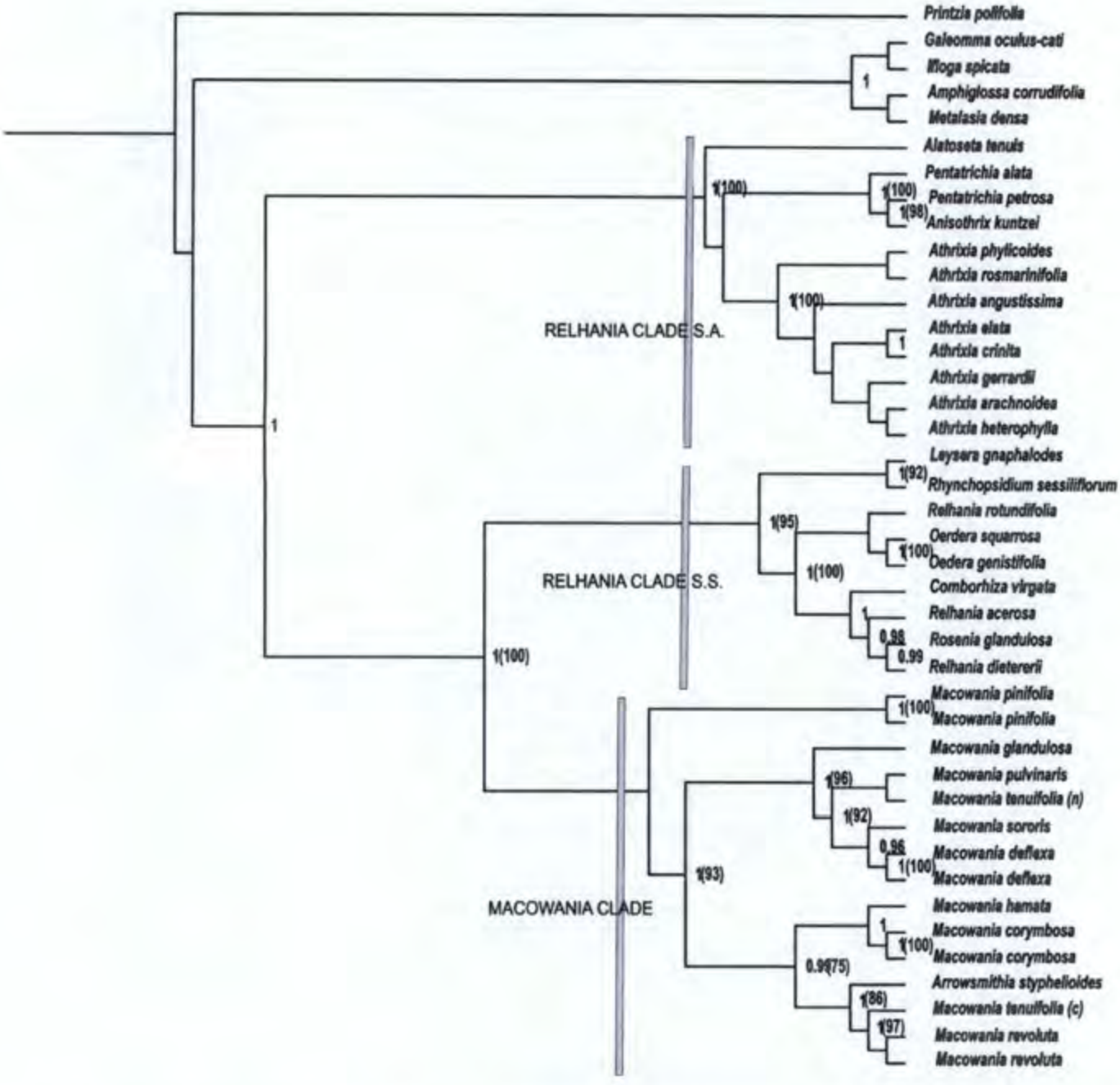

Fig. 6: 'Conflict decomposition analysis'. Combined Bayesian analysis of nuclear and plastid data with two accessions of *M. tenuifolia* (chloroplast (c) and nuclear (n)). PP values ≥0.95 are indicated followed by bootstrap percentages ≥75% in brackets.

or more of the three taxa were more often placed nearer to *M. deflexa* and *M. sororis* in the phylogeny, as these species have the lowest bootstrap proportions. The taxa were placed least next to *M. revoluta* and *A. styphelioides*. A bootstrap value of 85% supports the monophyly of all Macowania species, while a second node (BS = 82 %) supports the monophyly of *Macowania* excluding *M. pinifolia*. A bootstrap value of 85% supports the placement of *M. conferta* with the remaining Macowania species excluding M. *abyssinica* and *M. ericifolia* (Fig. 7), suggesting that *M. conferta* belongs in the *Macowania* core clade.

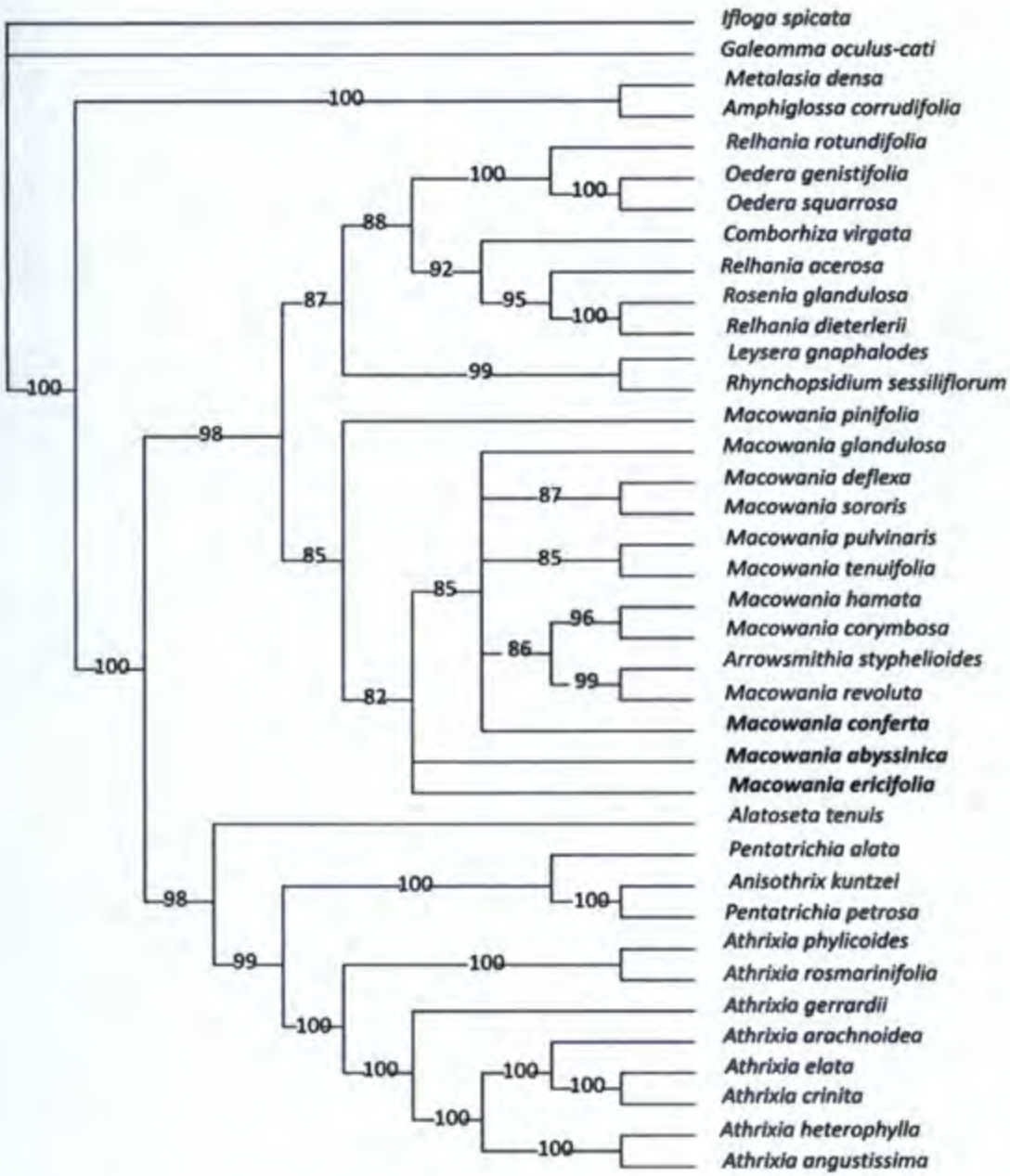

Fig. 7: Morphological constraint analysis. Results from the constrained parsimony bootstrap analysis of morphological characters. Only the positions of *M. conferta, M. abysinnica* and *M. ericifolia* were allowed to vary in this analysis. Values on branches are bootstrap percentages ≥ 75 %. Values of 100 % indicate clades which did not contain the unconstrained taxa in any of the bootstrap replicates. Positions of *M. conferta, M. abysinnica* and *M. ericifolia* are indicated in bold.

Mean age estimates for nodes of interest

The root node (node A) is estimated at 40.08 million years old with a 95% Highest Posterior Density (HPD) of 6.67 and 51.86 Ma, and a PP of 1 (Fig. 8). The 95% HPD for the age of *Relhania s.s. + Relhania s.a.* (node C – Relhaniinae) node is estimated to be between 16.17 and 30.88 Ma with a median of 23.23 Ma (PP= 1) and

encompasses the Oligocene and Miocene. The node encompassing the Gnaphalieae (node B) is estimated at 31.32 Ma (PP=1) with a 95% HPD of 21.75 and 41.49 Ma. The node defining the Gnaphalieae excluding the

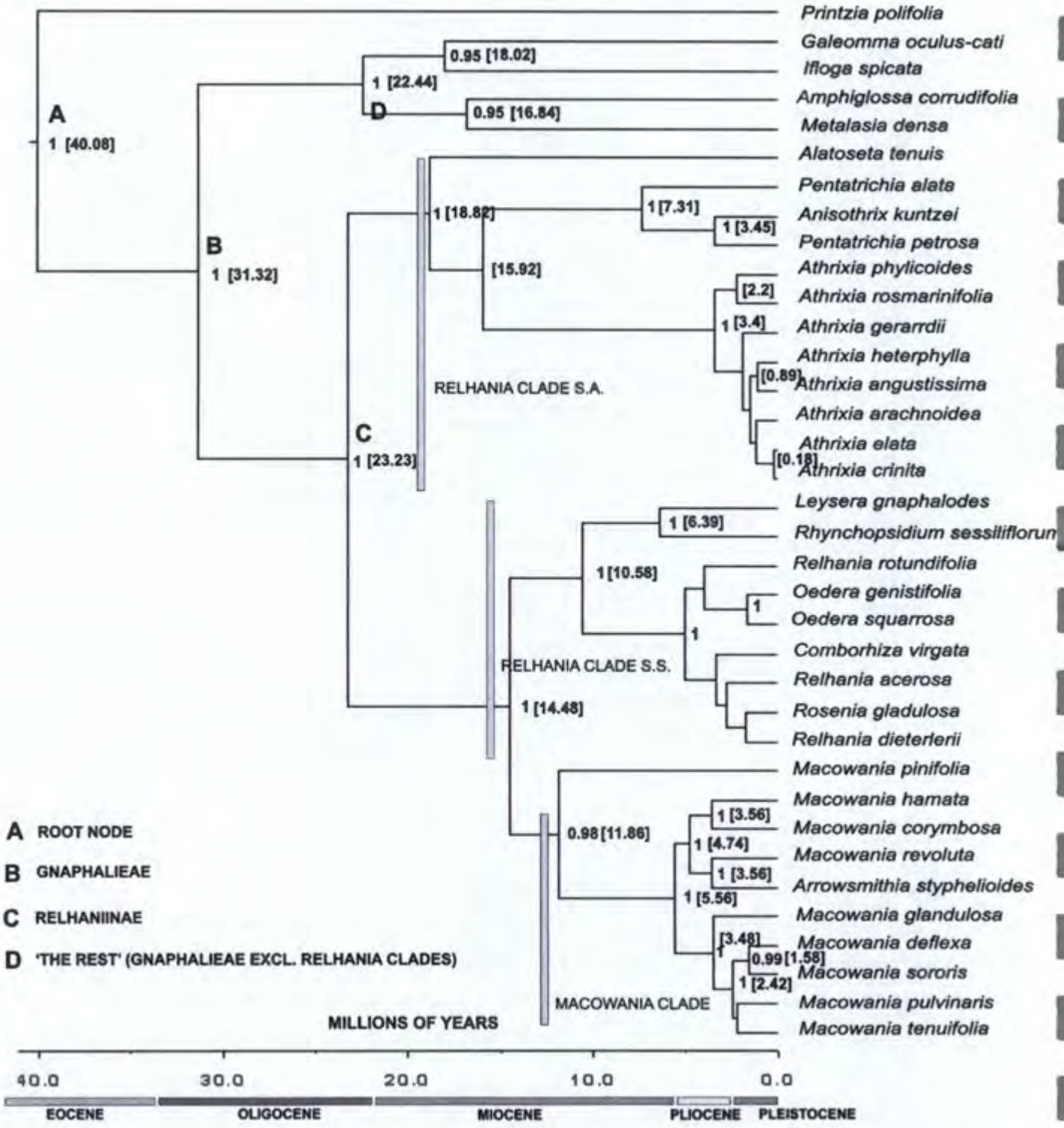

Fig. 8. BEAST dating analysis output with PP values followed by median age estimates. 95% HPDs for nodes of interest are reported in the results. PP values ≥0.95 are presented. Nodes are labelled A, B, C and D corresponding to the key provided.

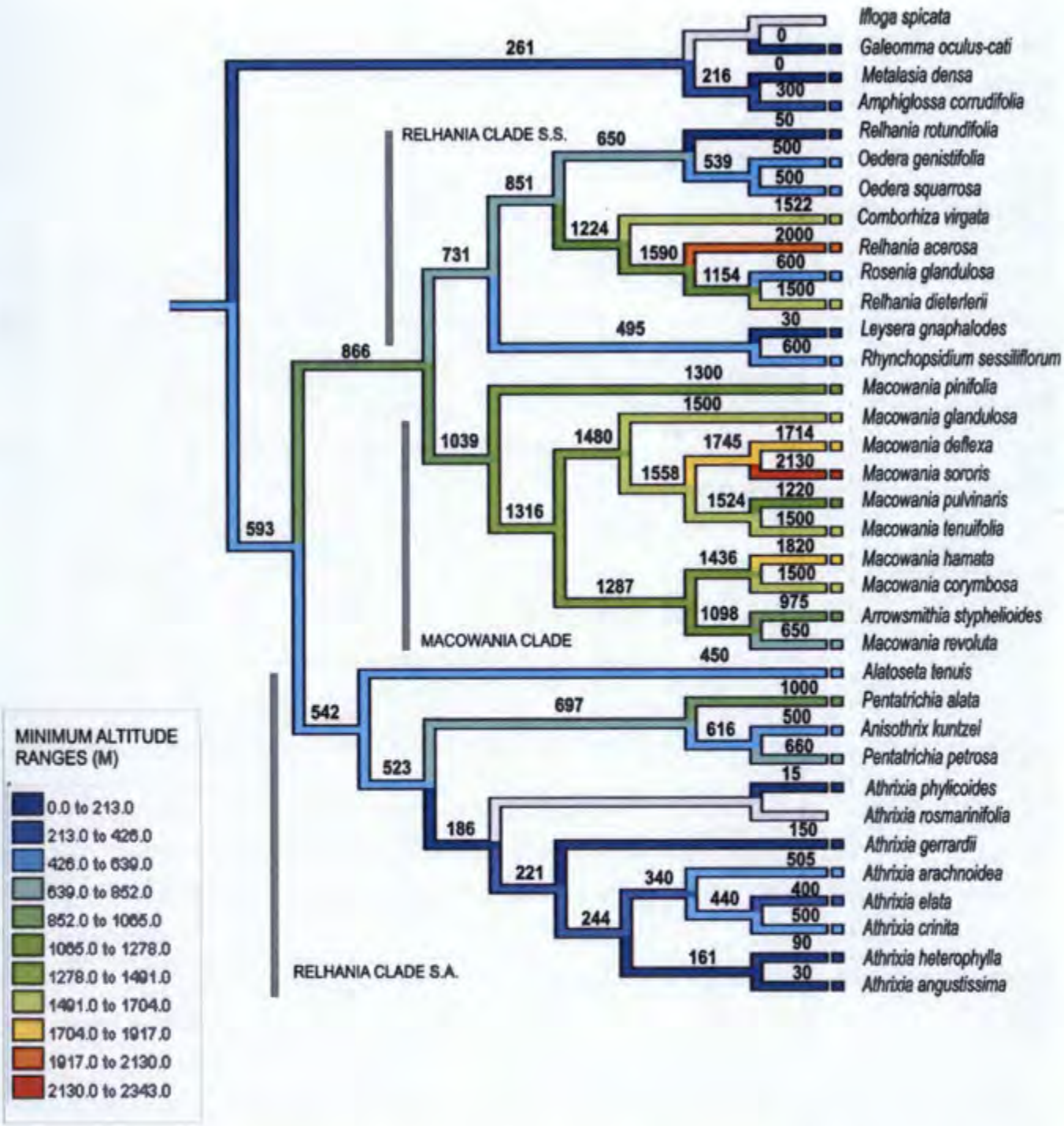

Fig. 9. Ancestral area reconstruction using squared change parsimony. Values represent minimum altitude value in meters. White branches represent missing data. The key provided represents minimum altitude ranges. Note that *Macowania* experiences, on average, the highest minimum altitudes.

Relhania clades (node D) is estimated at 22.44 Ma (PP=1) with a 95% HPD of 14.87 and 30.56. The divergence of the *Macowania* clade from the *Relhania* clade s.s. is estimated at between 9.42 and 20.44 (95% HPD) with a

median of 14.48 Ma (PP=1), encompassing the Miocene with *M. pinifolia* splitting from the rest of *Macowania* between 7.22 and 17.12 Ma (95% HPD), with a median of 11.86 Ma (PP=0.98). The rest of the *Macowania* clade experience a divergence event between 3.38 and 8.32 Ma (95% HPD), encompassing the Pliocene and Miocene with a median of 5.56 Ma (PP=1).

Ancestral area reconstructions

The ancestral nodes in the squared change parsimony analysis are all estimated to have low minimum altitudes, ranging from 261m for the node encompassing D from the dating analysis (Fig. 8) and 593m for the node separating the *Relhania* clade s.s. and *Macowania* from the *Relhania* clade s.a (Fig 9). The node separating *Macowania* from the *Relhania* clade s.s. is estimated at 867m and the node encompassing *Macowania* and *M. pinifolia* is estimated to be 1040m. The node separating the non-riparian *Macowanias* with the riparian *Macowanias* and *Arrowsmithia* is estimated at 1316m with the node encompassing the riparian *Macowanias* at 1288m and the non-riparian *Macowanias* at 1480m. Most species in *Macowania* are characterised by a shift to a higher mimimum altitude habitat or a shift within the same range than their ancestors with the exception of *M. revoluta* and *A. styphelioides.* Several species in the *Relhania* clade s.s., namely *R. acerosa, R. deiterlerii* and *C. virgata* experience a shift to a higher minimum altitude than those occupied by their ancestors. Within the *Relhania* clade s.a. only marginal minimum altitude range shifts occur. Out of the groups sampled, *Macowania* occupies the highest minimum altitude habitats on average, followed by the *Relhania* clade s.s.

The root node in the biome reconstruction indicates an endemic succulent karoo ancestry with subsequent taxa having ancestry in both the succulent karoo biome and Drakensberg grassland bioregion (Fig. 10). The node separating the *Relhania* clade s.s. from the *Relhania* clade s.a. (Fig. 10) is associated with the mesic highveld and Drakensberg grassland bioregions, respectively. The node separating *Macowania* and *M. pinifolia* from the *Relhania* clade s.s. depicts ancestral endemism in the Drakensberg grassland bioregion with regards to *Macowania* and *M. pinifolia,* and ancestry in the fynbos biome and Drakensberg grassland bioregion with regards to the *Relhania* clade s.s. Ancestral endemism in the Drakensberg grassland bioregion then appears again at the node separating the riparian (including *Arrowsmithia)* and non-riparian *Macowanias. M. tenuifolia* has endemism in the mesic Highveld bioregion. Further movements into the sub-escarpment bioregion are seen with regards to *M. corymbosa, M. glandulosa, M. sororis* and *M. pulvinaris,* with the latter also extending into the mesic Highveld bioregion. Ancestral endemism in the fynbos biome appears to occur twice in the *Relhania* clade s.s. with some movements into the succulent karoo biome.

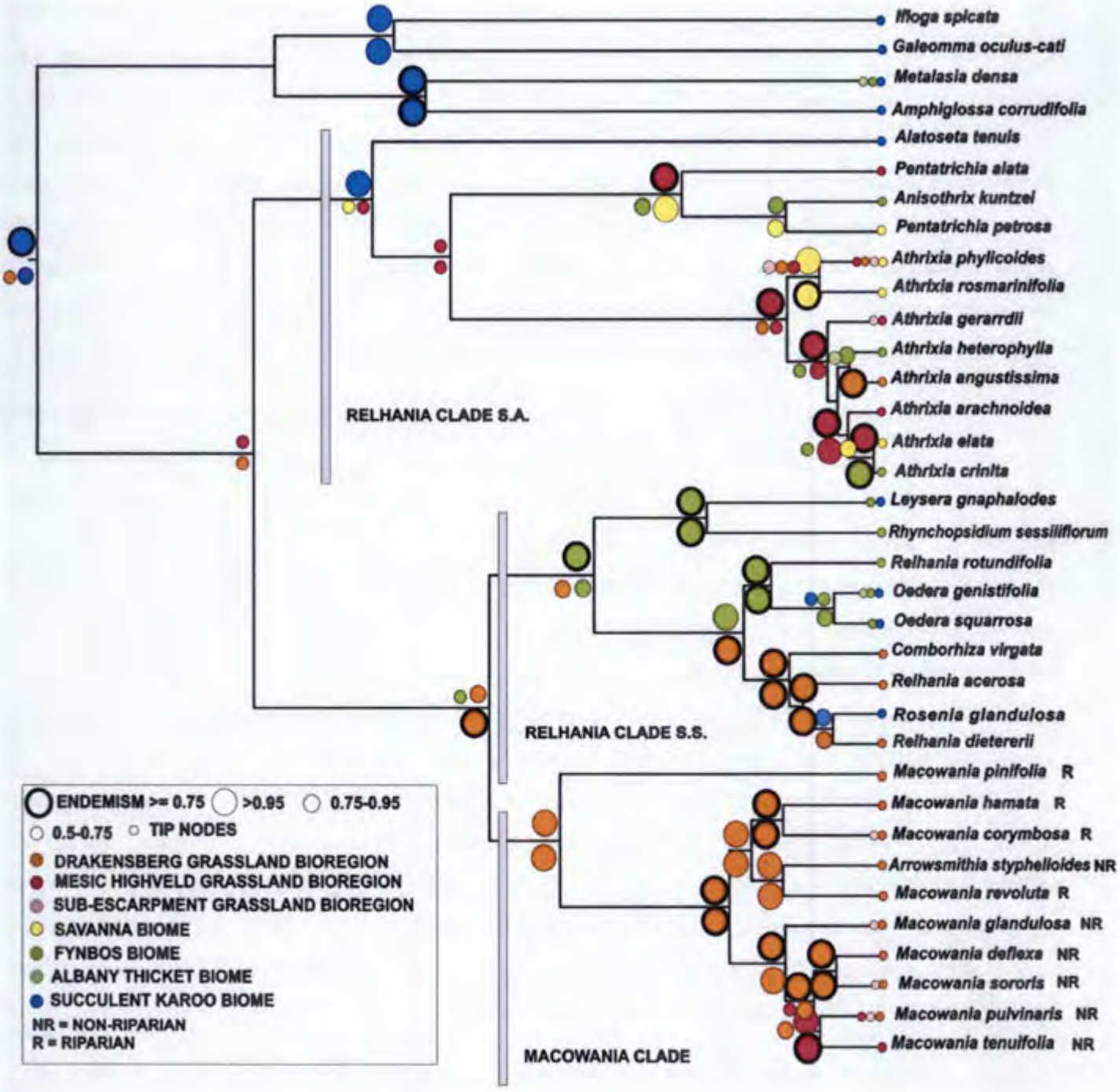

Fig. 10. Ancestral area reconstruction of biome/bioregion. Circles describe probabilities of ancestry in ancestral biome. Outlined circle represents ancestry with ≥0.75 endemism in that biome. Riparian (R) and non-riparian (NR) *Macowanias* including *Arrowsmithia* and *M. pinifolia* have been indicated. Note the ancestral endemism of *Macowania* in the Drakensberg grassland bioregion.

This study presents the first detailed phylogenetic analysis of the relhanioid assemblage of genera within Gnaphalieae. Within this clade, there is good support for three principal lineages: (i) a lineage dominated by species of *Pentatrichia* and *Athrixia*; (ii) a lineage dominated by *Oedera, Relhania* and *Rosenia*; and (iii) a lineage dominated by species of *Macowania*. Except for *M. pinifolia* whose position remains equivocal, the monophyly of *Macowania* is well supported, so long as *Arrowsmithia* is included. While sequence data were only obtained for nine of the twelve *Macowania* species, morphology also placed the remaining three species within *Macowania*, albeit with poor resolution. Overall, the relationships presented here are highly consistent with those reported by Bergh & Linder (2009). While Bergh & Linder (2009) investigated the southern African origin and radiation of Gnaphalieae, they did not investigate the ancestral habitats of the groups in this tribe.

There have been conflicting views on the placement of *M. pinifolia*. While some have argued that this species is an *Athrixia,* such as Hilliard & Burtt, (1985) who suggest that "*Athrixia pinifolia* is not a species of *Macowania*...in *Athrixia* it certainly seems a little out of place at first sight, but floral details tend to refute judgement...," others support its placement within *Macowania* (Kroner, 1980; Anderberg, 1991). This study has shown, with good support, that *M. pinifolia* is most closely related to *Macowania* and *Relhania* s.s. rather than *Athrixia*. *M. pinifolia* does not, however, possess the conspicuous, raised midrib on its lower leaf surface which is characteristic of *Macowania* (see Fig. 1 h,k,l compare with Fig. 1 g). It also lacks the glandular leaf surfaces that characterise *Macowania* and the ligules, like those of *M. ericifolia* and *M. abyssinica,* differ from most species in being white with mauve undersides. The leaves are also not revolute, which typifies the rest of the genus. Less conspicuous is a difference in ray floret achene morphology. Most *Macowania* species have achenes that are typically ten or fifteen-ribbed, while *M. pinifolia* shares a three-ribbed achene with *Athrixia* (Hilliard & Burtt, 1985). Anomalous taxa are often difficult to place on morphological grounds as they tend to share characteristics with several groups, and molecular phylogenetics can provide useful insights in these cases. For the present, the molecular evidence support the retention of *M. pinifolia* within *Macowania,* at least until its relationship within the *Macowania-Relhania* s.s. clade is resolved.

Arrowsmithia styphelioides, like *M. pinifolia*, has been of considerable interest to taxonomists owing to its morphological similarity to *Macowania*. Hilliard & Burtt (1985) address the considerable resemblance of this species to the genus, but note that it differs in its short, pungent ovate leaves (Fig 1 b), long woolly hairs on the upper half of its disc and ray florets, as well as its twenty-ribbed ray floret achene. The ovaries of the female-sterile disc flowers of *Arrowsmithia* are twelve-ribbed, in contrast to the rest of *Macowania*, in which the disc ovaries are ten-ribbed. Despite these morphological differences, nuclear DNA analysis strongly suggests placement of this species within Macowania, requiring the inclusion of *Arrowsmithia* in *Macowania* in order to maintain generic monophyly. The name *Arrowsmithia* (De Candolle, 1838) is, however, older than *Macowania* (Oliver, 1870). Thus, the nomenclatural rule of priority requires that all *Macowania* species be transferred to *Arrowsmithia,* which would result in eleven name changes (proposed before by Kroner (1980)). The transfer of

Macowania to *Arrowsmithia* would also necessitate reconsideration of the generic designation of *M. pinifolia* since the type of *Macowania* (*M. revoluta)* would be synonymised with *Arrowsmithia. M. pinifolia* was originally described as an *Athrixia (A. pinifolia* N. E. Brown in Kew Bulletin 26, 1895) and then transferred to *Macowania* by Kroner (1980). This was then refuted by Hilliard & Burtt (1985) but reinstated by Anderberg (1991), who drew on a chemical study by Bohlmann & Zdero (1977) showing that *M. pinifolia* shares a thymol-derivative compound with *Macowania* which is lacking in *Athrixia* (Bohlmann & Zdero, 1977). Given current information and the information provided by his study, the best option may be to transfer *M. pinifolia* to its own, monotypic genus. For now, the currently accepted names will remain.

The incongruent placement of *M. tenuifolia* was also addressed in this study. The 'conflict decomposition' analysis showed that the differences between the nuclear and chloroplast trees did in fact lie with this species. The choice was made to use only the nuclear data available for *M. tenuifolia* to avoid conflict in the tree. Hilliard & Burtt (1976) suggest that *M. tenuifolia* is closely allied to *M. pulvinaris* based on leaf anatomical characteristics such as narrow leaves with a very prominent, abaxial rounded midrib that extends into the apiculus, an adaxial groove above the midrib, as well as the presence of stomata on the leaf upper surface and the tendency in both species for their leaves to be held at length in the sub-erect position. These authors also suggest that these two species have an affinity in being habitat generalists with the largest ranges in the genus. In contrast, *M. revoluta* shares several morphological synapomorphies with *M. corymbosa*, including dark margins on the involucral bracts (Fig. 2 b, d,) and large leaves with stalked glands (Fig. 2 h) (Hilliard & Burtt, 1976), and these species were considered by these authors to be sister taxa. Morphology thus supports the nuclear DNA placement of *M. tenuifolia*. Since morphological features are the result of effects and interactions of multiple, unlinked genes, they are likely to give a good representation of species history. It was therefore decided to use the nuclear data for this taxon and it is represented only by ITS and ETS sequences in the dating analysis.

Apart from the morphological reasons, the plastid placement of *M. tenuifolia* as sister to *M. revoluta* seems highly unlikely. The most likely explanation for such incongruence is chloroplast capture (Wolfe & Elisens, 1995). This would have resulted in a hybrid that has the nuclear genes of *M. tenuifolia* from both pollen and ovule, and chloroplast genes from the ovule of *M. revoluta.* Based on current distributions, such a hybridisation event seems unlikely as the ranges of *M. tenuifolia* and *M. revoluta* are quite distant from one another, and this scenario would have needed the pollen from *M. tenuifolia* to transfer to *M. revoluta,* with the resulting seed moving back to the northern range of *M. tenuifolia.* Alternatively, if the conflict described by *M. tenuifolia* reflects the retention of ancestral polymorphisms (incomplete lineage sorting) then the chloroplast DNA would have had to been retained from before the divergence of the riparian and non-riparian clades. This also seems unlikely as the error in calculating phylogenetic trees when there is incomplete lineage sorting is more pronounced when the evolutionary time is short and the effective population size is large (Pamilo & Nei, 1988).

I would suggest that future investigations should repeat the sequencing of nuclear and chloroplast DNA for this species with more than one accession to confirm that the sequences are correct.

Macowania may be divided into two groups based on habitat, with *M. revoluta, M. corymbosa* and *M. hamata* being riparian, and *A. styphelioides, M. deflexa, M. glandulosa, M. pulvinaris, M. sororis* and *M. tenuifolia* being found in more rocky habitats at higher altitudes, although not exclusively so. On the strength of habitat preference, *M. conferta* (Drakensberg), *M. ericifolia* (North Africa) and *M. abyssinica* (North Africa) also belong in the latter group, all of these species favouring rocky situations at high altitude. There is also some morphological support for these associations, with Hilliard & Burtt (1976) noting that *M. conferta* resembles *M. sororis* and *M. glandulosa*, and that *M. ericifolia* resembles *M. tenuifolia*. Except for *A. styphelioides, M. pinifolia* and possibly the three species which were sampled only for morphology, the riparian and rocky habitat groups coincide with the two principal clades that make up the *Macowania* crown group (Fig. 10: riparian and non-riparian nodes). *M. revoluta* and *M. corymbosa* are identified as being closely related, which supports statements by Hilliard & Burtt (1976), though the identification of *M. hamata* and *M. corymbosa* as sisters contradicts comments by Hillard & Burtt (1976). Interestingly, *M. sororis* and *M. hamata* are the only species that share a continuous palisade layer of mesophyll across the leaf—all other *Macowania* species (excluding *M. pinifolia* and *A. styphelioides* for which the data is at this point unknown) do not have a continuous palisade layer (Hilliard & Burtt, 1976). This is interesting because it suggests that the trait has arisen twice within the genus as these two species do not come out as sister taxa. Alternatively, if this had been the ancestral state then it would have been lost several times which is not a parsimonious explanation.

Several *Macowania* species are characterised by Mucina & Rutherford (2006) as being endemic to certain vegetation units within the Drakensberg grassland region. *Arrowsmithia* and *M. revoluta* have endemism within the Amathole montane grassland which generally has sedimentary rocks (Beaufort group), mean annual precipitation (MAP) of 670mm and a generally low incidence of frost. The Drakensberg-Amathole Afromontane fynbos unit has *M. conferta* as an endemic taxon, with a MAP of 800-1820mm and Jurassic basalts (Drakensberg group) and several Karoo supergroup sedimentary rocks with frost occurring frequently (more than 40 days per year) (Mucina & Rutherford, 2006). *Macowania deflexa, M. hamata* and *M. sororis* are characterised as endemic to the uKhahlamba basalt grassland, consisting of basalts of the Drakensberg group and a MAP of 830-1820mm with frequent frost (55 days per year) (Mucina & Rutherford, 2006). The Lesotho highland basalt grassland contains endemic taxon *M. pulvinaris* and experiences around 575mm MAP as it is mostly in a rain shadow (Mucina & Rutherford, 2006). Frosts occur throughout winter and sometimes in summer at the highest elevations, and snow is common. The area is underlain by basalt lava flows of the Drakensberg group (Mucina & Rutherford, 2006). Information was not available for *M. corymbosa, M. tenuifolia* and *M. pinifolia.*

All of the divergence events in Macowania fall within the time period of the Pliocene uplift with a specialised adaptation to a high minimum altitude occurring most recently. The hypothesis that the topographic variation within the Drakensberg region appears to have stimulated the speciation of *Macowania* appears plausible as the species tend to occupy often distinct and confined ranges, as explained above. For example, *M. conferta* appears to occur only on mountain single mountain block (Ngeli mountain) and *M. sororis,* although rather extensive in the KwaZulu-Natal Drakensberg, occurs only within a confined minimum altitude range at the escarpment edge. Very few collections of *M. deflexa* have been made and this species is still poorly understood, but the available data and ancestral area reconstruction suggests that this species, too, occupies a restricted, high -altitude habitat. The MAP, geology and frost occurrence within different regions in the Drakensberg grassland probably influence the distributions within the genus greatly

Primary grassland originated during climate change in the Oligocene (34-23 Ma) and expanded when southern Africa experienced a glacial event that resulted in temperatures 5°C lower than the present, and favoured the replacement of forests and woodland by frost-tolerant grasslands (Bredenkamp *et al*, 2002). The uplift events experienced during the Miocene and Pliocene may have acted as a catalyst for radiation in *Macowania*, which is largely endemic to high-altitude, escarpment-edge habitats. The timing of the second (post-African II), more substantial uplift event during the Pliocene coincides with the split of the riparian and non-riparian *Macowania* clades and immediately precedes their radiation, implying that the increased topographic heterogeneity and increased river incision generated by uplift had a direct influence on the radiation of *Macowania*. Ancestral state reconstructions show that the ancestors of *Macowania* occupied lower altitudes ranges than do most of the present-day species, implying a recent movement into the high altitude zone, probably in association with uplift (Fig. 9). The appearance of higher altitude habitats, characterised by increased precipitation, colder air and reduced evaporation (Linder, 2003) may have provided a specialist niche within which *Macowania* speciated, with speciation being powered by disruptive selection operating along steep environmental gradients (see Linder 1985). Overall, the data suggest geographically-driven diversification which may be associated with different habitats within the DAC.

The most recent common ancestor of *Macowania* (excluding *M. pinifolia)* appears to have been endemic to the Drakensberg grassland bioregion. Subsequent to the existence of this common ancestor, there have been several migration events into other bioregions. The endemism of *M. tenuifolia* in the mesic highveld bioregion, for example, reflects dispersal accompanied by speciation, in which *M.* tenuifolia differentiated from the related *M. pulvinaris*. A role for allopatry in driving speciation is supported by the fact that the ranges of these species do not overlap. Similarly, the riparian *Macowania* clade can be separated into Eastern Cape (*M. revoluta)* and KwaZulu-Natal (*M. corymbosa* and *M. hamata)* elements whose divergence may reflect either dispersal into the Eastern Cape or a vicariance event (Fig. 10). While it is possible that uplift resulted in the formation of a geographic barrier and spatial separation of the KwaZulu-Natal and Eastern Cape riparian

groups, this remains speculative. *Arrowsmithia styphelioides* may represent a colonisation into a drier habitat while it may be speculated that the ancestral form at the divergence of *M. pinifolia* from the rest of *Macowania* may have been a damp habitat form. Although the ranges of the 95% HPDs on the divergence events are wide, it does appear as if the riparian group arose earlier than the drier habitat group, suggesting a riparian ancestry. It is believed that the rejuvenated drainage and erosion incised river gorges substantially in the east during uplift, resulting in increased river capture and exposure of bedrock. The increased slope resulted in rapid down-cutting by rivers producing heterogeneous topologies (McCarthy & Rubidge, 2005). Those species found at the highest altitudes may take advantage of the frost that occurs higher up during the dry winter to supplement moisture while those that occur along river beds or in seeps probably have access to water all year round. While the ancestral node separating the *Relhania* clade s.s. and *Macowania* from the *Relhania* clade s.a. suggests a Drakensberg grassland ancestor for the former two clades, many unsampled taxa from the *Relhania* clade s.s., which comprises more than 60 species, occur in fynbos and the succulent karoo and it is entirely likely that this clade may optimise to a winter-rainfall ancestor had more sampling taken place. This would then suggest that the distant ancestor of *Macowania* may have been dispersed via rivers from the winter rainfall areas into the grassland biome.

Conclusion

Phylogenetic analysis and morphological parsimony analysis demonstrates the monophyly of *Macowania,* subject to the inclusion of *A. styphelioides*, though the position of *M. pinifolia* remains uncertain and requires further investigation. Application of a relaxed-clock dates the radiation of *Macowania* as coincident with post-African II uplift during the Pliocene, identifying this event as having a direct influence on the radiation of *Macowania.* This is further supported by ancestral area reconstructions which identify *Macowania* as originating from a low-altitude ancestor, but radiating within the Afrotemperate, high-altitude Drakensberg zone. The 'stepping stone' hypothesis of northwards colonisation seems appropriate as an explanation for the distribution of the North African disjunct taxa. Future work entails sampling the two North African species and *M. conferta* for DNA as well as including more outgroup taxa to obtain a fuller picture of deeper relationships. The exact placement of *M. pinifolia* should also be investigated further. A more detailed investigation of substrate and geology, frost occurrence and MAP will probably provide further insight into the distribution of the genus and bioclimatic envelope modelling may also provide insight into the sequence of habitat evolution within *Macowania.*

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Appendix 1:

Table 3: Characters used in the morphology analysis of the species in this study. Characters are coded 0,1 or 2, and may also be polymorphic. Missing data is coded as ?.

1. (0) Perennial or (1) non-perennial
2. (0) Subterranean woody rhizome absent, (1) subterranean woody rhizome present
3. Flower colour; (0) pale yellow or white (1) deep yellow or (2) pink or purple
4. Rays female-fertile (0) rays - sterile (1)
5. (0) Brachyblasts present (1) brachyblasts absent
6. (0) Leaves imbricated (1) leaves not imbricated
7. (0) Leaves erect-spreading (1) leaves deflexed-recurved
8. (0) Leaves linear to linear-oblong (1) leaves broad
9. (0) Leaf margin entire (1) leaf margin apparently denticulate or sparsely serrate
10. (0) Leaf apex rounded, no mucro (1) leaf apex mucronate (2) leaf apex pungent
11. (0) Leaves glabrous (1) leaves woolly or thinly hispid
12. (0) Leaf margins (0) flat (1) revolute (2) involute
13. Heads (0) sessile, solitary (1) solitary and pedunculate or shortly pedunculate, (2) heads several
14. (0) Leaves glandular (1) leaves glandular-punctate or stalked (2) eglandular
15. (0) involucral bracts with spreading limb (1) involucral bracts with straight limb
16. Achenes of female ray floret; (0) 15-20 ribbed (1) 10 ribbed (2) 3 ribbed
17. (0) Involucral bracts glandular (1) Involucral bracts eglandular
18. (0) Involucral bracts not hairy (1) Involucral bracts woolly or thinly hispid
19. (0) Involucral bracts uniformly straw-coloured or light brown (1) bracts dark-margined
20. (0) Leaf palisade tissue interrupted by midrib (1) palisade tissue continuous
21. (0) Floret tubes eglandular (1) floret tubes glandular
22. Shrub (0) large (eg. > 1 m tall) (1) dwarf, < 1m tall.
23. Leaf width-length ratio; (0) 0-10 (1) 11-18 (2) 19-33
24. (0) Leaf lamina thicker at margins than midway from margin to midrib (1) leaves not thicker at margins than midway from margin to midrib
25. (0) Leaf midrib lying in a marked groove (1) midrib not so
26. (0) Leaf palisade tissue in one row (1) palisade tissue in two rows
27. (0) Pappus bristles shorter than floret tube (1) pappus bristles same length or longer than floret tube
28. (0) Leaves held for a long time in sub-erect position (1) leaves not held for a long time in sub-erect position

Table 4: Morphological matrix

Species Names	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Athrixia arachnoidea	0	1	2	?	1	1	0	0	0	1	1	1	0	1	0	2	0	1
A. capensis	0	1	2	?	1	1	0	0	0	1	1	1	0	1	0	2	0	1
A. crinita	0	1	2	?	1	1	0	0	0	1	1	1	0	1	0	2	0	1
A. fontinalis	0	0	2	?	1	1	0	0	0	1	?	1	0	?	0	2	?	?
A. gerrardii	0	0	2	?	1	1	0	0	0	1	1	1	0	1	0	2	0	1
A. heterophylla	0	1	2	?	1	1	0	0	0	1	1	1	1	1	0	2	0	1
A. phylicoides	0	0	2	?	1	1	0	1	0	1	1	0	0	2	0	2	1	1
A. rosmarinifolia	0	0	2	?	1	1	0	1	0	1	1	1	0	2	0	2	1	1
A. angustissima	1	0	2	?	1	1	0	0	0	1	1	1	0	2	0	2	1	1
A. elata	0	0	2	?	1	1	0	0	0	1	1	1	0	2	0	2	1	1
Alatoseta tenuis	1	0	2	?	1	1	0	0	0	1	1	0	0	0	1	?	?	?
Amphiglossa corrudifolia	0	0	0	?	0	1	0	0	0	1	0	2	0	2	1	?	?	1
Anisothrix kuntzei	0	0	1	?	1	1	0	1	0	0	0	0	0	0	0	?	1	0
Arrowsmithia styphelioides	0	0	1	1	1	1	0	0	0	2	1	1	2	2	1	0	1	1
Comborhiza virgata	0	1	1	?	0	1	0	0	0	1	1	0	0	0	1	?	1	?
Galeomma oculus-cati	1	0	0	?	1	1	0	1	0	0	1	0	2	2	1	?	0	1
Ifloga spicata	1	0	0	?	1	1	0	0	0	1	1	2	2	2	1	?	0	0
Leysera gnaphalodes	0	0	1	?	0	1	0	0	0	1	1	2	1	1	1	?	0	1
Macowania abyssinica	0	0	0	0	1	1	0	0	0	1	1	1	0	0	1	1	0	0
M. conferta	0	0	1	0	1	1	0	0	0	1	1	1	0	0	1	1	0	1
M. corymbosa	0	0	1	1	1	1	0	0	0	1	1	1	2	1	1	1	0	1
M. deflexa	0	0	1	0	1	1	1	0	0	1	1	1	0	2	1	1	?	1
M. ericifolia	0	0	0	0	1	1	0	0	0	1	1	1	1	0	1	1	0	0
M. glandulosa	0	0	1	0	1	1	0	0	0	1	1	1	0	01	1	1	0	?

M. hamata	0	0	1	0	1	1	0	0	0	1	1	1	0	0	1	1	?	1
M. pinifolia	0	0	0	0	1	1	0	0	0	1	0	0	1	1	0	2	1	0
M. revoluta	0	0	1	1	1	1	1	0	0	1	1	1	2	0	1	0	0	1
M. sororis	0	0	1	0	1	1	0	0	0	0	1	1	1	0	1	1	0	1
M. tenuifolia	0	0	1	0	1	1	0	0	0	1	1	1	1	2	1	1	0	1
M. pulvinaris	0	0	1	0	1	1	0	0	0	1	1	1	1	1	1	1	0	1
Metalasia densa	0	0	2	?	0	1	0	0	0	1	1	2	2	2	1	?	1	0
Oedera genistifolia	0	0	1	?	1	1	0	0	0	1	0	0	2	0	1	?	0	0
O. squarrosa	0	0	1	?	1	01	1	1	0	1	0	0	2	2	1	?	0	0
Pentatrichia alata	0	0	0	?	1	1	0	1	1	0	0	0	0	1	0	?	?	?
Pentatrichia petrosa	0	0	0	?	1	1	0	1	1	0	0	0	0	1	0	?	?	?
Philyrophillum brandbergense	0	0	1	?	1	1	0	1	1	0	0	0	0	1	0	?	?	?
Printzia polifolia	0	0	2	?	1	1	0	1	1	0	0	0	0	2	1	?	1	0
Relhania dieterlenii	0	0	0	?	1	0	0	0	0	1	1	2	0	0	1	?	0	0
R. rotundifolia	0	0	0	?	1	1	0	1	0	1	1	2	0	0	1	?	0	0
R. acerosa	0	0	0	?	1	1	0	0	0	2	0	0	0	0	1	?	0	0
Rhynchopsidium sessiliflorum	1	0	1	?	1	1	0	0	0	1	0	2	0	1	1	?	?	?
Rosenia glandulosa	0	0	1	?	0	1	0	0	0	1	0	2	0	1	1	?	0	0

19	20	21	22	23	24	25	26	27	28
0	?	?	?	?	?	?	?	?	?
0	?	?	?	?	?	?	?	?	?
0	?	?	?	?	?	?	?	?	?
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0	?	?	?	?	?	?	?	?	?
0	?	?	?	?	?	?	?	?	?

0	?	?	?	?	?	?	?	?	?
0	?	?	?	?	?	?	?	?	?
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