Phylogenetic studies in the genus

JAMESBRITTENIA

tribe Manuleae, family Scrophulariaceae

J. thunbergii

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James Britten for whom Otto Kuntze named the genus, was keeper of Botany at the British Museum during the late nineteenth century.

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ABSTRACT

Jamesbrittenia is a genus of 84 largely perennial sub-shrubs and herbs with a wide distribution in southern Africa (except *J. dissecta* in Sudan, Egypt and India). Plastid (*rps*16 and *psb*A-*trn*H) and nuclear (GScp) sequences were obtained for 42 species, mostly from the arid winter-rainfall west and southern regions of southern Africa. Applying both parsimony and Bayesian inference to combined molecular and morphological datasets, a phylogenetic hypothesis which is robust at the deeper nodes, was produced. This supports the monophyly of *Jamesbrittenia,* and also indicates the early divergence of *J. ramosissima* and three major clades. The first two clades have fewer species and are confined to the arid west, while the third clade includes the greatest diversity, both in number of species, morphological variation and geographic distribution. The divergence of these clades was estimated (NPRS and molecular clock) to have occurred mid-Miocene, while parallel radiations occurred during the Pliocene-Pleistocene, possibly triggered by the onset of drier, Mediterranean climate in the region. The optimization of ecological variables onto the phylogeny leads to the inference that *Jamesbrittenia* arose in the arid winter-rainfall west, on granite soil, and that diversification accompanied the shift to novel soil types and to regions of higher rainfall with seasonal variation. It was not possible to unequivocally establish the ancestral life history, but taking into account the mesic conditions of the Miocene, it was probably perennial. Annual life history has then arisen independently in the three major clades, presumably in response to drought. Conflict between plastid and nuclear phylogenies for one species (*J. pristisepala*) was interpreted as possibly due to historic hybridization. Caution is urged about hybridization of extant species for horticultural purposes. The inferred history of *Jamesbrittenia* was compared with other southern African genera, and a general similarity in temporal radiation was found. The life history pattern of a probably perennial ancestor, giving rise to annuals is, however, different from that found in *Zaluzianskya* and a clade of *Heliophila*.

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CHAPTER 1: **INTRODUCTION**

The flora of southern Africa is very diverse, the winter rainfall area containing a large proportion of this diversity. With over 20,000 species (Goldblatt and Manning, 2000; Germishuizen and Meyer, 2003) the southern African flora is large, but relatively poorly investigated in relation to its size (Golding, 2002). However, in recent decades attention has been paid to the Cape Floristic Region (CFR), which is roughly the area from the Olifants river mouth and Nieuwoudtville in the west to Port Elizabeth in the east, including, in a broad arc, the Cape fold mountains and the western Cape coastal platform. The CFR is the richest temperate flora in the world with about 9,000 species of vascular plants of which over 67% are endemic (Goldblatt and Manning, 2000). The winter rainfall area, which includes most of the CFR, extends along the south-western coast and near interior of southern Namibia, south through Namaqualand and the western Karoo to the southern coast of South Africa near Port Elizabeth, and is home to some 12,000 species (Goldblatt et al., 2002).

Molecular phylogenetic methods have provided novel insights into the diversification of the winter rainfall flora, particularly the timing and tempo of diversification, and patterns of habitat specialization. Several studies suggest that much of the species diversity in the western part of southern Africa is associated with late Miocene aridification (Table 1). Richardson et al. (2001) used molecular data to establish that *Phylica* originated on the African mainland and that the group experienced rapid radiation in the Cape 7 to 8 million years ago (mya). Goldblatt et al. (2002) investigated *Moraea* and found that it split from its sister genus *Ferraria* about 25 mya, with the main clades emerging before the end of the Miocene. As with *Phylica*, the proliferation of *Moraea* species at the south-western tip of South Africa coincided with increasing aridification and a shift to winter rainfall during the Pliocene. In *Moraea,* species occurring in the summer rainfall areas belong to clades nested within the main Cape radiation.

Within the arid winter-rainfall zone, diversification has been associated with shifts in rainfall, micro-habitat, altitude and substrate, as well as with changes

in life history. Hardy and Linder (2005) studied *Thamnochortus* (32 species) with the aim of inferring its likely ancestral habitat in the Cape flora. They infer a common ancestor to have been distributed in habitats much like those found in the south-western Cape mountains today. Major ecological trends included shifts to lower rainfall environments, to lower altitudes, and shifts from sandstone to limestone-derived alkaline soils. Verboom et al. (2003) investigated the grass genus *Ehrharta*, and showed that radiation of this genus was associated with a shift from a wet heathy Fynbos environment into increasingly more arid habitats, this shift coinciding with late Miocene climate change in the region. They showed that a pulse of rapid radiation during the late Miocene led to speciation rates of between 0.87 and 4.18 species per million years, and that this was associated with a shift to more arid, summer-dry habitats and a shift in bedrock from sandstone to granite and shale. Verboom et al. (2004) explored this pattern further and argued that that the shift to nutrient rich substrate facilitated the development of an annual life history, by allowing an increase in growth rate.

The role of key innovations has also been considered. Klak et al. (2004) investigated the Ruschioideae (Aizoaceae), a group containing over 1,500 species, which radiated between 3.8 and 8.7 million years ago, resulting in the particularly high per-lineage diversification rate of 0.77 – 1.75 species per million years. They speculate that this diversification occurred within an already arid habitat, but was linked to several key innovations, one of which was the development of cylindrical leaves. The majority of these species occur outside the CFR, but in the winter-rainfall area of southern Africa. Bakker et al. (2004, 2005) found that the large widespread genus *Pelargonium* (280 species) arose at the Oligocene – Miocene boundary, with a winter-rainfall lineage diverging early in the Miocene. This included a xerophytic clade (comprising almost half the species), which is considered to have evolved in the CFR in response to late Miocene and Pliocene aridification. Nested within this clade is a radiation of 80 species (section Hoarea), where the development of tunicate tubers may have been a key innovation.

Whilst the lineages making up the Cape flora have received extensive study, much less attention has been paid to taxa with a broader distribution in southern Africa. Linder et al. (2006) suggest that an understanding of the evolution of the broader southern African flora might be reached by studying individual taxa which make up this flora. They investigated *Melianthus*, and report that although the genus started to diversify during the Oligocene, more recent speciation during the late Miocene, occurred in response to general aridification and the establishment of the summer-dry climate in the central and western parts of southern Africa. Mummenhoff et al. (2005) conducted phylogenetic studies on seven genera of Brassicaceae endemic to southern Africa. Diversification of the three main clades within the *Heliophila* genus was relatively recent, being dated to the Pliocene - Pleistocene boundary. These three clades each had their origin in the winter-rainfall west, with subsequent dispersal eastwards, in one clade as far as the Drakensberg. Archibald et al. (2005a) produced the first phylogenetic hypothesis for a genus of the southern African Scrophulariaceae with their study of *Zaluzianskya*. They also investigated life history evolution and inferred an annual life history and an arid western distribution to be ancestral, the perennial life history being associated with dispersal into relatively mesic areas to the east. In view of the paucity of phylogenetic research on the broader southern African flora, this study examines the phylogeny and diversification of *Jamesbrittenia* O. Kuntze (tribe Manuleae, family Scrophulariaceae). This is a genus of shrublets and herbs with a broad distribution in southern Africa, but with a centre of diversity in the winter rainfall region. As currently defined, *Jamesbrittenia* contains 84 species (Table 2).

The systematics of the Scrophulariaceae has been notoriously difficult and has involved numerous changes, both at familial and tribal levels. Scrophulariaceae as currently delimited, is a large family of about 1,700 species (Angiosperm Phylogeny website, 2006). Olmstead and Reeves (1995) used molecular methods to investigate the family and found that it was paraphyletic, consisting of two non-sister clades. The clade containing *Jamesbrittenia* (known as "Scroph I") was in a clade with Bignoniaceae and Acanthaceae and is now known as Scrophulariaceae. Sister to this was a smaller clade, "Scroph II", which now takes the name Veronicaceae

(Olmstead et al., 2001). Within Scrophulariaceae, Manuleae Benth. has been distinguished from the tribe Selagineae mainly by the possession of multiple versus single ovules, but Kornhall et al. (2001) used molecular phylogenies to show that Selagineae arose within Manuleae, and that solitary ovules arose more than once. Their analyses also showed that the genus *Limosella* L. was included in Manuleae. This was surprising, as it was the only genus in the tribe having a cosmopolitan distribution, the others being largely restricted to southern Africa. Kornhall and Bremer (2004) propose that Manuleae (excluding *Antherothamnus* N. E. Br. and *Camptoloma* Benth.) and Selagineae, together with the genus *Limosella,* be combined in a tribe called Limoselleae, that name having precedence. As currently defined, the tribe Manuleae *sensu* Bentham is characterized by having the posticous corolla lobes external in the bud, synthecous anthers, and a septicidal capsule that opens further by loculicidal splits. The tribe contains 17 genera including *Jamesbrittenia* O. Kuntze*, Lyperia* Benth.*, Sutera* Roth and *Zaluzianskya* F. W. Schmidt (Hilliard, 1994).

Generic limits within Manuleae have been contentious, and this is true of *Jamesbrittenia*, whose membership has changed substantially since the first publication of the name by Otto Kuntze in 1891 (Kuntze, 1891). Hilliard (1994) undertook a full revision of the tribe Manuleae Benth. In the course of this work she increased the number of species in the genus *Jamesbrittenia* from one to 83. In so doing, she described 21 new species, and made new combinations for 61 existing species (Hilliard, 1992). She defined *Jamesbrittenia* by the following: calyx tube lobed almost to the base, corolla tube abruptly and asymmetrically expanded below the limb, two pairs of stamens which are included in the corolla tube, decurrent posticous stamens, and at least the posticous filaments with hairs. Many species in Manuleae fell within this new circumscription of *Jamesbrittenia*, most of these being previously included in the genera *Lyperia* and *Sutera*. Bentham (1876) describes his genus *Lyperia* as having a corolla tube that is "expanded, gibbous and incurved", which describes the bump in the corolla tube so characteristic of *Jamesbrittenia*. Hilliard (1994) distinguishes *Lyperia* from *Jamesbrittenia* by these characteristics: stems narrowly winged by decurrent leaf bases, stigma spear shaped, not minutely bifid. She transferred 25

species from *Lyperia* to *Jamesbrittenia*. *Sutera* Roth section Chaenostoma, as now circumscribed by Hilliard, has stamens exserted. Nineteen species with included stamens were transferred from *Sutera* to *Jamesbrittenia*. Ten species with the basionym *Chaenostoma* Benth. were also transferred to *Jamesbrittenia*. These species would meanwhile have been sunk in *Sutera* (Phillips, 1951). The remaining *Jamesbrittenia* species have basionyms in *Manulea* L. (four species), *Buchnera* L., *Cycnium* Benth., and *Erinus* L. (one species each).
The 84th species of *Jamesbrittenia* was discovered as recently as 2002. *J. bergae* P. Lemmer has beautiful, brilliant red flowers and is known only from the type locality in Limpopo province, South Africa (Lemmer, 2003).

The majority of *Jamesbrittenia* species occur in southern Africa, particularly South Africa and Namibia (Table 2, Figure 1). Fourteen species extend north into Angola, Botswana, Zimbabwe, Zambia, Mozambique and Malawi. The only species occurring outside this area, *J. dissecta* is an insignificant weed of Egypt, Sudan, India and Bangladesh (Hilliard, 1994). Most species are shrubs or shrublets, about fifteen being annuals. *Jamesbrittenia* species grow in a wide range of habitats, from coastal scrub through grasslands and savannas to arid deserts, although here they often occupy sheltered, slightly damper micro-habitats. They are absent from the oligotrophic habitats of the CFR, preferring richer soils. Populations are usually small, in some cases, consisting of tens of plants. This may be due to very particular soil or aspect requirements. For example, *J. bergae* grows only on ferrocrete outcrops and has a very limited distribution (Lemmer, 2003), *J. fimbriata* is found in association with banded ironstone, and *J. stellata* and *J. calciphila* are found only on limestone in the southern and western Cape (Hilliard 1994). Many species grow in crevices among rocks, often between granite boulders. Shale, basalt and coastal sands are also favoured.

Hilliard (1994) divided *Jamesbrittenia* into two groups (Table 2) based on testa surface structure, species in which the testa cells were separated by visible cross walls (Group 1) being distinguished from those without such cross walls (Group 2). Group 1 was further subdivided according to whether the cells' walls were smooth (Group 1.a) or "knotted" (Group1.b). Within this

framework, she subdivided the genus even further by grouping "assemblages of seemingly related species", but admits that she was only able to define one of these (Group 1.b.1 - the group which lacks glistening glands) by mutually exclusive characters. Hilliard's delimitation of species is based on meticulous observation of morphology, with particular attention being paid to indumentum characters. *Jamesbrittenia* species can be "hairy and smelly" or encrusted with gem-like crystalline outgrowths called "glistening glands". Hilliard uses the presence or absence of glandular trichomes or glistening glands on various parts of the plant in the circumscription of species. For example, *J. aspalathoides* is distinguished from the very similar *J. calciphila* by the absence of glandular hairs from the stem. Despite the taxonomic importance of these trichomes, their adaptive significance in *Jamesbrittenia* is poorly understood. Wagner et al. (2004) reviewed the functions of trichomes in general. These include adaptation to brighter, drier, hotter conditions and resistance to macro- and micro-herbivory. Recently, interest in the histochemistry of trichome secretions has increased (Kolb and Muller, 2004), particularly in economically important plants like *Nicotiana tabacum* (Wagner, 2001). Glistening glands occur in a number of genera in Manuleae (*Sutera, Manulea, Lyperia*), but their function has not been investigated.

Jamesbrittenia has received little phylogenetic study to date. Kornhall (2004) sequenced six species of *Jamesbrittenia* in his study of Manuleae, and based on this limited sampling, considered *Jamesbrittenia* to be monophyletic and "basal" within Manuleae. His results resolve *J. megadenia* as sister to an unresolved clade containing *J. dissecta, J. microphylla, J. atropurpurea, J. foliolosa* and *J. filicaulis.* Besides Kornhall, only Archibald et al. (2005a) have published any sequence information on *Jamesbrittenia*, but they only sequenced *J. adpressa* as an outgroup in their study on *Zaluzianskya*.

This study is the first to investigate inter-specific relationships within *Jamesbrittenia*, using molecular data. Its broad aims are to generate a phylogeny of the genus and to use this to investigate the diversification of the genus within southern Africa. This project forms part of a broader study, the focus here being mainly on the winter rainfall taxa. The group is interesting because of the wide habitat diversity that it exhibits and its broad distribution

throughout southern Africa. It is also interesting because of its horticultural potential: flowers are often striking, with strong honey-guide markings (cover picture), individual blooms may measure up to 30 mm across and the plants may be very floriferous. *Jamesbrittenia grandiflora* and *J. jurassica* are already available overseas as garden plants, and several hybrid forms are under patent. Moreover, the South African National Biodiversity Institute (SANBI) has a mutually advantageous agreement with the Ball Company in the U.S.A. to make South African plants available for horticultural development. As a result of this arrangement, informal hybridization experiments are conducted at Kirstenbosch Botanic Gardens, and plants showing horticultural potential are passed on to the Ball Company for further development. The responsible horticulturist at Kirstenbosch reports that most casual crosses within *Jamesbrittenia* produce viable seed (A. Harrower, personal communication). This implies that genetic barriers between species are not firmly established. Hilliard (1994) also reports that hybridization in the wild appears to be common. Thus there is a concern that if hybrid plants are widely cultivated, they may introgress back into wild populations and the weak species barriers that are the result of geographic isolation will break down. This may then lead to the loss of species (Levin et al., 1996). It is hoped that increased knowledge about the evolution of the genus and relationships between the species will inform decisions about horticultural breeding.

The specific aims of this project are:

1. To test whether *Jamesbrittenia* is monophyletic.
2. To investigate relationships amongst the species and to evaluate whether the main lineages coincide with Hilliard's morphology-based groups.
3. To date historical lineage divergence. In particular, do the winter rainfall groups show a signature of recent speciation associated with late Tertiary climate change?
4. To reconstruct the biogeographical history of the group. In particular, did it originate in the summer-rainfall east, or the winter-rainfall west, and how does this compare with the pattern found in *Zaluzianskya, Pelargonium*, *Melianthus* and *Heliophila* ?

5. To reconstruct ancestral habitats (rainfall and soils) and the evolution of annual life history. Specifically, how many times has annualness evolved and does it represent an adaptation to extreme seasonal drought?

CHAPTER 2: **METHODS**

Sampling

Forty two species of *Jamesbrittenia* were sampled in this study (Table 3), this representing roughly half of the species in the genus. Twenty six species were sampled on a spring collecting trip to the northern Cape and southern Namibia, while a further seven species were sampled from the southern Cape during October-November 2005. It was not possible to collect summer rainfall species in the field during the course of this study but these will be sampled during 2006 / 2007 and added to the analyses. To augment the available field collections, twelve accessions, including seven species not collected in the field, were obtained from Kirstenbosch Botanic Garden, where they are in cultivation. Six species from the summer-rainfall areas of eastern South Africa were among the Kirstenbosch plants. The inclusion of some replicate accessions made it possible to test the monophyly of those species and to compare wild and cultivated specimens. Specimens of two further species from central and eastern South Africa were acquired from other collectors. Outgroups from the family Scrophulariaceae, particularly the tribe Manuleae, were also sampled. Altogether 61 accessions, representing 42 species of *Jamesbrittenia* and 11 outgroup species were sequenced for this study (Table 3). All accessions used are represented by a voucher specimen, housed in the Bolus Herbarium (BOL), University of Cape Town. Included in Table 3 are three outgroup taxa for which *rps*16 sequences were downloaded from GenBank.

Molecular data

In order to infer phylogenetic history from DNA sequences, the gene regions chosen should include informative variation among species. For purposes of species level studies, non-coding DNA regions are typically used (Soltis et al., 1992) because their presumed neutrality allows for a faster accumulation of mutations and hence greater phylogenetic signal. Although Chase et al. (2000) found that, in Asphodelaceae, the coding gene *rbc*L contained similar levels of variation to the non-coding *trn*L-F intron and spacer, this probably reflected a faster substitution rate in the fewer variable sites in *trn*L-F. Genetic markers should also be selected from different genomes, as

phylogenies that are supported by more than one genome are considered more likely to reflect the true species history. This is because individual gene trees are not necessarily identical to the species tree, due to the effects of incomplete lineage sorting as well as variation in modes of inheritance (Brower et al., 1996; Wolfe and Randle, 2004; Nichols, 2001). While chloroplast DNA is more abundant in the cell and is inherited from only one parent, the nuclear genome reflects bi-parental inheritance and has the potential to reveal past hybridization and reticulate evolution (Sang et al., 1997). One nuclear and two chloroplast markers were used in this study.

In order to select a chloroplast marker, the following regions were amplified using the polymerase chain reaction (PCR): the *trn*L-*trn*F intergenic spacer (Taberlet et al., 1991), the *psb*A-*trn*H intergenic spacer (Sang et al., 1997), the *rps*16 intron (Oxelman et al., 1997) and part of the *mat*K gene (Hilu and Liang, 1997). The *trn* L-*trn*F region gave very pale bands and only amplified five out of nine samples. In contrast, the *mat*K region gave some strong product, but the sequence was long and lacked sufficient variation, being a coding region. These two chloroplast markers were rejected as *rps*16 and *psb*A-*trn*H had been shown to give useful results. The *rps*16 intron was chosen because it amplified easily, it was reasonably long (700-900 base pairs) and it contained useful variation. This intron sequence has 85% to 95% pairwise similarity scores in Solanaceae and Poaceae, which makes it suitable for phylogenetic studies below the level of family. The primers are based in the flanking exons or the adjacent conserved extremes of the intron (Oxelman et al., 1997). The *psb*A-*trn*H intergenic spacer region was chosen as the second chloroplast marker. It was shorter than *rps*16 with 400-500 base pairs, but had similar variability. The *psb*A-*trn*H locus contains an intergenic spacer in an evolutionarily plastic region of the chloroplast genome and has been used successfully to assess inter specific relationships in the genus *Paeonia* (Sang et al., 1997).

In selecting a nuclear marker, PCR was attempted with the following: ITS5 – ITS4, 18KCR – ITS2 (Baldwin, 1992), 5S - NTS (Cox et al., 1992), and GScp (Emshwiller and Doyle, 1999). None of these gave single bands. For ITS and 5S this implied the presence of multiple copies, but for GScp – which is

reported to be a single / low copy gene (Emshwiller and Doyle, 1999), non-specific priming provides an alternative explanation. For GScp, bands which appeared consistently in almost all samples were excised, gel-cleaned and sequenced. A BLAST search in GenBank matched one of these bands to GScp in the related family Gesneriaceae, and it was selected as the nuclear marker for use in this study. GScp is chloroplast-expressed isozyme of glutamine synthetase, which is responsible for the secondary assimilation of ammonia products during photorespiration (Emshwiller and Doyle, 1999). This nuclear encoded gene is a member of a multigene family, but diverged long ago (before the split between the Monocot/Eumagnolioid versus Eudicot clades) from other members which are active in the cytosol. Unlike the cytosolic-expressed members of the family, the chloroplast-expressed isozyme appears to occur as a single copy in most plants that have been investigated. The region amplified contains four non-coding introns. In *Oxalis* the substitution rate for GScp was higher than for ITS, with substitutions occurring in both coding and non-coding regions (Emshwiller and Doyle, 1999).

Morphological data

Morphological data may be combined with DNA sequences for phylogenetic analysis by appending it to a DNA matrix. Eldenäs and Linder (2000) and Verboom et al. (2003) consider that morphological characters are useful in aiding resolution near the tips of a tree. A matrix of morphological characters (Table 4, Appendix 1) was constructed, based on personal observation of specimen material as well as species descriptions from Hilliard (1994), which were confirmed by examining voucher specimens. Indumentum characters are used extensively because they are variable and Hilliard often relies on the nature and distribution of the indumentum to distinguish species within *Jamesbrittenia*. As far as possible qualitative characters, expressed as "present / absent", were chosen to avoid the problem of establishing appropriate data cut-off points when using quantitative characters (Stevens, 1991). Morphological data were not scored for the outgroup because both Eldenäs and Linder (2000) and Pennington (1996) show that morphological data are more useful in resolving relationships near the tips of the tree, while resolving deeper relationships with morphological data can be confounded by

convergent evolution and homoplasy (Verboom et al., 2006). For instance it was felt that the "glandular hairs" of *Jamesbrittenia* were too different from the hairs of *Sutera* to permit accurate homology assessment.

DNA extraction and amplification

Fresh leaf material was collected into silica gel for rapid drying and later processing. A modified CTAB protocol was used to extract total DNA (Doyle and Doyle, 1987). About 0.2 g dried leaf was ground in liquid nitrogen, with a pinch of sterile sand to aid maceration, and a little PVP40 (polyvinylpyrrolidone) to reduce the effects of secondary phenolic compounds, taking care to keep the material frozen, as DNAses become active when the material thaws, and freeze-thaw cycles also cause DNA strands to fracture. The samples were then incubated with 700 µl 2x CTAB extraction buffer and 1 µl mercaptoethanol, at 65°C for an hour. An equal quantity of chloroform : isoamyl alcohol (24:1, v/v) was added and, after centrifuging, the supernatant liquid was withdrawn to separate the extract from cell debris. DNA was precipitated with ice cold isopropanol in a -20°C freezer overnight and the DNA compacted into a pellet by centrifugation. The clean dry pellet of DNA was dissolved in 50 µl sterile water. The extract was checked for the presence of sufficiently large DNA fragments by electrophoresis on a 1% agarose gel, to which a trace of ethidium bromide had been added, allowing visualization of the DNA in ultra violet light.

Polymerase chain reaction (PCR) amplification was carried out in 30 µl volumes, prepared on ice as follows: 14.65 µl sterile water, 3 µl 10 x Supertherm buffer (Bioline Ltd, London, U.K.), 6 µl 25 mM magnesium chloride, 1.2 µl dNTP containing 2.5 mM of each nucleotide, 1 µl each of forward and reverse primer (10 µM), 0.15 µl Supertherm TAQ polymerase (5u/µl, Bioline Ltd, London, U.K.), and 3 µl DNA template. For GScp amplification, which proved difficult, 0.6 µl 100% DMSO (dimethyl sulphoxide) was added to the reaction mix (Buckler et al., 1997). For the chloroplast markers, standard primers *rps*16F, *rps*16R (Oxelman et al., 1997), *psb*A and *trn*H (Sang et al., 1997), were used with one tenth dilution of the DNA extract. In contrast, GScp amplification required the use of undiluted DNA extract as

the template. Because of limited success with standard GScp primers (Emshwiller and Doyle, 1999), *Jamesbrittenia* -specific forward and reverse primers were designed and prepared, based on sequences of *Jamesbrittenia* species which had been successfully amplified using standard primers. These primers were: GS38F 5' TGA GCC (CT)TT CTT GTT TCG TG 3'; GS784R 5' ATA CTT GTT A(AG)T GAT TTT GCC 3' and GS681R 5' AGC TTG TTC TGT TAT TCT CTG 3'. The new primers, particularly GS38F-GS681R, improved amplification success, giving a single product (whereas the standard primers gave multiple bands), but PCR was not successful for all species. Initial amplification was carried out on a GenAmp 2700 (Applied Biosystems, Foster City, CA, USA) thermal cycler, using the following program: an initial denaturation period of 2 minutes at 94°C followed by 30 cycles, each consisting of one minute at 94°C (denaturation), one minute at 52°C (primer annealing – *rps*16 and *psb*A-*trn*H) or 50-54°C (GScp) and 2 minutes at 72°C (primer extension), followed by a final extension period of seven minutes at 72°C. Each PCR batch was accompanied by a control where sterile water replaced the DNA template. PCR products were cleaned with GFX purification kit (Amersham Biosciences, Little Chalfont, UK); and gel bands were cleaned with QIAquick DNeasy Plant Minikit gel extraction kit (Qiagen GmBH, Hilden, Germany), both used according to the manufacturer's instructions. Sequencing was accomplished with the same primers in separate forward and reverse strands, using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Sequences were read by Genecare, a private company in Cape Town, using an Applied Biosystems 3130 Genetic Analyzer with AB DNA Sequencing Analysis Software v5.2 (Applied Biosystems, Warrington, UK).

Alignment of sequences

The forward and reverse sequences were assembled and edited using the program Seqman II version 2.04 (Lasergene Software, DNAStar Inc, Madison, WI, USA). The consensus sequences were aligned in Megalign (Lasergene Software, DNAStar Inc, Madison, WI, USA), final adjustment being done by eye. Characters were equally weighted and gaps were treated as missing. Homology was usually easy to determine in the ingroup, but presented some

problems in the outgroup; therefore the following bases in the *psb*A –*trn*H sequences were replaced with "?" and treated as missing data: *J. ramosissima* 345-383, *Hemimeris racemosa* 210-491, *Colpias mollis* 251-491, *Diascia longicornis* 251-380, *Oftia africana* 251-362, *Teedia pubescens* 251-358, *Manulea adenocalyx* 251-298, *Manulea schaeferi* 251-301, *Sutera subsessilis* 251-354, *Sutera hispida* 251-377, *Lyperia tristis* 251-387 and *Lyperia violacea* 354-397. Since positions 715 – 748 in the *rps*16 sequences were unalignable in the outgroup and contained no variation in the ingroup, they were discarded from analyses.

Indels were scored using simple gap coding (Simmons and Ochotorena, 2000) and the results were appended to the DNA matrix. Indel scoring was conservative and those next to homopolymers were ignored because they tend to vary even within species, so that the length of flanking indels may not be meaningful for inter-specific comparisons. The aligned matrix was analyzed in PAUP* version 4b10 (Swofford, 2003), using an iMac G3 computer.

Analyses

The datasets for each gene region were analyzed separately, using parsimony. Searches were done heuristically with no Maxtrees limitation, 10,000 random addition replicates, holding 10 trees at each step, TBR branch swapping and Multrees in effect. Support was assessed using 500 bootstrap replicates (Felsenstein, 1985), and involved a heuristic search with a simple addition sequence, TBR branch swapping, Multrees in effect and Maxtrees set to 500. The strict consensus trees obtained from the *rps*16 and *psb*A-*trn*H chloroplast loci showed no incongruence and a combined plastid analysis was carried out with only those taxa for which GScp sequences were available (Table 3). This combined plastid tree was compared with the topology obtained from the GScp analysis in order to identify instances of incongruence. Statistical testing e.g. the Incongruence Length Difference test (Farris et al., 1994; Mason-Gamer and Kellogg, 1996), were not carried out as these tests have been found to be overly harsh, meaning they tend to reject combinability too easily (Dolphin et al., 2000; Yoder et al., 2001).

The combined molecular data for all taxa excluding two accessions that were found to have conflict between plastid and nuclear loci, were analyzed using parsimony as outlined above. A similar analysis was carried out for these taxa, using the total evidence of molecular plus morphological data.

Bayesian analysis was carried out with all four data partitions, using the program "Mr Bayes" version 3.1 (Huelsenbeck, 2000; Huelsenbeck and Ronquist, 2001). As with Maximum Likelihood, Bayesian inference incorporates a model, but model parameters are allowed to vary during the analysis; the final parameter (and tree) estimates, reflecting the posterior probability distribution, are a function of the set of prior probabilities and the likelihood function. The posterior probability of a tree is the probability that the tree is correct, assuming that the model is correct. Using simulations, Huelsenbeck and Rannala (2004), investigated the effect of applying various models during Bayesian analysis and found that when the underlying evolutionary model was simple, the result was not affected by applying an overly complex model during analysis, but that applying a simple model when the real model was complex, led to incorrect posterior probabilities. They recommend that the model used should be as complex as possible, while still allowing parameters to be identified. In this analysis a GTR + Γ + I model was specified, based on the fact that under-parameterization is a potential problem with Bayesian searches. Three separate Bayesian analyses were conducted, using this model, in order to ensure that all regions of the tree space had been visited and that the settings used led to convergence. Each analysis ran with one cold and three heated Monte Carlo Markov chains, for a million generations, sampling a tree every hundredth generation. Graphs plotting the log likelihood against generation time from the three analyses were examined and were found to converge, indicating that all chains had visited all tree space. The period before stationarity was reached was discarded as "burn in"; that is, trees 1-1,500 were discarded in each analysis. Since all three runs yielded similar results, the remaining trees from the three analyses were combined to form a single 50% majority rule consensus tree. The resulting posterior probabilities represent the probabilities of nodes being encountered during the sampling.

The plastid and combined data trees were rooted on a clade of *Hemimeris* + *Colpias* + *Diascia*, as this clade has been identified as being sister to the rest of Scrophulariaceae in a higher level analysis by Kornhall (2004). As none of the outgroup species were successfully PCR-amplified for GScp, the GScp tree was rooted on the most basal lineage within *Jamesbrittenia*, as inferred on the basis of the plastid analysis.

Dating

The idea of using the divergence of DNA molecules over time to date evolutionary events was first proposed by Zuckerkandl and Pauling (1965). Adaptations of this idea of using DNA sequences to date lineages are now widely used (e.g. Magallon, 2004; Mummenhoff et al., 2005; Klak et al., 2004). The two major requirements for dating lineages from DNA sequences, using a strict molecular clock, are reliable calibration information and rate homogeneity. Fossils may be used to date calibration nodes in a phylogenetic tree. This will give a most recent time limit because it is always possible that an older fossil may yet be discovered (Heads, 2005). Geological uncertainty about absolute dates of fossils will also add to the error associated with any subsequent dates derived from the calibration point (Graur and Martin, 2004).

Unfortunately there are no direct fossil dates available for the family Scrophulariaceae, so a secondary calibration had to be used. For this purpose information was used from Wikström et al. (2001), where many nodes on the Angiosperm phylogenetic tree were dated using NPRS (Sanderson, 1997) and fossil evidence for the split between Fagales and Cucurbitales as 84 million years ago (mya). The appropriate node is No 291, describing the divergence of *Scrophularia* and *Verbascum* in the family Scrophulariaceae. This divergence is estimated to have occurred either 31 mya (parsimony, ACCTRAN; maximum likelihood) or 25 mya (parsimony, DELTRAN) with a standard error 5 million years (myr). It should be noted that this standard error refers only to the bootstrap analysis which was carried out to test for noise introduced by the stochastic process of substitution, so errors associated with the dating of the fossils which are not accounted for, become compounded (Graur and Martin 2004). In order to use these dates

it was necessary to incorporate the *Scrophularia* - *Verbascum* node into the *Jamesbrittenia* phylogeny, for which purpose appropriate sequences were sought in GenBank. No GScp or *psb*A-*trn*H sequences were available, but *rps*16 sequences for *Scrophularia arguta* (AJ431061), *S. peregrina* (AJ609139) and *Verbascum arctura* (AJ609128) were downloaded. They were readily aligned with the other *rps*16 sequences and were incorporated into the dataset. They were included in total evidence parsimony and Bayesian phylogeny inference analyses. For dating purposes one of the most parsimonious trees from the total evidence dataset was chosen at random, but only *rps*16 data were used in the calculation of branch lengths.

Modeltest (Posada and Crandall, 1998) was used to identify the model of sequence evolution most consistent with the *rps*16 dataset in the context of the chosen parsimony topology, and to estimate the optimal values of the parameters associated with this model. With these parameter values fixed, the negative log-likelihood scores of the tree were obtained, with and without a molecular clock enforced. Comparison of these scores using a log-likelihood ratio test (Felsenstein, 1981) revealed a significant difference, suggesting that a molecular clock was not justified; therefore, two approaches were used to obtain an ultrametric tree. The first was the application of nonparametric rate smoothing (NPRS, Sanderson, 1997) which assumes that rates are phylogenetically autocorrelated, and smoothes the rate change over the tree. NPRS was accomplished in the program "r8s" (Sanderson, 2002) using the POWELL algorithm, as recommended in the manual. The age of the *Scrophularia –Verbascum* divergence calibration node was stipulated (without the standard error), and the ages of various nodes of interest were obtained. The variance associated with these node age estimates was established by running a bootstrap analysis with 100 replicates, using the likelihood settings obtained from Modeltest, and constrained by the tree used above. These 100 bootstrap trees were made ultrametric in "r8s" using NPRS, and profiled for each node separately, which calculated the mean age and the associated standard deviation of that node. This was done using both Wikström et al.'s 31 mya and 25 mya dates for the *Scrophularia – Verbascum* calibration node.

Because the phylogram for this tree showed that some of the outgroup taxa had particularly long branches, suggesting that these might be responsible for rejection of the molecular clock and, fearing that NPRS smoothing might distort the other branch lengths (Hugall and Lee, 2004), a second approach was used. Long branches leading to six outgroup taxa (*Hemimeris racemosa, Manulea adenocalyx, M. schaeferi, Sutera hispida, S. subsessilis* and *Lyperia tristis :* see Fig. 9) were pruned from the tree (Welch and Bromham, 2005), and the log likelihood ratio test for rate heterogeneity was repeated. Since the result was no longer significant, a clock was enforced. In order to estimate the error associated with these node ages, 100 bootstrap replicates were obtained, using likelihood, with the clock enforced and constraining the topology. Mean age and associated standard deviation for the relevant nodes were calculated using Microsoft Excel. This was again done for both Wikström dates.

Ancestral areas and character evolution

Ancestral species distributions were reconstructed using Dispersal-Vicariance analysis (Ronquist, 1997) as implemented in the program DIVA. The commonly held view is that speciation is usually allopatric, the result of a vicariance event: a widespread ancestral population is divided by some barrier to gene flow, and the two populations then accumulate mutations independently until they are so different that they are recognized as separate species. This requires the ancestor of a genus to have been as widespread as the current distribution of the whole genus, with the descendant species occupying smaller and smaller areas. This does not match the observed distribution of species, which requires at least some dispersal and, or, local extinctions. The program DIVA allows for dispersal and extinction events, but penalizes them in relation to vicariance. It maximizes information of ancestral areas by allowing more than one area at each step. DIVA requires a fully resolved tree and a current species distribution matrix. The Bayesian tree, with polytomies arbitrarily resolved and replicate accessions and outgroups removed, was used. Distribution data were taken from Hilliard (1994) and specimens at BOL, NBG and PRE. Areas were arbitrarily defined and are shown in Figure 2. These roughly approximate established bio-climatic

zones, particularly in the west where most of the study species occur. The matrix for species distribution is given in Appendix 2.

The program MacClade (Maddison and Maddison, 1992) was used to optimise a variety of life history, morphological characters and habitat states onto the phylogeny using parsimony. The Bayesian 50% consensus topology, with polytomies arbitrarily resolved and replicate accessions and outgroups removed, was used as it was considered a reasonable estimate of the species phylogeny. However, alternative resolutions of weakly supported nodes were considered in the interpretation of results (Losos, 1994).

Rainfall information for species distributed in South Africa was obtained from Schultze et al (1997), while rainfall data for Namibian species were inferred from maps in Fullard (1971). Rainfall quantity was divided into three classes, which corresponded very roughly with three regions in the distribution of the species sampled: arid areas receiving less than 300 mm per annum, wet areas receiving more than 600 mm per annum, and an intermediate area receiving 300 to 600 mm p.a. Rainfall states for species were deduced from their distributions, acquired from Hilliard (1994), and specimens at BOL, NBG and PRE. These three states were treated as ordered and Wagner parsimony was used in the optimization (Farris, 1970). The same sources were used to allocate the species to three states according to whether rain fell mostly in winter, summer or all year round. These states were treated as unordered and analysed using Fitch parsimony (Fitch, 1971).

Preferred soil types for *Jamesbrittenia* species were taken from Hilliard (1994), herbarium notes and personal observation. Where soil type was not mentioned, it was deduced from geological maps of South Africa and southern Namibia. For instance, species growing in Lesotho were scored "basalt". A problem is that collector's notes often confuse soil particle size with bedrock type, e.g "sand" versus "granite". (See recommendations by Linder, 2005). In this study "sand" was allocated a soil type where this was reasonable (e.g. limestone on the Agulhas plain), otherwise it was interpreted

as representing dry riverbed / wash localities, which are often favoured by *Jamesbrittenia.*

Life history states were taken from Hilliard (1994) and personal observation. Some *Jamesbrittenia* species show a capacity, typical of many desert plants, to flower when still quite small, but continuing to grow as long as conditions permit, and this may blur the distinction in life history strategy in some cases. The life history states that were used in this study were conservative in relation to annualness. Rainfall, soil type and life history states are listed in Table 5, with the matrix showing distribution of states in Appendix 3. States were optimised onto the tree using ACCTRAN, which favours early changes and later reversals, and also DELTRAN, which favours later changes, closer to the tips of the tree. Alternative resolutions of weakly supported nodes were also considered.

CHAPTER 3: **RESULTS**

Phylogeny

Plastid data

The *rps*16 dataset for 64 taxa contained 777 characters of which 117 (15%) were parsimony informative. The strict consensus of 218 equally parsimonious trees is shown in Figure 3, where fifteen nodes have bootstrap support of over 80%. The *psb*A-*trn*H dataset for 61 taxa contained 437 characters of which 63 (14%) were parsimony informative. The strict consensus of 97,260 equally parsimonious trees is shown in Figure 4, where nine nodes have bootstrap support of over 80%. Tree scores are shown in Table 6. Comparison of the *rps*16 and *psb*A-*trn*H topologies reveals no hard conflict. Although there are some discrepancies in the arrangement of species in the sub-groups within clade C (Figures 3 & 4) none have reciprocal bootstrap support > 80%. Larger datasets give more reliable results, but data should only be combined if there is no incongruence between them (de Queiroz et al., 1995). Considering the degree of congruence between the two plastid datasets, it was reasonable to combine them.

The *rps*16 data resolve *Jamesbrittenia* as monophyletic (87% bootstrap support) and *J. ramosissima* as sister (97% bootstrap support) to the rest of the species, which form three main clades (A, B and C), each with over 90% bootstrap support (Figure 3). The *psb*A-*trn*H data resolve the same three major clades with good support, but the monophyly of *Jamesbrittenia* is not resolved as *J. ramosissima*, clade A, and clade B+C form a polytomy with some of the outgroups (Figure 4). Neither the *rps*16 nor the *psb*A-*trn*H data support the monopohyly of tribe Manuleae, including *Teedia* + *Oftia* (both plastid datasets), *Scrophularia* and *Verbascum* (*rps*16), in a polytomy with members of Manuleae. This arrangement does not, however, contradict the possibility that Manuleae is monophyletic, and may be resolved with further sampling of manuleoid and non-manuleoid outgroups. Of the species represented by replicate accessions, only the two *J. grandiflora* accessions are shown to be monophyletic, and then only in the *psb*A-*trn*H tree (Figure 4). Replicate accessions of *J. pristisepala, J. calcipohila, J. tenuifolia, J. fruticosa* and *J. canescens* are all resolved as paraphyletic.

GScp nuclear data

Although PCR was attempted for all accessions, GScp sequences were obtained for only 28 species of *Jamesbrittenia* (excluding *J. ramosissima*), and not for any outgroup species. The dataset contained 585 characters of which 61 (10%) were parsimony informative. Eighteen equally parsimonious trees were recovered, with seven nodes having bootstrap support over 80% (Figure 5 a). This phylogeny is largely consistent with that obtained from the combined plastid data for the 28 GScp taxa (Figure 5 b). Except for the placement of *J. pristisepala*, the same three main clades identified by plastid data (A, B and C) are resolved with strong support (>85% bootstrap). The plastid data include *J. pristisepala* within clade C (100% bootstrap support), while the GScp data place it in clade A with over 90% support. This reciprocally well supported conflict was considered "hard incongruence" and *J. pristisepala* was excluded from further combined analyses.

Morphological data

The morphological dataset contained 16 characters, all parsimony informative, and yielded 4,952 equally parsimonious trees. The strict consensus tree (Figure 6) is poorly resolved; although ten nodes are resolved, (compared with 14 in the *psb*A tree), only one has bootstrap support over 80%. No nodes in the morphology tree conflict with the topologies obtained from the molecular data.

Combined data

In view of the conflict over the position of *J. pristisepala*, this species was omitted from analyses of the combined morphological, nuclear and plastid data. With *J. pristisepala* excluded, parsimony and Bayesian analyses of these data give similar results.

The strict consensus tree obtained from parsimony analysis of the combined plastid and nuclear loci is shown in Figure 7 a. The monophyly of *Jamesbrittenia* is well resolved (with 87% bootstrap support), and *J. ramosissima* is again shown to be sister to the rest of the genus, with 99% bootstrap support. Clades A, B and C each have 100% bootstrap support, while, within clade A, a subclade of three annual species (*J. thunbergii, J. racemosa* and *J. pedunculosa)* has 84% bootstrap support. Within clade C, sub-clade E (*J. kraussiana, J. microphylla, J. foliolosa* and *J. aspalathoides*

from Kirstenbosch) has strong (94% bootstrap) support, while a clade containing the wild accession of *J. calciphila*, the Kirstenbosch accession of *J. stellata* and both accessions of *J. tenuifolia* has 88% support. Of the species represented by replicate accessions, the two *J. grandiflora* accessions group together with strong (90%) support, but no other species represented by replicate accessions are monophyletic. Similar results were obtained when the morphological data were combined with all three molecular loci (Figure 7 b). The monophyly of *Jamesbrittenia* has 89% support, and the sister relationship of *J. ramosissima* has 98% support. Clades A, B and C are resolved with bootstrap support greater than 95%. Within clade A, a sub-clade of seven species lacking glistening glands is resolved with 86% support, while the annual, *J. aridicola*, is sister to the sub-clade of three annuals which resolves with 52% support. In clade C sub-clade E is again resolved, albeit with lower support of 70%, and sub-clade D is resolved with 78% support. Because of better resolution within clades A and C, this four partition dataset was used in the Bayesian analysis.

The 50% majority rule consensus of the trees retained from three separate Bayesian runs results in a relatively well resolved tree which is shown in Figure 8. Monophyly of Manuleae is not resolved, due to the inclusion of *Verbascum* in a polytomy with *Jamesbrittenia* and a *Sutera – Manulea – Lyperia* clade. However, the monophyly of *Jamesbrittenia* is well supported, with a posterior probability of 1.0. *Jamesbrittenia ramosissima* is again shown to be sister to the rest of the genus, and the same major clades, A, B and C, are recovered, each with posterior probability of 1.0. Clade A comprises *J. aridicola* as sister to a clade comprising a sub-clade of three annual species and another sub-clade of seven species of herbaceous dwarf shrublets which are very glandular hairy, but lack glistening glands. Clade B comprises four annual species, all from western southern Africa. Clade C is the largest of the lineages within *Jamesbrittenia* and includes all sequenced species from the southern Cape and the eastern parts of South Africa, as well as species from the western part of southern Africa. Clade C includes sub-clade D (posterior probability 1.0) which consists of species, often growing on limestone, as well as sub-clade E (also posterior probability 1.0) (*J. kraussiana, J. foliolosa, J. microphylla* and the cultivated *J. aspalathoides)*.

Molecular Dating

The Akaike Information Criterion identified the General Time Reversible model with rate variation among sites to be optimal for the *rps*16 dataset in the context of an appropriate topology (one of the most parsimonious trees obtained with the combined dataset). Under this model, with the parameters identified as optimal (base frequencies and rate matrix estimated from the data, invariant sites = 0, $\alpha = 0.7993$), a molecular clock was rejected by the likelihood ratio test ($-2\Delta\ln L=117$, df=60, $p<0.001$), indicating that rates varied among sequences. The phylogram of the unpruned tree is shown in Figure 9. When six outgroup taxa with particularly long branches were removed, the clock was no longer rejected ($-2\Delta\ln L=59$, df=54, $P>0.05$). This showed that the variation in substitution rate between sequences was reduced and the likelihood of the substitution behaving in a clock-like manner had increased. The major differences between results using the two methods are illustrated in Figure 10 a & b. The very short branches in Figure 10 b compared with Figure 10 a, reflect the younger ages obtained by using the enforced clock method of dating, compared with NPRS. The ages of eight nodes (identified in Figure 10), with associated variance, for both secondary calibration dates, using both methods for obtaining the ultrametric tree are shown in Table 7. A result is not shown for node 1 using NPRS, because 36 of the 100 bootstrap trees had a branch length of zero leading to this node, and the results obtained from the remaining 64 trees would have been biased towards a younger age. Nevertheless, the estimated age of the genus *Jamesbrittenia* (node 1) can be inferred as lying somewhere between the calibration date (31 or 25 mya) and the split of *J. ramosissima* from the rest of the genus (27.3±1.3 or 22.1±1.1 mya).

Biogeography

The full results of the DIVA analysis are shown in Table 8 and the unions of these are plotted onto the tree in Figure 11. The DIVA analysis would not run with the entire tree, so the A and B + C clades were analysed separately, with the basal node remaining unresolved. As expected with a DIVA analysis, the deeper nodes suggest multiple ancestral areas. The ancestor at the root node of the clade containing clade B + clade C is inferred to occur in all areas,

as is the ancestor of clade C, while the ancestor of clade B is inferred to have occurred only in southern Namibia. Within clade A, the ancestral areas are restricted to southern Namibia, Namaqualand, northern Cape and central Karoo. Bearing in mind the fact that polytomies have been arbitrarily resolved, clade C shows sub-clades centred in southern Namibia, the Port Elizabeth – Transkei area, the central Karoo, the Drakensberg and the southern Cape, implying local radiations.

Ecology: Optimisation of habitat and characters

Rainfall

The optimisation of rainfall volume onto the phylogenetic hypothesis is shown in Figure 12. The ancestral state is arid, with rainfall of less than 300 mm per annum. Within clade C there is a change to moderate rainfall (300-600 mm p.a.), with two shifts to high (>600 mm) rainfall and two reversions to aridity. The ancestral condition is winter-rainfall. Within clade C, summer and year-round rainfall species are interspersed amongst the winter-rainfall species. These results are robust as the deeper nodes, and most nodes leading to changes, have posterior probabilities greater than 0.9.

Soil

The soil types associated with *Jamesbrittenia* species are mapped onto the tree in Figure 13. The inferred ancestral soil type in all three major clades is granite. Soil preference shifts to sand early within clade C, at a well supported node (posterior probability>0.9). Another well supported node gives rise to a sub-clade on igneous basalt and dolorite, and another on shale. Finally, the well supported sub-clade D is associated with limestone, including a sub-clade on shale. Within clade A, there are changes to shale and limestone. Two polymorphic species are mapped as equivocal. Alternative resolutions of the tree at weakly supported nodes do not alter this basic pattern.

Life history

The optimisation of life history onto the phylogeny is shown in Figure 14. The base of the tree is equivocal; under ACCTRAN the annualness evolves in the ancestor of *Jamesbrittenia* with two subsequent reversals (in clade A and in clade C) and an even later reversal back to annualness (in *J. adpressa*). In contrast, DELTRAN infers a perennial ancestor, with annualness evolving four

times, twice in clade A, once in clade B and again in *J. adpressa*. The distribution of the character "xerophytic leaves" (Table 3, Number 5, that is: small - often less than 3 mm, thick texture with no visible veins, fascicled, no glandular hairs) is shown in Figure 14. It has evolved within a perennial clade and is present in all members of sub-clade D, and in three other species.

CHAPTER 4: **DISCUSSION**

Marker selection and phylogeny

The three molecular loci and the morphological data used in this study were of varying utility. The two plastid gene regions, amplified easily and contained sufficient variation to give trees of reasonable resolution, except near the tips. Although *rps*16 resolves *Jamesbrittenia* as monophyletic, while *psb*A-*trn*H does not, the trees do not conflict, and both resolve the four main clades within the genus (*J. ramosissima* sister to clades A, B and C). Agreement between plastid markers is expected because the chloroplast genome is non-recombining and is inherited as a unit. The larger quantity of plastid DNA compared with nuclear DNA may facilitate extraction and amplification. The nuclear locus GScp was more difficult to amplify, and with standard primers, gave many bands, possibly due to multiple copies or, alternatively, due to non-specific primer binding. Although PCR was attempted with all accessions, only 28 were successful, even using the newly designed primers. The GScp sequences from these 28 species of *Jamesbrittenia* produced a tree similar to that obtained with combined plastid data for the same taxa, except in respect of the position of *J. pristisepala*. This indicates that the band amplified by the newly designed primers, is probably homologous across the taxa sampled. No GScp sequences could be obtained from outgroup species, and this may require designing further primers.

Of the three molecular markers (details in Table 6), the *rps*16 intron provided the greatest number of parsimony informative characters, and resolved the most nodes in the strict consensus tree, as well as the highest number of nodes with bootstrap support over 80%, perhaps due to its greater length. Although the strict consensus obtained from the *psb*A-*trn*H intergenic spacer was less well resolved and had less support than *rps*16, it nevertheless contained good resolution. Likewise, GScp data contained useful variation and, although the tree is smaller, it contains good resolution. The polytomies near the tips of the trees obtained with molecular data, suggest that there has been insufficient time for large genetic distances to be established between the species due to the recentness of their diversification (see below). The strict consensus of the most parsimonious trees obtained using morphological

data alone was poorly resolved; however, when the morphological data are added to the combined molecular data, the number of nodes in the strict consensus increases from 29 to 36, although nodes with bootstrap support over 80% drops from 17 to 16. This increase in resolution may be because morphological and molecular data provide phylogenetic resolution at different hierarchical levels, and are complementary (Pennington, 1996; Eldenäs and Linder, 2000).

The position of *J. pristisepala* is very different in the combined plastid tree and the nuclear GScp topology (Figure 5 a & b). The nuclear data include *J. pristisepala* in clade A with >90% bootstrap support, while the plastid data place it in clade C with similar strong support. This is "hard incongruence" (Wolfe and Randle, 2004) and indicates that the plastid and nuclear genes used have different histories in this species, the reticulate pattern reflecting either incomplete lineage sorting, hybridisation, horizontal gene transfer or chloroplast capture (Verboom et al., 2006). Incomplete lineage sorting occurs when there has been insufficient time for ancestral polymorphisms to disappear and the nodes of the species tree and the gene trees do not coincide exactly (Nichols, 2001). This seems an unlikely explanation of the conflict involving *J. pristisepala* as the conflict is across multiple, deep, well supported nodes, which suggests that there has been sufficient time for ancestral polymorphisms to disappear. Hilliard (1994) suggests that hybridisation is common within the genus. She mentions in particular that *J. pristisepala* appears to hybridise with *J. stricta, J. breviflora* and *J. jurassica,* inferring this from the existence of intermediate morphological forms. In *Solanum* however, molecular data do not support hybridisation as the cause of morphologically intermediate forms (Miller and Spooner, 1996). That hybridisation occurs is, however, confirmed by the horticulturist at Kirstenbosch Botanical Gardens who reports that most casual crossing experiments are successful and that F1 hybrids grow easily and produce fertile seed (A. Harrower, personal communication). This identifies hybridisation as the likely cause of incongruence in *Jamesbrittenia*. It is however surprising that the conflict is between such divergent lineages (clade A and clade C) and between groups that are geographically so widely separated (clade A in the arid north west, clade C, largely in the southern and

eastern regions). The examples of supposed natural hybridisation mentioned by Hilliard (1994) all have range overlap, and all occur in clade C. There have been no horticultural attempts to produce hybrids using the rather pungent species in clade A. It is unfortunate that the particular specimen of *J. pristisepala* which yielded the GScp sequence was in cultivation at Kirstenbosch, where accidental cross-pollination (by an insect?) may have occurred in the greenhouse. To eliminate this possibility, a second, wild specimen of *J. pristisepala* was sampled using PCR: unfortunately, while amplification of this material was successful for the plastid gene regions, it was not successful for GScp. Repeat amplification and sequencing of the cultivated specimen to eliminate operational error was not done. In view of this, both accessions of *J. pristisepala* were excluded from analyses, but a final interpretation requires that the conflicting pattern be repeated with a specimen sampled from the wild. Fourteen species of *Jamesbrittenia* yielded only plastid sequences (Table 3), and perhaps caution should be exercised in the interpretation of the phylogeny, lest these also contain conflict between plastid and nuclear genomes.

Testing the monophyly of the tribe Manuleae was not a specific objective of this study. However, it is worth noting that although the data presented are consistent with the monophyly of Manuleae, it is not clearly supported, the plastid data including *Oftia, Teedia, Verbascum* and *Scrophularia* in a clade with the genera from Manuleae, while the total evidence Bayes tree, includes *Verbascum* within the Manuleae clade. Various authors (Olmstead and Reeves, 1995; Olmstead et al., 2001; Oxelman, Kornhall et al., –paper 1 in Kornhall 2004) show Scrophulareae (including *Scrophularia* and *Verbascum*) as sister to Manuleae, while this study includes at least *Verbascum* in a polytomy with *Lyperia* and other outgroup members of Manuleae. It is interesting that traditional morphology-based taxonomy identifies the tribe Scrophulareae as being centred in the Northern Hemisphere, compared with the largely southern African distribution of the Manuleae, suggesting separate geographical origins for the two tribes. However, the limited sampling of Kornhall and Bremer (2004) removes *Antherothamnus* (southern Africa) from Manuleae and places it in a clade with *Scrophularia* and *Verbascum* with

100% bootstrap support. The circumscriptions and monophyly of both tribes need to be tested with more extensive sampling.

In the context of the taxa sampled and, given that outgroup sequences could not be obtained for the nuclear marker, the monophyly of *Jamesbrittenia* is well supported (*rps*16 with 87% bootstrap, total evidence with 89% bootstrap, or posterior probability = 1.0). In order to demonstrate monophyly of a genus there should be extensive outgroup sampling. Unfortunately the sequences obtained by Kornhall (2004) have not been deposited in GenBank, so it was not possible to make use of his extensive outgroup sampling. Kornhall et al. (2001) however, identify a clade containing *J. megadenia* and *J. filicaulis* among more than 30 other genera of Scrophulariaceae in a tree rooted on Oleaceae. Since these species span the node between clades B and C resolved in this study, at least this portion of *Jamesbrittenia* is monophyletic.

All analyses placed *J. ramosissima* as sister to the remainder of the genus. This is not surprising as there are three morphological characters in which *J. ramosissima* differs from the generic circumscription according to Hilliard (1994): first, the calyx tube is not lobed to the base, but only for about two thirds of its length; second, the posticous filaments are not decurrent on the corolla tube; and third, the filaments are glabrous, and do not meet the diagnostic criterion of having "at least posticous filaments with hairs". Hilliard (1994) also mentions that this species is unique in *Jamesbrittenia* by having "distinctive, short-stalked glistening glands". It is curious that this lineage is represented by a single species. Either speciation has not occurred, extinction rates have been high, or other related species have not yet been sampled. It is possible that once all species of *Jamesbrittenia* are sampled, some may form a clade with *J. ramosissima*. Hilliard places *J. ramosissima* in a group with *J. pedunculosa, J. hereroensis, J. tenella* and *J. fragilis*. The latter three species have not yet been sampled, but the molecular data, do not uphold a close relationship with *J. pedunculosa*, this species being embedded in clade A with strong support. Given its morphological distinctiveness, it seems unlikely that other species in *Jamesbrittenia* will group with *J.*

ramosissima. It is even possible that molecular data will eventually show that it is closer to one of the other genera in Manuleae.

A key feature of all the phylogenetic hypotheses generated in this study, which sampled 42 (mainly winter-rainfall) of the 84 *Jamesbrittenia* species, is the recovery of three well supported (bootstrap > 95%, posterior probability =1.0) clades (in addition to *J. ramosissima*), that accommodate most of the species diversity in *Jamesbrittenia*.

Clade A contains, on current sampling, eleven species, four annual and seven perennial. The latter constitute Hilliard's group 1.b.1, which is the only group for which she identified a clear synapomorphy: namely a total lack of glistening glands. Species in this group (*J. maxii, J. megaphylla, J. bicolor, J. fruticosa, J. sessilifolia, J. amplexicaulis* and *J. major*) all have large hairy leaves, usually with three conspicuous veins from the base, and typically white flowers with modest markings around the throat. The four annuals differ in having glistening glands as well as glandular hairs. Within the annuals, *J. aridicola* is sister to a clade consisting of *J. thunbergii*, *J. racemosa* and *J.pedunculosa.* The former two look similar, both have mauve flowers with very dramatic markings (see cover picture), while *J. racemosa* and *J. pedunculosa* both lack glistening glands on the capsule. *J. pedunculosa* is unusual in the genus, in having yellow flowers with a short corolla tube and a dark spot in the throat. The short tube caused Hilliard (1994) to group it with *J. ramosissima*, a relationship which is not upheld by molecular data. The flowers of *J. pedunculosa* bear a striking resemblance to *Hemimeris* with which it is sympatric. The similarity in the flowers may be due to convergence as they possibly share a pollinator.

Clade B is composed of four annual species: *J. primuliflora, J. fimbriata, J. glutinosa* and *J. megadenia*. These species all have flowers with strong markings and an indumentum of hairs which are much stouter than in the rest of the genus.

Clade C contains the remainder of the species sampled, and exhibits much greater diversity, particularly in growth form, leaf shape and indumentum, than

clades A or B. For example, *J. adpressa*, is a prostrate annual with yellow flowers and deeply dissected leaves, *J. grandiflora,* is a shrub with large blue flowers and large soft leaves, and *J. breviflora* is an alpine plant with flowers ranging from terracotta to rose-pink. Clade C exhibits the greatest internal resolution and includes sub-clade D, which consists of eight species with a preference for limestone substrates. The flowers of the species in sub-clade D are morphologically divergent, ranging from brown leathery (*J. atropurpurea*) through brown with white edges to the corolla lobes (*J. albomarginata*), mauve with orange or brown throat markings (*J. stellata*), to deep blue (*J. tenuifolia*). In contrast to the variation in flower form and colour, these species all have leaves which are relatively small (sometimes microphyllous), thick and fleshy, with no visible veins, but usually covered in glistening glands, often looking "varnished". Sub-clade E consists of *J. kraussiana, J. microphylla, J. foliolosa* and the cultivated *J. aspalathoides*. This group is consistently recovered with good support (total molecular data 94% bootstrap, Bayesian posterior probability = 1.0), but does not appear to have any morphological features in common, e.g leaves range from flat and soft (*J. kraussiana*) to microphyllous (*J. microphylla*). The conflict between the position of wild and cultivated J. aspalathoides requires further investigation, preferably re-sampling. Clade C is certainly under-sampled and, with more species included, more structure may be revealed. Analyses by Kornhall (2004) imply that the northern hemisphere *J. dissecta* is included in this clade.

Taxonomic implications

It has been suggested (Wheeler, 2004) that there is a trend towards abandoning traditional taxonomy, particularly monographs and revisions, in favour of molecular phylogenetics. This project shows that these fields are interlinked, and complement each other. The revision of Manuleae by Hilliard (1994) informed the choice of plants for this molecular study, whilst conversely, this study, in resolving relationships within the genus, provides the basis for improved taxonomy of the group. Before 1990 some species sampled here were not yet recognised, while others were included in four or more genera and it is most unlikely that they would have been studied as a group. While Hilliard's morphological studies allowed her to identify the

bounds of the genus accurately, they left ambiguity regarding the definition of infrageneric groupings. Specifically, none of the groups characterised by testa texture (groups 1.a.1-3, groups 1.b.1-2 and groups 2.1-2.5, Table 2) coincides with the phylogeny (Figure 14), indicating that the testa character is not useful. However, the indumentum characters are sometimes correct, for instance Hilliard's group 1.b.1 which lack glistening glands is upheld by the phylogeny.

Molecular data are increasingly offered as a route to defining species, monophyly usually being a requirement (Sites and Marshall, 2003). Of the few cases in this study where multiple accessions were included, some resolve as monophyletic (e.g. *J. grandiflora*), but others, like *J. aspalathoides,* are paraphyletic. This is not entirely surprising. Given the recentness of divergence, one might expect incomplete lineage sorting, as biological speciation usually precedes genealogical speciation (Hudson and Coyne, 2002). This highlights the limited utility of molecular data in delimiting species where these are recently diverged.

Molecular data, however, may support taxonomic decisions made on morphological grounds. Hilliard (1992) recognised *J. aridicola* as a new species distinguishing it from *J. megadenia* by smaller glandular hairs and more deeply notched corolla lobes. The molecular data place the two species in clades A and B respectively, showing that the distinction between them is significant. This is an example of meticulous revision of a genus being most useful, and the morphological species concept being supported by molecular evidence. *J. aridicola* has range overlap with *J. megadenia*, which was probably the cause of earlier confusion. On the other hand, although Hilliard (1992) recognised *J. fimbriata* as a new species, distinguishing it from *J. primuliflora* by the longer hairs in the throat, which extend out onto the corolla lobes, the DNA data suggest a very close relationship (combined molecular 99% bootstrap, posterior probability = 1.0). This is closer than for other species where two accessions were sequenced, suggesting that speciation may be incomplete.

Molecular Dating

Dating using molecular methods is open to much criticism, mostly due to the difficulty of accommodating rate heterogeneity, and to errors associated with calibration. Heads (2005) points out that any calibration based on fossil evidence must be considered a minimum age only, because older fossils may always be found subsequently. The dates used here are derived indirectly using a fossil calibration (Wikström et al., 2001), so uncertainties about stratigraphical dating and the minimum age proviso are relevant, as well as multiple sources of error associated with their clock analysis. These include uncertainty associated with tree topology, stochastic sampling effects due to use of a finite length of sequence and the method used to convert the branch length tree to an ultrametric tree. Hugall and Lee (2004) comment that bootstrapping leads to unjustifiably low associated errors as the method only considers stochasticity in the substitution model and ignores much larger sources of uncertainty, such as variation in character sampling, uncertainty in tree topology, and calibration accuracy. These sources of error affect the reliability of the age of the *Scrophularia – Verbascum* node and become compounded when that node is used in further age estimations (Graur and Martin, 2004). However, by using ACCTRAN, DELTRAN and ML branch length–based estimates (Wikström et al., 2001), the branch length misspecification is to some extent accommodated. In Table 7 two dates: 31 mya and 25 mya, provided by Wikström et al. (2001), are used for each of two dating methods in deriving possible dates for historical divergence events in *Jamesbrittenia*. The standard error of 5 myr (which is variance associated with bootstrapping, but not stratigraphy), associated with the Wikström dates has not been taken into account during these rate smoothing and molecular clock calculations, so the errors presented here are underestimates. In a comparison of age estimation using direct and indirect calibration, Linder et al. (2003) determined the age for the origin of Restionaceae, using fossil pollen, and found that it was substantially older than the secondary calibration date suggested by Wikström et al. (2001), so the *Jamesbrittenia* dates based on this calibration may be too young. Rate heterogeneity within phylogenies can present problems. Sanderson and Doyle (2001) found that there were marked differences in diversification rates between lineages, herbaceous plants having higher rates. Archibald et al. (2005b) found that annual species

of *Coreopsis* had longer branch lengths than perennial species. Despite differences in life history amongst the species of *Jamesbrittenia*, only the annual *J. thunbergii* has a slightly longer branch (Figure 9), the sequences being generally clock-like.

The two methods used here give different ages, NPRS ages generally being older than when the molecular clock is enforced using Maximum Likelihood, a result also found in other studies (Mummenhoff et al., 2005; Linder et al., 2005). Sanderson and Doyle (2001) found that NPRS aggravated the conflict between molecular ages and the fossil record, which they surmised was due to rates of molecular evolution changing abruptly and not being phylogenetically autocorrelated, as assumed in NPRS. Hugall and Lee (2004) found that methods of creating ultrametric trees (especially NPRS) resulted in trees with elongated branches, which leads to over-estimation of the age of nodes. Consistent with these observations, six outgroup taxa with particularly long branches were excluded in this study and this resulted in younger age estimates (Table 7).

Linder et al. (2005) investigated the effects of under-sampling with various dating methods. They found that under-sampling had the greatest effect with NPRS: 10% sampling resulted in age estimates half of that obtained with 100% sampling. In this study 42 out of 84 *Jamesbrittenia* species are included, and as the relationship between under-sampling and age under-estimation is logarithmic, sampling intensity should not be a major cause of age discrepancies here. However, the calibration node is among the outgroup species and sampling in that region of the tree is sparse, and is decreased further when the six taxa with long branches are excluded. Linder et al. (2005) also found that age estimation varied with the method used: molecular clock enforcement and Penalised Likelihood gave youngest node ages, with least dependence on sample size or distance of node from the calibration point. In contrast, when using NPRS, both these factors increased the age estimate. The data presented in Table 7 confirm that distance from the calibration node increases the divergence between the estimates obtained with the two methods: node 6 is estimated to be approximately five times older with NPRS than with the molecular clock. This

suggests that the younger ages obtained by using the molecular clock may be more realistic.

In spite of the problems with error estimations and inconsistencies between methods, the data give a broad picture of diversfication in *Jamesbrittenia*. In this study (Table 7) it was found that the genus *Jamesbrittenia* first diverged approximately 30 million years ago, in the early Miocene; close to the time of the *Scrophularia – Verbascum* split. The *J. ramosissima* ancestor diverged soon afterwards, between 20.3 and 27.4 mya, and the three main lineages had all diverged by the mid-Miocene, approximately 10 mya. Most present day species diversity in clades A, B and C is the result of recent parallel radiations dated late Miocene – Pliocene – Pleistocene.

Biogeography

Jamesbrittenia species occur throughout southern Africa, with one weedy species extending northwards to Sudan, Egypt and India. The plants usually grow in relatively small populations, which are often widely separated. A few species of *Jamesbrittenia* have a very wide distribution (e.g. *J. huillana* occurs from southern Namibia, to Angola, Zambia, Zimbabwe, eastern Transvaal, Natal and the Eastern Cape), but most species are much more restricted, some growing only in single localities (e.g. *J. megaphylla, J. amplexicaulis*).

This study used the program DIVA (Ronquist,1997) to investigate the possible ancestral geographical distribution of the genus. The strengths of a DIVA analysis are that multiple ancestral areas are allowed, anticipating speciation by vicariance (Ronquist, 1997). However, if the current distribution of species is in fact due more to dispersal than to vicariance, then the rationale of DIVA to penalise dispersal and not vicariance, will lead to incorrect inferences. The results of the DIVA analysis done here suggest a series of very widespread ancestors, whose ranges seem unlikely in the context of the very restricted distributions of most extant species. It may be noted that the DIVA analysis was unable to determine the ancestral distribution for the basal node in *Jamesbrittenia*, the same problem being recorded by Mummenhoff et al. (2005) for *Heliophila*.

Although the DIVA analysis did not resolve a range for the ancestor of all the *Jamesbrittenia* species, it indicates localised divergence for the major clades: clade A in the north west (Namibia, Namaqualand and northern Cape), clade B in southern Namibia and clade C widespread, but including clade D in the southern Cape, thus implying local radiations. The fact that the three earliest divergences (*J. ramosissima*, clades A and B) each give rise to lineages restricted to the west (north west Cape, Namaqualand, southern Namibia) implies an origin for the genus there, even though this is not supported by DIVA. It appears that clades A and B diversified in this ancestral area, while clade C experienced dispersal which led to the invasion of new areas to the south and east, with subsequent speciation.

During this study only half of the species of *Jamesbrittenia* were sampled, and these were mostly from the western and southern parts of the range. It is highly likely that this bias could have influenced the result of the DIVA analysis. It is unclear what effect the inclusion of eastern and tropical species would have on biogeographic inferences. Only one species, *J. dissecta*, occurs outside southern Africa. As a result of Kornhall's (2004) work, this is inferred to fall within clade C (see above) and, if so, it would add evidence to the supposition that the current distribution of species in clade C is the result of dispersal.

Radiation of *Jamesbrittenia*

The results of the biogeographic analysis and the molecular dating suggest that the common ancestor of *Jamesbrittenia* occurred in the west (or had a wide distribution) during the Miocene, when conditions were thought to have been warmer and wetter than at present (Linder, 2003). Reconstruction of habitat variables implies that this ancestor was associated with granite soils and was adapted to rain in winter with a summer-dry season. Hardy (2006) notes that parsimony reconstruction of ancestral habitats may be "fraught with pitfalls", one of which is the inference of ancestral environments that, according to the geological record, did not exist at the time. Such a situation arises here with the inference of an ancestral winter-rainfall habitat in the Miocene, since it is thought that Mediterranean climate with dry summers only became established in southern Africa in the Pliocene (Cowling and

Richardson, 1996). An alternative scenario, though less parsimonious, is that *Jamesbrittenia* ancestrally occupied summer- or all-year rainfall habitats, with subsequent parallel switches to winter rainfall environments when these conditions arose in the Pliocene. Frumhoff and Reeve (1994) point out that parsimony optimisation will accurately reveal the timing and direction of historical transitions between character states only if the rate of character change within lineages is low relative to the rate of cladogenesis, which may not be the case for ecological variables.

The phylogenetic data suggest that in the early to mid- Miocene, *J. ramosissima* (or its ancestor) adapted to life in the valleys of the Gariep river system. The lack of speciation in the lineage leading to *J. ramosissima* has already been commented on. Although it is currently restricted to the Gariep catchment, it is not a riverine plant, preferring to grow among boulders, well above the water level. The persistence of *J. ramosissima* cannot therefore be attributed to stable, mesic conditions usually enjoyed by riverbank species (c.f. *Phylica*, Richardson et al., 2001). During the mid-Miocene the ancestors of clades A, B and C diverged probably in the north-west, with subsequent dispersal of clade C, resulting in a wide distribution in southern Africa. Radiation of clades A, B and C appears to have been relatively recent, possibly being associated with adaptation to novel edaphic environments and late Miocene climate change, when increasing aridification caused the mesic woodlands to shrink, making novel habitats available to arid adapted plants. The putative ancestor of Clade A is postulated to have favoured an arid climate of low winter-rainfall. This reconstruction is questionable, however, since it is likely that the climate was generally moister in the early Miocene and the current summer-arid climate of the west coast is thought to be of relatively recent origin. Due to the growth of the Antarctic ice-sheet during the mid-Miocene sea water temperatures fell, resulting in the establishment of the south Atlantic high. This caused the climate to become drier, particularly in the west, and when the Drake Passage opened and the cold Benguela current was established, at the Miocene-Pliocene boundary, the climate in the south-west became Mediterranean, with dry summers (Linder, 2003). The relatively sudden burst of speciation that took place in clade A towards the end of the Miocene, may have been triggered by this

climate change. Edaphically, clade A shows a general preference for soils of granite origin, and likely radiated on these substrates. However the restriction of some isolated neo-species to shale and limestone ("schwartzkalk") soils, suggests edaphic specialisation. Speciation in clade B has been less spectacular, resulting in only four species. It seems to have followed a similar pattern with an ancestor favouring granite soils in a summer-arid habitat. One species, *J. megadenia* has adapted to limestone and shale from the ancestral granite. Of the three major clades in *Jamesbrittenia*, clade C shows the most spectacular radiation. The diversification of this group is likely associated with the ability to disperse widely and adapt to a variety of habitats. The data suggest that the dispersal of this clade into novel habitats was accompanied by changes in leaf and flower morphology. Two bursts of speciation are apparent, one at the Miocene-Pliocene boundary, the other much more recent, possibly less than a million years ago. The first, between two and ten million years ago, is associated with the occupation of new soil types from the ancestral granite substrate, together with new rainfall regimes, the latter involving both increased amounts of precipitation as well as changes in rainfall seasonality. The clade appears to have originated on granite, with subsequent shifts to shale, alluvial sand, and basalt, and finally to limestone. This shift to limestone derived soils, is a pattern similar to that found by Hardy and Linder (2005) for *Thamnochortus*. It seems as if there have been a series of sub-radiations, each onto a distinctive soil type, and these may well become more apparent with further sampling. A similar series of nested radiations has been reported for *Pelargonium* (Bakker et al., 2005). The radiation of species on limestone may have been facilitated by the exposure of dispersal routes as sea levels fell during the late Pliocene. When these dispersal routes disappear, the habitats become insular, promoting differentiation (Wiens, 2004a).

The nature of species and speciation has enjoyed wide ranging discussion in the literature (Wiens, 2004b; Riesenberg et al., 2006; Levin, 1993, 2000, 2006; Sites and Marshall, 2003). In *Jamesbrittenia*, the timing of radiation reflects a correlation with adaptation to novel climatic and edaphic conditions, possibly linked to Pliocene-Pleistocene climate change. However, the

speciation mechanisms involved are not clear. Levin (1993) suggests that speciation does not follow from the gradual divergence of races, but rather occurs when small populations are isolated at the fringe of the distribution of a more widespread progenitor. Small populations are more likely to experience change due to fixation of random neutral mutations (genetic drift). Most populations of *Jamesbrittenia* are relatively small and they tend to be quite far, sometimes many kilometres, apart. Also, the seeds are small (less than a millimetre) and lack adaptations for long distance dispersal like wings, tufts of hairs or edible coverings, so gene flow is probably quite restricted. The current distributions of the species in clade A, for example, fit this pattern, with one species (*J. maxii*), being widespread and the remainder having much more restricted ranges. Within the neospecies, divergent selection is likely to result in adaptation to the new habitat, with morphological change developing later. The development of reproductive isolating mechanisms may also occur, but would not be essential if the new species remained geographically isolated (Wiens, 2004b). Within *Jamesbrittenia* there may be extreme habitat specificity and the habitats are often localised and discontinuous, for example river beds or limestone outcrops. Adaptation of incipient species to these new habitats would be driven by natural selection. A possible example of this is the relationship between *J. canescens* and *J. barbata*. The former is widespread in Namibia and Botswana, often growing in dry river beds, and showing wide morphological variation, while *J. barbata* has a very restricted range, growing in harsh dry conditions on the edge of the Namib desert. The molecular data place the species together, while morphologically, the flowers of *J. barbata* are very similar to the yellow morphs of *J. canescens*, but *J. barbata* has developed a distinctive covering of white glistening scales, presumably the result of selection in conditions of harsh sun and wind. Levin (2000) suggests that under strongly divergent ecological pressures, morphological divergence is not matched by divergence in neutral molecular markers, with the result that morpho-speciation occurs with less-than-expected genetic divergence.

Evolution of annualness and leaf form

The results of this study are ambiguous about which life history strategy is plesiomorphic in *Jamesbrittenia*. Subject to the incomplete sampling of

species, the ancestor of the genus is reconstructed as being either annual (ACCTRAN) or perennial (DELTRAN). Considering that the basal node antedates the origin of aridification, perennial life history seems likely to be ancestral in *Jamesbrittenia*. Annualness is usually considered to be an adaptation to aridity (Evans et al., 2005; Robbins et al., 1965). Therefore it is surprising that in clade A, a clade restricted to desert environments, the perennial habit (*J. maxii, J. major, J. bicolor, J. sessilifolia, J. fruticosa, J. amplexicaulis* and *J. megaphylla*) arose from annuals, in an arid environment. This result though unexpected, is not unique, this also being found in *Oenothera* (Evans et al., 2005). Evans et al. (2005) investigated the influence of climatic factors on life history evolution in *Oenothera,* and found that temperature during the summer non-growing season, as well as water availability during the winter growing season, were more important determinants of the evolution of an annual life history, than the intensity of summer drought. This was interpreted as a capacity to become semi-dormant during the arid summer, and it is possible that *Jamesbrittenia* adopts a similar strategy, but this requires further study.

The perennial species in clade A all have large (20-30 mm) expanded leaves, that seem atypical of arid adapted plants. However, the leaves of these species are covered in dense glandular hairs, which may afford protection against desiccation by increasing the boundary layer and reducing transpiration (Charest-Clark, 1984). Large leaves are poor convectors of heat and may have elevated temperatures under high radiation loads (Chabot and Hicks, 1982). It is significant, therefore, that these plants grow in micro-habitats which usually involve shade from boulders; or they may have access to underground water in dry stream beds, where overheating can be overcome by transpirational cooling (Parkhurst and Loucks, 1972). In contrast, a synapomorphy that is associated with the radiation of species onto limestone, where they grow in exposed situations in full sun, is small xerophytic leaves. These leaves are usually less than 3 mm long, fascicled, and lack large glandular hairs (although they are often covered in glistening glands). They are always thick textured, almost succulent, and the veins are not visible. All members of clade D have this kind of leaf, and it has arisen on two other occasions in Clade C. It may represents an adaptation for

conserving water because, although the group does not occupy environments of extreme aridity, plants often grow in well-drained soils, on dry north facing slopes. Small leaf size is associated with higher convection coefficients (Parkhurst and Loucks, 1972), and reduces the risk of overheating in these exposed conditions.

Context: comparison to other studies

Early naturalists sought a single phenomenon, acting on the entire flora, to explain the floral diversity of the Cape region. Linder (2005), however, argues that the diversity of the Cape flora may be the result of recruitment of many lineages over a long period, followed by *in situ* diversification. He suggests that within lineages, the earliest species to diverge should be found in mesic habitats, with the most recent large radiations being situated in the more arid west. This has been found for *Ehrharta* (Verboom et al., 2003), *Thamnochortus* (Hardy and Linder, 2005), *Pelargonium* (Bakker et al., 2005), and *Erica* (McGuire and Kron, 2005). *Moraea* in contrast, appears to have originated in the south west, with more recent radiations being centred in the summer rainfall east (Goldblatt et al., 2002). Within a broader south African context, Archibald et al. (2005a) investigated the genus *Zaluzianskya*, especially in respect of life history evolution. They found the annual life history was closely associated with rainfall of less than 500 mm p.a. and that the annual condition was plesiomorphic. They deduce that *Zaluzianskya* evolved in the west of southern Africa after aridification was established, and subsequently diversified into the more eastern parts of the sub-region, adopting a perennial life history there. They did not attempt to date the evolutionary history and note that "the assumption that the distributions of current populations, and the distributions of current precipitation regimes, reflect those found in the past" may not be correct. Mummenhoff et al., (2005) found a similar pattern in "clade C" of *Heliophila*, where the ancestral life history was annual and the ancestral area was centred in the arid Richtersveld. As species evolved and invaded more mesic habitats to the east, including the Drakensberg, there were reversions to perennial life history. *Jamesbrittenia*, appears to have a similar geographical pattern to the latter two genera, with the earliest divergences centred in the west and more recent radiations extending south and east. The life history, however is

equivocal. The data do not exclude the possibility of a shift from annual to perennial life history in *Jamessbrittenia*, but unlike *Zaluzianskya* and *Heliophila* "clade C", perennial *Jamesbrittenia* species are common in the arid west.

The most recent studies of diversification in the Cape flora have sought to investigate the role of Miocene climate change. The timing of dated radiations in the southern African flora (Table 1) implies that bursts of speciation were not contemporaneous, although most current day species diversity appears to have arisen within the last 30 million years. Bakker et al. (2004) urged more use of molecular methods to date radiations, with the particular aim of establishing whether these radiations occurred simultaneously, in a concerted response to a changing environmental or climatic factor. Mummenhoff et al. (2005) suggest that a meta-analysis of the data from many different lineages is required before realistic comparisons can be made. Considering the wide range of dates, and the large variances that result from different methods of age estimation this appears desirable. Nevertheless, the timing of radiation in *Jamesbrittenia*, (I-10 mya) is consistent with the suggestion that speciation was associated with changing climate during the Pliocene-Pleistocene, particularly increased aridity with dry summers in the west, while some species diversified to take advantage of more mesic, summer rainfall climate to the east.

Conservation

Eighty three species of *Jamesbrittenia* are endemic to southern Africa, many species having very restricted distributions. These attractive and unusual plants deserve to be conserved. Development of horticultural cultivars and their translocation represents a threat to the integrity of wild populations as most species in the genus have evolved recently and do not appear to have had time to develop the incompatibilty mechanisms, that are necessary to ensure reproductive isolation. That this process takes time, was shown by Moyle et al. (2004). They investigated the relationship between reproductive isolation and genetic distance and found no significant correlation in two out of three genera. They also show that reproductive isolation lags behind the geographic isolation of species, and this is what might constitute a very real

threat to the biodiversity in *Jamesbrittenia*. Levin et al. (1996) record instances where introgression following naturalisation of cultivated hybrids has resulted in the extinction of species, and this could well occur if horticultural hybrids of *Jamesbrittenia* become widely cultivated in areas where natural species grow. It might, however, be good for *Jamesbrittenia* to be brought into the limelight by horticultural development, so that a greater awareness of this genus is created, which would hopefully be reflected in conservation-friendly attitudes. But this would be better achieved by selecting for cultivars within existing species than by producing hybrids, unless the hybrids could be selected for incompatibility with existing species.

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Table 1. Dates of origin and / or radiation of southern African lineages, as reported in the literature.

Taxon	Estimated age (mya)	Geological time	Author
Heliophila	2 - 5	Pliocene - Pleistocene	Mummenhoff et al. (2005)
Melianthus	2 - 20	Miocene	Linder et al. (in press)
Aizoaceae, subfamily Ruschioideae	3.8 - 8.7	Pliocene	Klak et al. (2004)
Phylica	7 - 8	Late Miocene	Richardson et al. (2001)
Ehrharta	4.5 - 21.7	Miocene	Verboom et al. (2003)
Moraea	25	Early Miocene	Goldblatt et al. (2002)
African Restionaceae	64 - 71	Oligocene	Linder et al. (2003)
Thamnochortus	14 - 30	Early Miocene	Linder et al. (2005)
Pelargonium	30	Oligocene - Miocene	Bakker et al. (2005)
Pelargonium section *Hoarea*	10	Mid Miocene	Bakker et al. (2005)

Table 2. Distribution ranges, principal flowering periods and growth forms of species included in *Jamesbrittenia*, as summarized from data presented in Hilliard (1994) and showing her morphology-based groups. Distribution areas codes: Ang=Angola; Bot=Botswana; CP=Cape Province; Les=Lesotho; Mal=Malawi; Moz=Mozambique; Nam=Namibia; Nat=Natal; OFS=Orange Free State; Swa=Swaziland; Tvl=Transvaal; Zam=Zambia; Zim=Zimbabwe (Note that South African provincial boundaries are pre-1994).

Species	Hilliard group	Distribution	Flowering records	Habit
J. fodina (Wild) Hilliard	1.a.1	Zim	All year	Shrublet
J. giessii Hilliard	1.a.1	Nam	Feb	Subshrub
J. angolensis Hilliard	1.a.1	Ang	Jul-Aug	Subshrub
J. carvalhoi (Engl.) Hilliard	1.a.1	Moz	Jun-Oct	Shrub or subshrub
J. candida Hilliard	1.a.1	Tvl	Oct-Jan	Suffrutex
J. grandiflora (Galpin) Hilliard	1.a.1	Tvl Swa	Mar-Jul	Subshrub
J. macrantha (Codd) Hilliard	1.a.1	Tvl	Jun-Oct	Subshrub
J. albobadia Hilliard	1.a.1	Mal Zim	Jan-Oct	Suffrutex
J. burkeana (Benth.) Hilliard	1.a.1	Bot Tvl Swa Nat	All year	Suffrutex
J. accrescens (Hiern) Hilliard	1.a.1	Tvl	Jan-Oct	Suffrutex
J. dentatisepala (Overkott) Hilliard	1.a.1	Les Nat	Jul-Jan	Dwarf shrublet
J. elegantissima (Schinz) Hilliard	1.a.1	Ang Bot Zam Zim Nam	All year	Perennial herb
J. zambesica (R.E.Fries) Hilliard	1.a.2	Zim	Jun	Suffrutex
J. atropurpurea (Benth.) Hilliard	1.a.2	CP Tvl OFS Les Bot Nam	All year	Shrublet
J. huillana (Diels) Hilliard	1.a.2	Ang Nam Zam Zim Tvl Sw Nat CP	All year	Shrublet
J. namaquensis Hilliard	1.a.2	CP	May-Sep	Dwarf shrublet
J. incisa (Thunb.) Hilliard	1.a.3	CP	May-Sep	Dwarf shrublet
J. tortuosa (Benth.) Hilliard	1.a.3	CP	All year	Dwarf shrublet
J. tysonii (Hiern) Hilliard	1.a.3	CP	All year	Dwarf shrublet
J. filicaulis (Benth.) Hilliard	1.a.3	OFS Les CP Nat	Oct-May	Dwarf shrublet
J. albanensis Hilliard	1.a.3	CP	All year	Shrublet
J. phlogiflora (Benth.) Hilliard	1.a.3	CP	All year	Shrublet
J. maritima (Hiern) Hilliard	1.a.3	CP	All year	Perennial herb
J. kraussiana (Benth.) Hilliard	1.a.3	Nat CP	All year	Perennial herb
J. pinnatifida (L.f.) Hilliard	1.a.3	CP	All year	Perennial herb
J. argentea (L.f.) Hilliard	1.a.3	CP	All year	Shrub
J. integerrima (Benth.) Hilliard	1.a.3	Nam CP	All year	Shrublet or perennial herb
J. albiflora (Verdoorn) Hilliard	1.a.3	Tvl CP OFS	All year	Dwarf shrublet
J. tenuifolia (Bernh.) Hilliard	1.a.3	CP	All year	Dwarf shrub
J. foliolosa (Benth.) Hilliard	1.a.3	CP	All year	Dwarf shrublet
J. zuurbergensis Hilliard	1.a.3	CP	Jun-Oct	Shrub
J. microphylla (L.f.) Hilliard	1.a.3	CP	All year	Dwarf shrublet
J. aspalathoides (Benth.) Hilliard	1.a.3	CP	All year	Dwarf shrublet
J. calciphila Hilliard	1.a.3	CP	Aug-Jan	Dwarf shrublet
J. stellata Hilliard	1.a.3	CP	Jul-Jan	Dwarf shrublet
J. albomarginata Hilliard	1.a.3	CP	All year	Dwarf shrublet
J. merxmuelleri (Roessler) Hilliard	1.a.3	Nam CP	May-Oct	Dwarf shrublet
J. fruticosa Benth. Hilliard	1.b.1	Nam CP	Aug-Sep	Shrublet
J. maxii (Hiern) Hilliard	1.b.1	Ang Nam CP	All year	Shrublet
J. sessilifolia (Diels) Hilliard	1.b.1	Nam	All year	Shrublet
J. major (Pilger) Hilliard	1.b.1	Nam CP	Jun-Oct	Perennial herb
J. megaphylla Hilliard	1.b.1	Nam CP	Aug-Sep	Herb, possibly annual
J. bicolor (Dinter) Hilliard	1.b.1	Nam	Aug-Nov	Shrublet
J. amplexicaulis (Benth.) Hilliard	1.b.1	CP	May-Sep	Perennial herb
J. racemosa (Benth.) Hilliard	1.b.2	CP	Jul-Oct	Annual herb
J. thunbergii (G.Don) Hilliard	1.b.2	CP	Jun-Oct	Annual herb

J.maxii (Hiern)Hilliard #	TV 805	Northern Cape, Aggeneys farm	1.b.1
J.megadenia Hilliard #	TV 823	S.Namibia, Between Fish R canyon + Klein Karas	1.b.2
J.megaphylla Hilliard	TV 859	Namaqualand, 1.5km S of Vioolsdrif, Kosies river bed	1.b.1
J.merxmuelleri (Roessler)Hilliard #	TV 866	Namaqualand, 9km S of Alexander Bay, roadside	1.a.3
J.microphylla (L.f.)Hilliard	NB1453	Eastern Cape, Sundays river mouth	1.a.3
J.pallida (Pilger)Hilliard	TV 843	S.Namibia, S of Solitaire, granite koppie S.	2.1
J.pedunculosa (Benth.)Hilliard	TV 871	Namaqualand, Goegap nature reserve	2.4
J.primuliflora (Thellung)Hilliard #	TV 830	S.Namibia, Fish River bed near Seeheim	1.b.2
J.pristisepala (Hiern)Hilliard	TV 1029	Natal Drakensberg, Garden Castle	2.3
J.pristisepala (Hiern)Hilliard #	KB 5	Kirstenbosch Garden (source locality unknown)	2.3
J.racemosa (Benth.)Hilliard	TV 878	Namaqualand, Grootvleipas, granite koppie	1.b.2
J.ramosissima (Hiern)Hilliard	TV 808	Northern Cape, Groot Pella, gorge	2.4
J.sessilifolia (Diels)Hilliard #	TV 854	S.Namibia, 109km S of junction w. tar on road to Rosh Pinah from Aus, koppie	1.b.1
J.stellata Hilliard #	MH 38	Western Cape, Cape Point, N of Buffels Bay	1.a.3
J.stellata Hilliard	ADH1702	Bohnen reserve, Stilbaai, Southern Cape	1.a.3
J.tenuifolia (Bernh.)Hilliard	ADH1714	S.Cape, Great Brak	1.a.3
J.tenuifolia (Bernh.)Hilliard	TV 915	S.Cape, Sedgefield, SW shore of Swartvlei. Stabilised dune behind pub	1.a.3
J.thunbergii (G.Don)Hilliard #	TV 882	Namaqualand, 25km E of van Rhynsdorp	1.b.2
J.tortuosa (Benth.)Hilliard #	TV 785	Western Cape, Prince Albert, lower Swartberg Pass	1.a.3
J.tysonii (Hiern)Hilliard #	DGE	Northern Cape, Kimberley area	1.a.3
Lyperia tristis (L.f.) Benth.	TV 875	Namaqualand, 25km N of Kamieskroon, roadside in rocks	
Lyperia violacea (Link exJaroscz) B	MH 32	S.Cape, Ezeljachtspoort, George area	
Manulea adenocalyx Hilliard	TV 800	Namaqualand, N7, 10K north of Klawer	
Manulea schaeferi Pilg.	TV 822	S.Namibia, Holoog berg, dry riverbed	
Oftia africana (L.) Bocq.	MH 40	Western Cape, Cape Town, Rhodes Mem contour path	
Scrophularia arguta (Soland)*	AJ431061	GENBANK	
*Scrophularia peregrina L.**	AJ609139	GENBANK	
Sutera hispida (Thunb.) Druce	MH 37	Western Cape, Cape Point, N of Buffels Bay	
Sutera subsessilis Hilliard	MH 33	Western Cape, Kouebokkeveld, Tandfontein farm	
Teedia pubescens Burch.	MH 34	Western Cape, Kouebokkeveld, Tandfontein farm	
*Verbascum arctura L.**	AJ609128	GENBANK	

Table 4. List of morphological characters, with state definitions.

Char. No.	Character description and state definition
1	Petals bifid or retuse = 1, petals rounded = 0 Although the impression in the field was that flowers were of two distinct types, later examination of voucher material showed some species to be polymorphic.
2	Leaves ovate = 1, obovate = 0 This was very distinctive, some species have "normal" looking leaves, others are cuneate with the widest part distal. In *J.microphylla*, the leaves were too small to judge the shape, in *J.adpressa*, they were too dissected. These are recorded as polymorphic.
3	Leaves petiolate = 1, not petiolate = 0
4	Leaves with three main veins from the base = 1, venation either a single midrib or not clear due to thick texture = 0
5	Leaves mesophytic = 1, leaves xerophytic= 0 The xerophytic leaves are generally smaller (often< 3 mm), but they also have a thick texture, the venation is not visible, and they never have glandular hairs. They may be covered in glistening glands, often having a "varnished" appearance. They are also fascicled.
6	Glandular hairs present on the stem = 1, no glandular hairs on stem = 0
7	Glistening glands present on the stem = 1, no glistening glands on the stem = 0
8	Glandular hairs present on the dorsal leaf surface = 1, glandular hairs absent from dorsal leaf surface = 0
9	Glistening glands present on the dorsal leaf surface = 1, not present = 0
10	Glandular hairs present on the lower leaf surface = 1, not present = 0
11	Glistening glands present on the lower leaf surface = 1, not present = 0
12	Glandular hairs present on the calyx = 1, not present = 0
13	Glistening glands present on the calyx = 1, not present = 0
14	Glandular hairs present on the capsule = 1, not present = 0
15	Glistening glands present all over the capsule = 2, glistening glands present only on the sutures of the capsule = 1, no glistening glands on the capsule = 0
16	Extraordinarily stout hairs present = 1, not present = 0

Table 5. Variables and states used in life history and ecology optimizations, as applied in Appendix 3 and illustrated in Figures 12, 13, and 14.

Variable	State	State description
Rainfall	1	0 – 300 mm
	2	300 – 600 mm
	3	600 + mm
Rainy season	1	Summer
	2	Winter
	3	All year round
Preferred soil	1	Granite
	2	Limestone
	3	Sand
	4	Shale
	5	Basalt, Dolerite
Life History	0	Perennial
	1	Annual

Species	Hilliard group	Distribution	Flowering records	Habit
J. aridicola Hilliard	1.b.2	Nam CP	Mar-Oct	Annual herb
J. megadenia Hilliard	1.b.2	Nam CP	Apr-Jun	Annual herb
J. glutinosa (Benth.) Hilliard	1.b.2	Nam CP	Apr-Dec	Annual herb
J. primuliflora (Thell.) Hilliard	1.b.2	Nam	All year	Annual or perennial herb
J. fimbriata Hilliard	1.b.2	Nam	May	Annual or perennial herb
J. acutiloba (Pilger) Hilliard	2.1	Nam	Apr-Jul	Shrublet
J. dolomitica Hilliard	2.1	Nam	All year	Subshrub
J. heucherifolia (Diels) Hilliard	2.1	Ang Nam	All year	Dwarf shrublet
J. pallida (Pilger) Hilliard	2.1	Nam	Dec-Jul	Dwarf shrublet
J. fleckii (Thell.) Hilliard	2.1	Nam	All year	Perennial herb
J. lyperioides (Engl.) Hilliard	2.1	Nam	All year	Subshrub
J. pilgeriana (Dinter) Hilliard	2.2	Nam	All year	Dwarf shrublet
J. barbata Hilliard	2.2	Nam	All year	Dwarf shrublet
J. chenopodioides Hilliard	2.2	Nam	All year	Perennial herb
J. canescens (Benth.) Hilliard	2.2	Nam CP	All year	Subshrub / perennial herb
J. crassicaulis (Benth.) Hilliard	2.3	CP	Nov-Mar	Dwarf shrublet
J. stricta (Benth.) Hilliard	2.3	Tvl OFS Les CP	Nov-Apr	Dwarf shrublet
J. pristisepala (Hiern) Hilliard	2.3	OFS Les CP	Nov-Apr	Dwarf shrublet
J. lesutica Hilliard	2.3	Les	Dec-Mar	Dwarf shrublet
J. silenoides (Hilliard) Hilliard	2.3	Tvl	Oct-Feb	Herbaceous perennial
J. beverlyana (Hilliard & Burtt) Hilliard	2.3	Les	Feb	Perennial herb
J. ramosissima (Hiern) Hilliard	2.4	Nam CP	Mar-Oct	Shrublet
J. hereroensis (Engl.) Hilliard	2.4	Nam	Mar-May	Annual herb
J. tenella (Hiern) Hilliard	2.4	Nam CP	Mar-Sep	Annual herb
J. fragilis (Pilger) Hilliard	2.4	Nam	Dec-Jul	Annual herb
J. pedunculosa (Benth.) Hilliard	2.4	CP	May-Dec	Annual herb
J. breviflora (Schltr.) Hilliard	2.5	Nat CP Les	Oct-Mar	Perennial herb
J. jurassica (Hilliard & Burtt) Hilliard	2.5	Les	Jan-Apr	Perennial herb
J. aspleniifolia Hilliard	2.5	CP Les	Oct-Mar	Shrublet
J. multisecta Hilliard	2.5	CP	Nov-Mar	Perennial herb
J. aurantiaca (Burchell) Hilliard	2.5	Nam Bot Tvl OFS Nat Les CP	Oct-Mar	Perennial herb
J. montana (Diels) Hilliard	2.5	Zim Tvl Nat	Sep-Feb	Perennial herb
J. concinna (Hiern) Hilliard	2.5	Bot Nam	Jan-Sep	Annual herb
J. micrantha (Klotsch) Hilliard	2.5	Mal Zam Zim Bot Tvl Swa Moz Nat	Jun-Nov	Perennial herb
J. adpressa (Dinter) Hilliard	2.5	Nam CP	May-Sep	Annual herb
J. myriantha Hilliard	2.5	Zim	Jul-Sep	Annual herb
J. dissecta (O.Kuntze)	2.5	Egypt Sudan India	Dec-May	Annual herb
J. bergae P.Lemmer	Not applicable	Tvl	Jan	Perennial shrublet

Table 3. List of species sampled, together with voucher details (TV=G.A.Verboom, MH=M.Herron, NB=Nicola Bergh, ADH=A.D. Harrower, KB collected at Kirstenbosch) or GenBank sequence accession numbers. All localities are in South Africa, unless labelled Namibia. All accessions were sampled for both *rps* 16 and *psb* A-*trn* H except those marked *, for which only *rps* 16 sequences were obtained from GenBank. Only accessions marked # amplified GScp.

Species	Voucher	Collection locality	Hilliard's Group No.
Colpias mollis E.Mey ex Benth.	TV 873	Namaqualand,Goegap nature reserve	
Diascia longicornis (Thunb.) Druce	TV 888	Namaqualand, van Rhyns pass, shale cutting	
Hemimeris racemosa (Houtt.) Merr.	TV 803	Namaqualand, N7, 10K north of Klawer	
J. albomarginata Hilliard #	MH 36	Western Cape, E. of Pearly Beach, on vegetated dunes	1.a.3
J.adpressa (Dinter)Hilliard #	TV 829	S.Namibia, Fish River bed near Seeheim	2.5
J.amplexicaulis (Benth.)Hilliard #	TV 870	Namaqualand, between O'okiep & Carolusberg, dry river bed	1.b.1
J.aridicola Hilliard #	TV 806	Northern Cape, Aggeneys farm	1.b.2
J.aspalathoides (Benth.)Hilliard #	ADH1695	Southern Cape, Kamannassi mountains	1.a.3
J.aspalathoides (Benth.)Hilliard	TV 906	S.Cape, 5km W of Malgas on road to Swellendam	1.a.3
J.atropurpurea (Benth.)Hilliard #	ADH1151	Kirstenbosch Garden (source locality unknown)	1.a.2
J.barbata Hilliard #	TV 831	S.Namibia, 6km N of Bethanie, dry river bed	2.2
*J.bicolor (*Dinter)Hilliard	TV 856	S.Namibia, few km N of Witputz, dry rivulet	1.b.1
J.breviflora (Schltr.)Hilliard	TV 776	Natal Drakensberg, Cathedral Peak	2.5
J.calciphila Hilliard #	ADH1679	Rein's Nature Reserve near Mossel Bay	1.a.3
J.calciphila Hilliard	TV 910	S.Cape, Still Bay	1.a.3
J.canescens (Benth.)Hilliard	TV 817	S.Namibia, Ai-Ais, Riverbed N of campsite	2.2
J.canescens (Benth.)Hilliard #	TV 818	S.Namibia, Ai-Ais, Riverbed N of campsite	2.2
J.filicaulis (Benth.)Hilliard	KB449	Kirstenbosch Garden (source locality unknown)	1.a.3
J.fimbriata Hilliard #	TV 847	S.Namibia, Koppie, banded ironstone. S of Sossusvlei Mountain Lodge	1.b.2
J.fleckii (Thell.)Hilliard #	TV 835	S.Namibia, Gaub pass, betw Solitaire & Kuiseb, E of road	2.1
J.foliolosa (Benth.)Hilliard #	ADH 552	Southern Cape, Kamannassi mountains	1.a.3
*J.fruticosa (*Benth.)Hilliard	TV 864	Namaqualand, 12 km N of Steinkopf, granite hillside, not high	1.b.1
*J.fruticosa (*Benth.)Hilliard	ADH 676	Kirstenbosch Garden (source locality unknown)	1.b.1
J.glutinosa (Benth.)Hilliard	TV 814	S,Namibia, 5km E of Ai-ais,roadside	1.b.2
J.grandiflora (Galpin)Hilliard	TV 1048	Mpumalanga, Nelsberg pass, W. of Barberton - shale	1.a.1
J.grandiflora (Galpin)Hilliard #	ADH1155	N. Transvaal, Sekukhuniland	1.a.1
J.huillana (Diels)Hilliard #	TV 825	S.Namibia, E of N7 on road to Karasberg	1.a.2
J.incisa (Thunb.)Hilliard #	TV 885	Northern Cape, 55km from Calvinia on rd to Sutherland, dolerite koppie	1.a.3
J.integerrima (Benth.)Hilliard #	TV 851	S. Namibia, Klein Aus, granite koppie	1.a.3
J.jurassica (Hilliard&Burtt)Hilliard	KB 3	Kirstenbosch Garden (source locality unknown)	2.5
*J.kraussiana (*Bernh.)Hilliard #	ADH/W126	Eastern Cape, Kei Valley	1.a.3
*J.lyperioides (*Engl.)Hilliard	TV 842	S.Namibia, Gamsberg pass, roadside cutting	2.1
J.major (Pilger)Hilliard #	TV 815	S.Namibia, 5km E of Ai-ais,roadside	1.b.1

Table 6. Statistics associated with datasets used in Parsimony analyses and of the trees obtained from those analyses.

Dataset	No of characters	No of parsimony informative characters	No of most parsimonious trees obtained	No of nodes in strict consensus tree	No of nodes in consensus with bootstrap > 80%	Tree length	Consistency index	Retention index
*rps*16 (64 taxa)	777	117	218	20	15	295	0.74	0.92
*psb*A-*trn*H (64 taxa)	437	63	97,260	14	9	143	0.79	0.96
GScp (28 taxa, marked # in Table 3)	585	61	18	18	7	185	0.78	0.92
Morphology (50 taxa, no outgroups)	16	16	4,952	10	1	46	0.37	0.87
*rps*16 & *psb*A –*trn*H (28 GScp taxa only)	1,214	66	2	13	6	100	0.96	0.99
*rps*16, *psb*A –*trn*H & GScp (62 taxa excl. *J.pristisepala*)	1,799	234	32,790	29	17	597	0.77	0.94
Plastid, nuclear & morphology (62 taxa)	1,815	250	9,310	36	16	679	0.66	0.90

Table 7. Dates (mean ± std. dev.) of divergence times (mya) in *Jamesbrittenia,* calculated using two methods (NPRS and molecular clock with some long branch outgroups removed), and two calibration dates, the maximum and minimum inferred age for the split of *Verbascum* and *Scrophularia* (Wikström et al 2001). Node numbers as indicated in Figure 10.

Node no.	NPRS		Clock	
	31 mya	25 mya	31 mya	25 mya
1.	Not calculated	Not calculated	30.2 ± 0.2	24.3 ± 0.7
2.	27.4 ± 1.3	22.1 ± 1.1	25.2 ± 2.6	20.3 ± 2.1
3.	23.9 ± 1.5	19.3 ± 1.2	19.2 ± 2.9	15.5 ± 2.3
4.	7.0 ± 2.1	5.7 ± 1.7	5.8 ± 1.9	4.7 ± 1.5
5.	20.3 ± 2.1	16.3 ± 1.7	11.5 ± 3.3	9.3 ± 2.6
6.	6.3 ± 4.5	5.1 ± 3.7	1.2 ± 0.9	1.0 ± 0.7
7.	9.9 ± 3.0	8.0 ± 2.4	3.2 ± 1.1	2.6 ± 0.9
8.	5.5 ± 2.9	4.5 ± 2.4	0.7 ± 0.4	0.6 ± 0.4

1. Split of *Jamesbrittenia* ancestor from rest of Scrophulariaceae
2. Split of *J.ramosissima*
3. Split of clade A
4. Diversification of clade A
5. Split between clades B & C
6. Diversification of clade B
7. Diversification of clade C
8. Diversification of clade D

Table 8. Ancestral ranges in *Jamesbrittenia* as inferred using DIVA. Results show a variety of options for some nodes; no significance is attached to the sequence in which the results are reported. The union of all alternative reconstructions at each node is indicated in Figure 11. Area codes: A=Namibia, B=Namaqualand, C=N.Cape, D= central Karoo, E=Klein Karoo, F=SW & S.Cape, G=Port Elizabeth & Transkei, H= eastern South Africa.

Nodes defined by the common ancestor		Ancestral areas
J. aspalathoides	*J. atropurpurea*	E, CF, DF, CDF, EF, CEF, DEF, CDEF, CFG, DFG, CDFG, EFG, CEFG, DEFG, CDEFG
J. tenuifolia	*J. calciphila*	F
J. tenuifolia	*J. atropurpurea*	E, EF
J. huillana	*J. atropurpurea*	AF, AEF, FH, AFH, EFH, AEFH
J. albomarginata	*J. stellata*	F
J. albomarginata	*J. atropurpurea*	F
J. merxmuelleri	*J. atropurpurea*	BF
J. breviflora	*J. jurassica*	H
J. breviflora	*J. atropurpurea*	BFH
J. tysonii	*J. filicaulis*	C
J. incisa	*J. filicaulis*	C
J. tortuosa	*J. filicaulis*	CDE
J. tortuosa	*J. atropurpurea*	BDEF, BCDEF, BDEFH, BCDEFH
J. microphylla	*J. foliolosa*	G
J. kraussiana	*J. foliolosa*	G
J. kraussiana	*J. filicaulis*	BDEFG, BCDEFG, BDEFGH
J. adpressa	*J. grandiflora*	AH, CH, ACH
J. adpressa	*J. foliolosa*	BDEFGH, ABDEFGH, BCDEFGH, ABCDEFGH
J. canescens	*J. barbata*	A
J. fleckii	*J. barbata*	A
J. fleckii	*J. grandiflora*	BDEFGH, ABDEFGH, BCDEFGH, ABCDEFGH
J. integerrima	*J. barbata*	BCDEFGH, ABCDEFGH
J. pallida	*J. lyperioides*	A
J. pallida	*J. integerrima*	BCDEFGH, ABCDEFGH
J. glutinosa	*J. megadenia*	A
J. primuliflora	*J. fimbriata*	A
J. primuliflora	*J. megadenia*	A
J. primuliflora	*J. lyperioides*	ABCDEFGH
J. maxii	*J. fruticosa*	A
J. sessilifolia	*J. fruticosa*	A
J. megaphylla	*J. fruticosa*	A
J. major	*J. fruticosa*	A
J. amplexicaulis	*J. fruticosa*	AB
J. bicolor	*J. fruticosa*	A, AB
J. racemosa	*J. pedunculosa*	B
J. thunbergii	*J. pedunculosa*	B, BD, BCD
J. aridicola	*J. pedunculosa*	ABC, ABCD

Appendix 1. Distribution of morphological character states (as defined in Table 4).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
808	*J.ramosissima*	0	1	1	0	1	1	1	1	1	1	1	1	1	0	2	0
835	*J.fleckii*	01	1	1	0	1	1	0	1	1	1	1	1	1	0	2	0
842	*J.lyperioides*	01	1	1	0	1	1	0	1	0	1	1	1	0	1	0	0
843	*J.pallida*	01	1	1	0	1	1	0	1	0	1	1	1	1	0	0	0
851	*J.integerrima*	0	1	1	0	1	0	1	1	1	0	1	1	1	0	2	0
830	*J.primuliflora*	0	1	1	0	1	1	0	1	1	1	1	1	1	1	0	1
847	*J.fimbriata*	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0
814	*J.glutinosa*	01	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1
823	*J.megadenia*	1	1	01	0	1	1	0	1	0	1	1	1	0	1	0	1
806	*J.aridicola*	1	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1
854	*J.sessilifolia*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
805	*J.maxii*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
815	*J.major*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
856	*J.bicolor*	0	1	0	0	1	1	0	1	0	1	0	1	0	0	0	0
859	*J.megaphylla*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
864	*J.fruticosa*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
AH676	*J.fruticosa*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
871	*J.pedunculosa*	0	1	1	0	1	1	0	1	1	1	1	1	0	0	1	1
878	*J.racemosa*	1	1	01	0	1	1	0	1	1	1	1	1	0	1	1	1
882	*J.thunbergii*	1	1	1	0	1	1	0	1	0	1	1	1	0	0	0	0
870	*J.amplexicaulis*	0	1	0	1	1	1	0	1	0	1	0	1	0	0	0	0
NB1453	*J.microphylla*	01	01	0	0	0	1	1	0	0	0	1	0	1	0	1	0
AH552	*J.foliolosa*	01	0	1	0	0	1	1	0	0	0	1	1	1	0	1	0
W126	*J.kraussiana*	01	1	1	0	1	1	1	1	0	0	1	1	1	0	2	0
829	*J.adpressa*	0	01	1	0	1	0	0	0	1	0	1	0	1	0	1	0
1048	*J.grandiflora*	01	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0
AH1155	*J.grandiflora*	01	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0
AH1695	*J.aspalathoides*	1	0	0	0	0	0	1	0	0	0	1	0	1	0	2	0
906	*J.aspalathoides*	1	0	0	0	0	0	1	0	0	0	1	0	1	0	2	0
825	*J.huillana*	0	0	0	0	0	1	1	0	1	0	1	0	1	0	2	0
AH1151	*J.atropurpurea*	0	0	0	0	0	0	1	0	0	0	1	0	1	0	2	0
866	*J.merxmuelleri*	0	0	0	0	0	1	0	0	1	0	1	0	1	0	2	0
AH1679	*J.calciphila*	1	0	0	0	0	1	1	0	1	0	1	0	1	0	2	0
910	*J.calciphila*	1	0	0	0	0	1	1	0	1	0	1	0	1	0	2	0
AH1702	*J.stellata*	01	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0
M38	*J.stellata*	01	0	0	0	0	1	1	0	0	0	1	0	1	0	0	0
AH1714	*J.tenuifolia*	01	0	0	0	0	1	1	0	0	0	1	0	1	0	2	0
915	*J.tenuifolia*	01	0	0	0	0	1	1	0	0	0	1	0	1	0	2	0
885	*J.incisa*	0	1	01	0	1	1	0	1	0	1	1	1	0	0	0	0
DGE	*J.tysonii*	0	1	1	0	1	1	0	1	1	1	1	1	1	0	1	0
K449	*J.filicaulis*	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0
TVPA2	*J.tortuosa*	0	0	0	0	0	1	0	1	1	1	1	1	1	0	1	0
KB3	*J.jurassica*	0	1	0	0	1	1	1	1	1	1	1	0	1	0	0	0
M36	*J.albomarginata*	0	0	0	0	0	1	1	0	0	0	1	0	1	0	2	0
817	*J.canescens-maroon*	0	0	0	0	1	1	1	0	1	0	1	1	1	0	2	0
818	*J.canescens-yellow*	0	0	0	0	1	1	1	0	1	0	1	1	1	0	2	0
831	*J.barbata*	0	1	0	0	1	0	1	0	1	0	1	0	1	0	2	0
1029	*J.pristisepala*	01	1	0	0	1	1	1	1	1	1	1	1	1	0	2	0
KB5	*J.pristisepala*	01	1	0	0	1	1	1	1	1	1	1	1	1	0	2	0
TVBre	*J.breviflora*	0	1	1	0	1	1	0	1	0	1	1	1	1	0	2	0

Appendix 2: Distribution of Jamesbrittenia species used for DIVA analysis. Areas are shown on the map in Figure 2.

AREAS	A	B	C	D	E	F	G	H
J.ramosissima	1	0	1	0	0	0	0	0
J.fleckii	1	0	0	0	0	0	0	0
J.lyperioides	1	0	0	0	0	0	0	0
J.pallida	1	0	0	0	0	0	0	0
J.integerrima	1	0	1	0	0	0	0	0
J.primuliflora	1	0	0	0	0	0	0	0
J.fimbriata	1	0	0	0	0	0	0	0
J.glutinosa	1	0	0	0	0	0	0	0
J.megadenia	1	0	0	0	0	0	0	0
J.aridicola	1	0	1	0	0	0	0	0
J.sessilifolia	1	0	0	0	0	0	0	0
J.maxii	1	0	1	0	0	0	0	0
J.major	1	0	0	0	0	0	0	0
J.bicolor	1	0	0	0	0	0	0	0
J.megaphylla	1	0	0	0	0	0	0	0
J.fruticosa	1	1	0	0	0	0	0	0
J.pedunculosa	0	1	0	0	0	0	0	0
J.racemosa	0	1	0	0	0	0	0	0
J.thunbergii	0	1	1	1	0	0	0	0
J.amplexicaulis	1	0	0	0	0	0	0	0
J.microphylla	0	0	0	0	0	1	1	0
J.foliolosa	0	0	0	1	0	1	1	0
J.kraussiana	0	0	0	0	0	0	1	1
J.adpressa	1	0	1	0	0	0	0	0
J.grandiflora	0	0	0	0	0	0	0	1
J.aspalathoides	0	0	0	0	1	1	1	0
J.huillana	1	0	0	0	0	0	0	1
J.atropurpurea	0	0	1	1	1	0	0	0
J.merxmuelleri	0	1	0	0	0	0	0	0
J.calciphila	0	0	0	0	0	1	0	0
J.stellata	0	0	0	0	0	1	0	0
J.tenuifolia	0	0	0	0	1	1	1	0
J.incisa	0	0	1	0	0	0	0	0
J.tysonii	0	0	1	1	0	0	0	0
J.filicaulis	0	0	1	1	0	0	0	0
J.tortuosa	0	0	0	1	1	0	0	0
J.jurassica	0	0	0	0	0	0	0	1
J.albomarginata	0	0	0	0	0	1	0	0
J.canescens	1	0	1	0	0	0	0	0
J.barbata	1	0	0	0	0	0	0	0
J.breviflora	0	0	0	0	0	0	0	1

Appendix 3: Matrix for optimization of variables and ecological conditions as defined in Table 5.

	Rainfall volume	Rainfall season	Life history	Soil
J.ramosissima	1	2	0	1
J.fleckii	1	2	0	13
J.lyperioides	1	2	0	12345
J.pallida	1	2	0	1
J.integerrima	1	2	0	1345
J.primuliflora	1	2	1	13
J.fimbriata	1	2	1	1
J.glutinosa	1	2	1	13
J.megadenia	1	2	1	1
J.aridicola	1	2	1	134
J.sessilifolia	1	2	0	13
J.maxii	12	2	0	134
J.major	1	2	0	13
J.bicolor	1	2	0	2
J.megaphylla	1	2	1	23
J.fruticosa	1	2	0	1
J.pedunculosa	1	2	1	1
J.racemosa	1	2	1	1
J.thunbergii	1	2	1	4
J.amplexicaulis	1	2	0	13
J.microphylla	2	3	0	3
J.foliolosa	2	3	0	34
J.kraussiana	23	3	0	34
J.adpressa	1	2	1	3
J.grandiflora	3	1	0	45
J.aspalathoides	2	3	0	24
J.huillana	123	12	0	12345
J.atropurpurea	12	2	0	4
J.merxmuelleri	1	2	0	23
J.calciphila	2	3	0	2
J.stellata	2	23	0	2
J.tenuifolia	2	3	0	23
J.incisa	1	2	0	45
J.tysonii	1	1	0	45
J.filicaulis	23	1	0	45
J.tortuosa	1	2	0	4
J.jurassica	3	1	0	5
J.albomarginata	2	2	0	2
J.canescens	1	2	0	34
J.barbata	1	2	0	3
J.breviflora	3	1	0	5

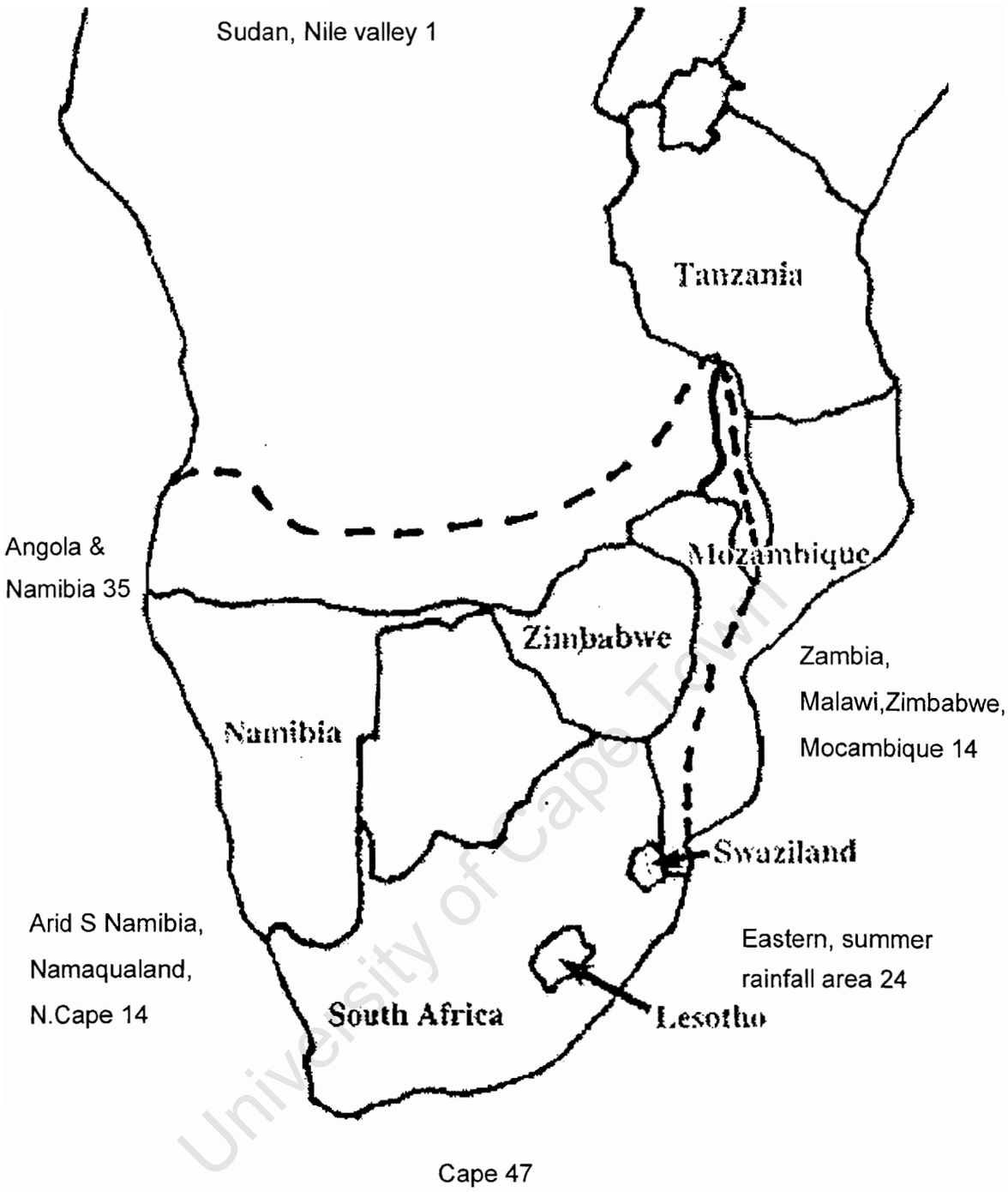

Figure 1. Distribution of *Jamesbrittenia.* All except one of the 84 species occur in southern Africa.

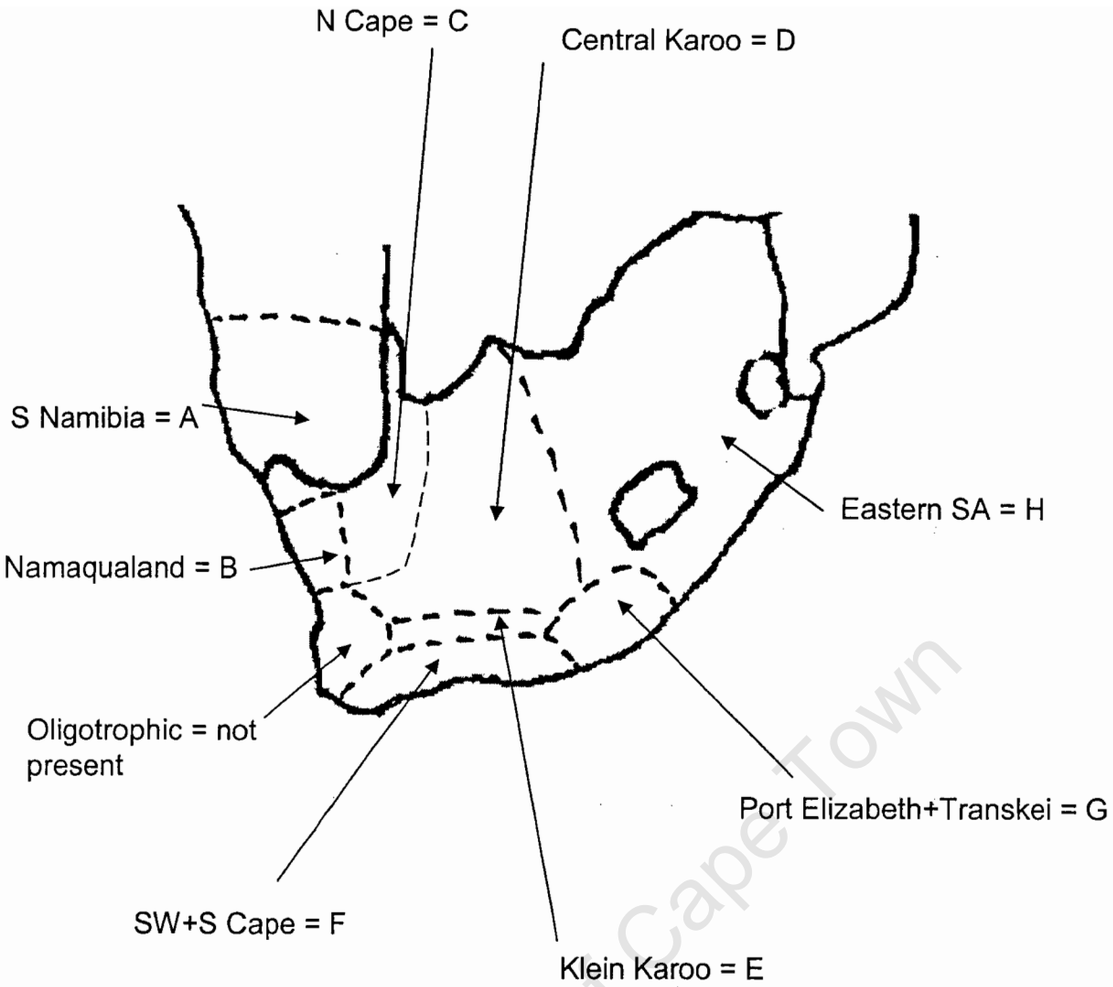

Figure 2. Map of southern Africa showing the areas used in DIVA analysis.

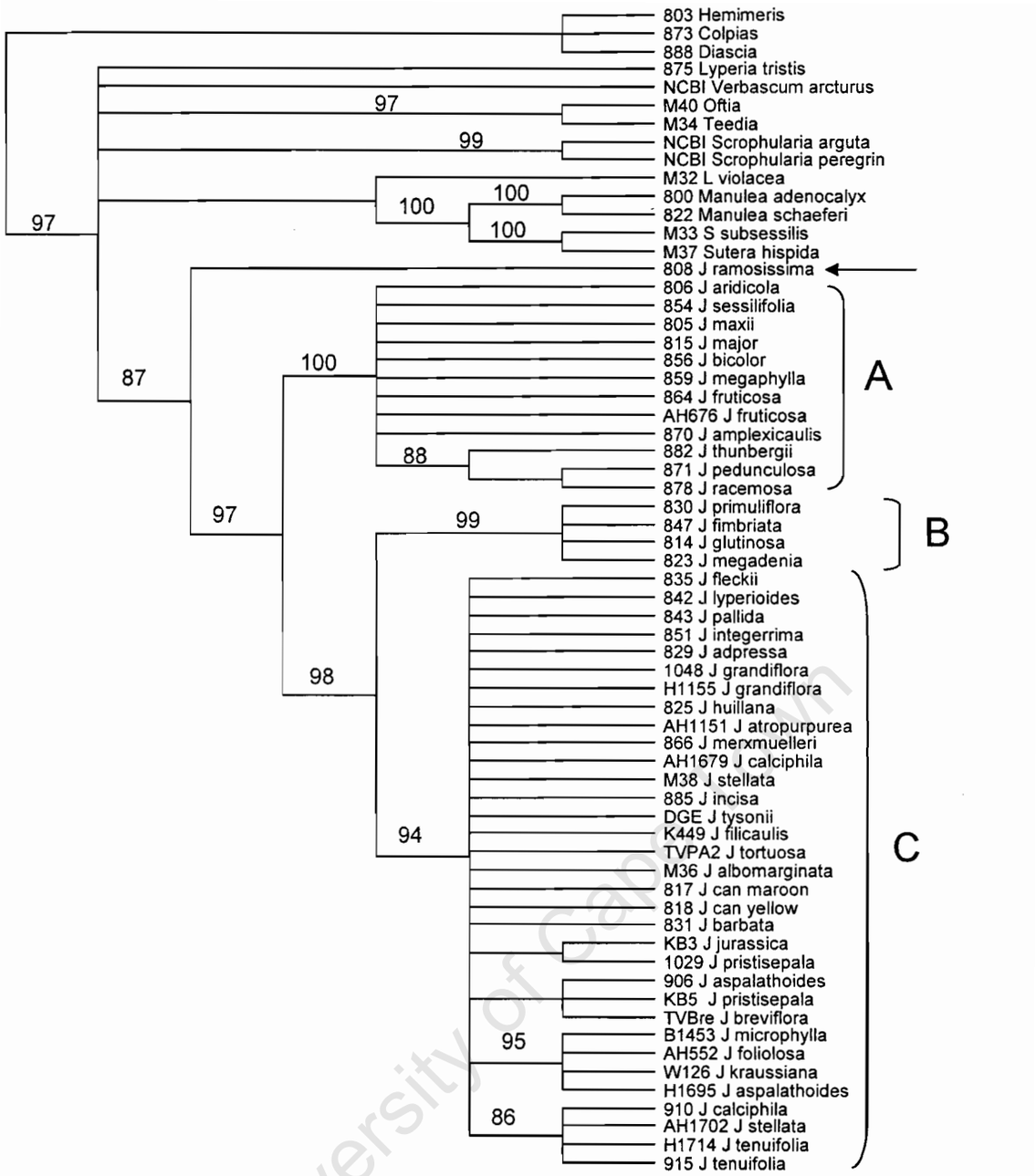

Figure 3. Strict consensus of 218 trees produced by parsimony analysis of the *rps*16 dataset. Numbers above nodes indicate bootstrap support >80%. Marked clades are referred to in the text. Each accession is indicated by a species name and voucher number (M=Margaret Herron, AH,H=Adam Harrower, TV or number only=Verboom, K,KB,W=Kirstenbosch, B=N.Bergh).

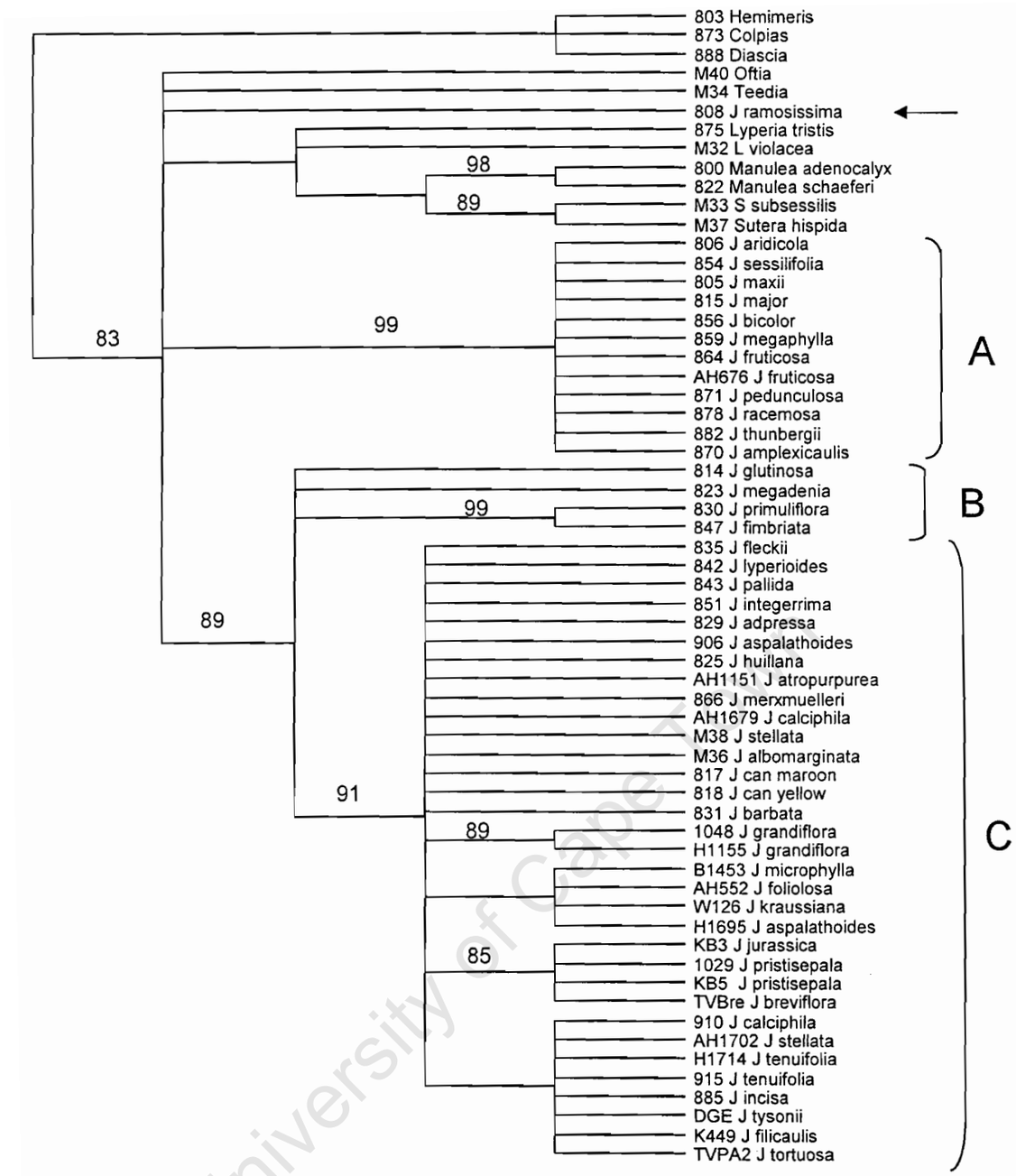

Figure 4. Strict consensus of 97,260 trees produced by parsimony analysis of the *psb*A-*trn*H dataset. Numbers above nodes indicate bootstrap support >80%. Marked clades are referred to in the text. Each accession is indicated by a voucher number and species name.

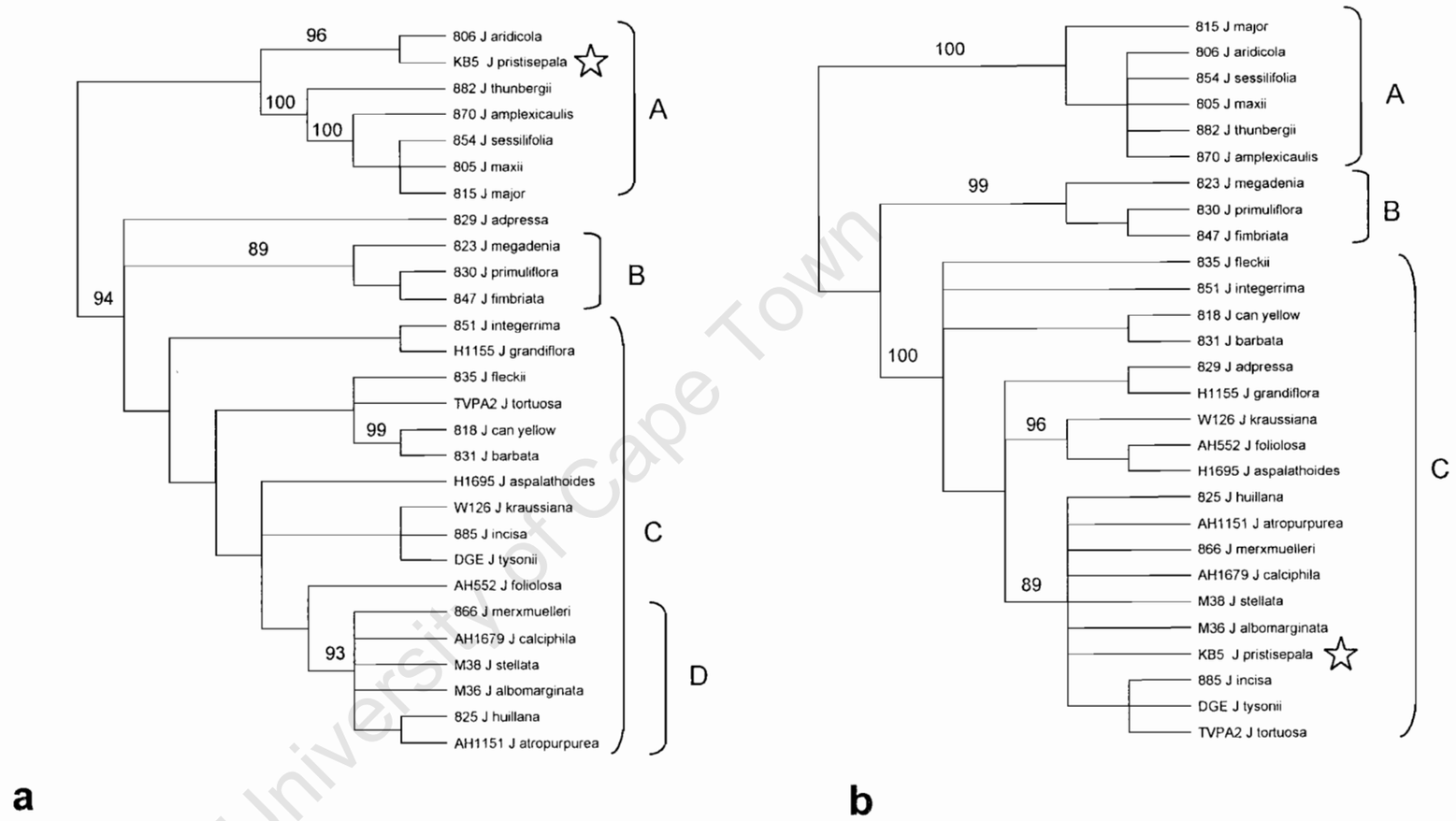

Figure 5. **a.** Strict consensus of 18 most parsimonious trees retrieved with GScp data. **b**. Strict consensus of the two trees obtained with combined plastid data, containing only those taxa for which GScp sequences are available. *J.pristisepala* is marked with a star. Bootstrap support >85% is shown. Numbered clades are referred to in the text.

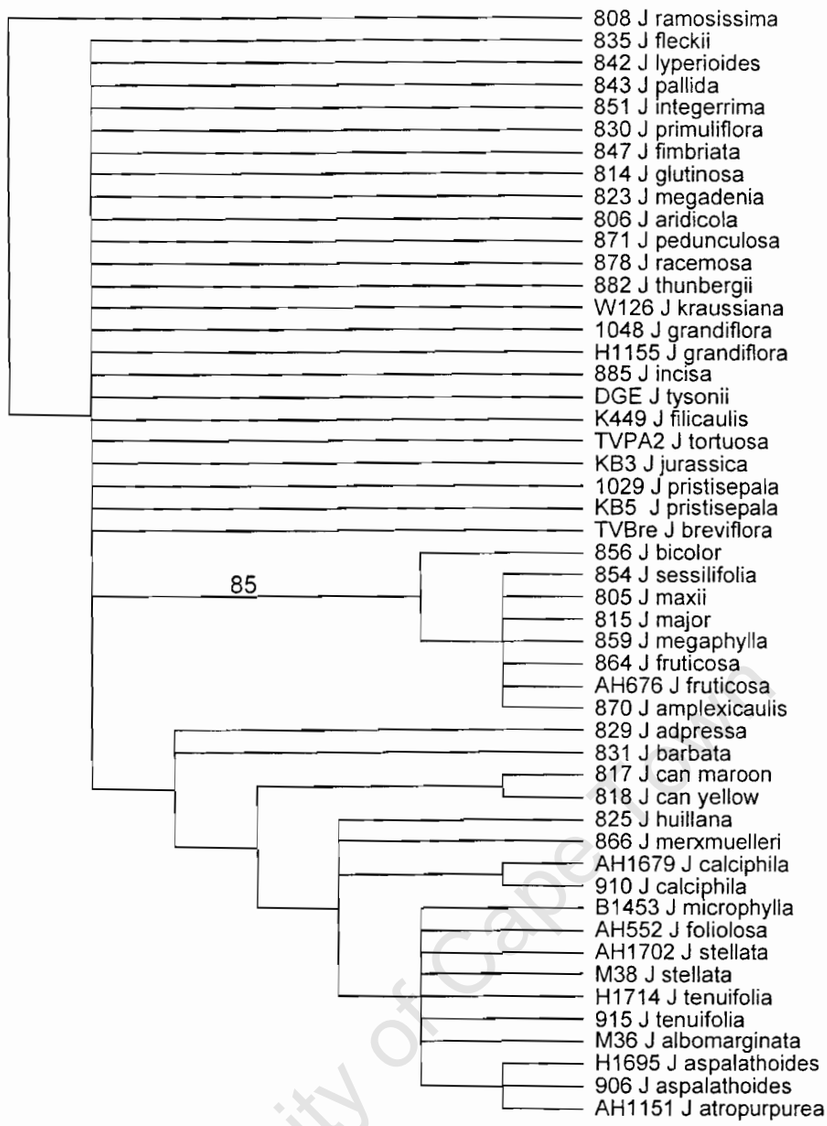

Figure 6. Strict consensus of 4,952 trees produced by parsimony analysis of the morphological dataset. Numbers above the nodes indicate bootstrap support >80%. Each accession is indicated by a voucher number and species name.

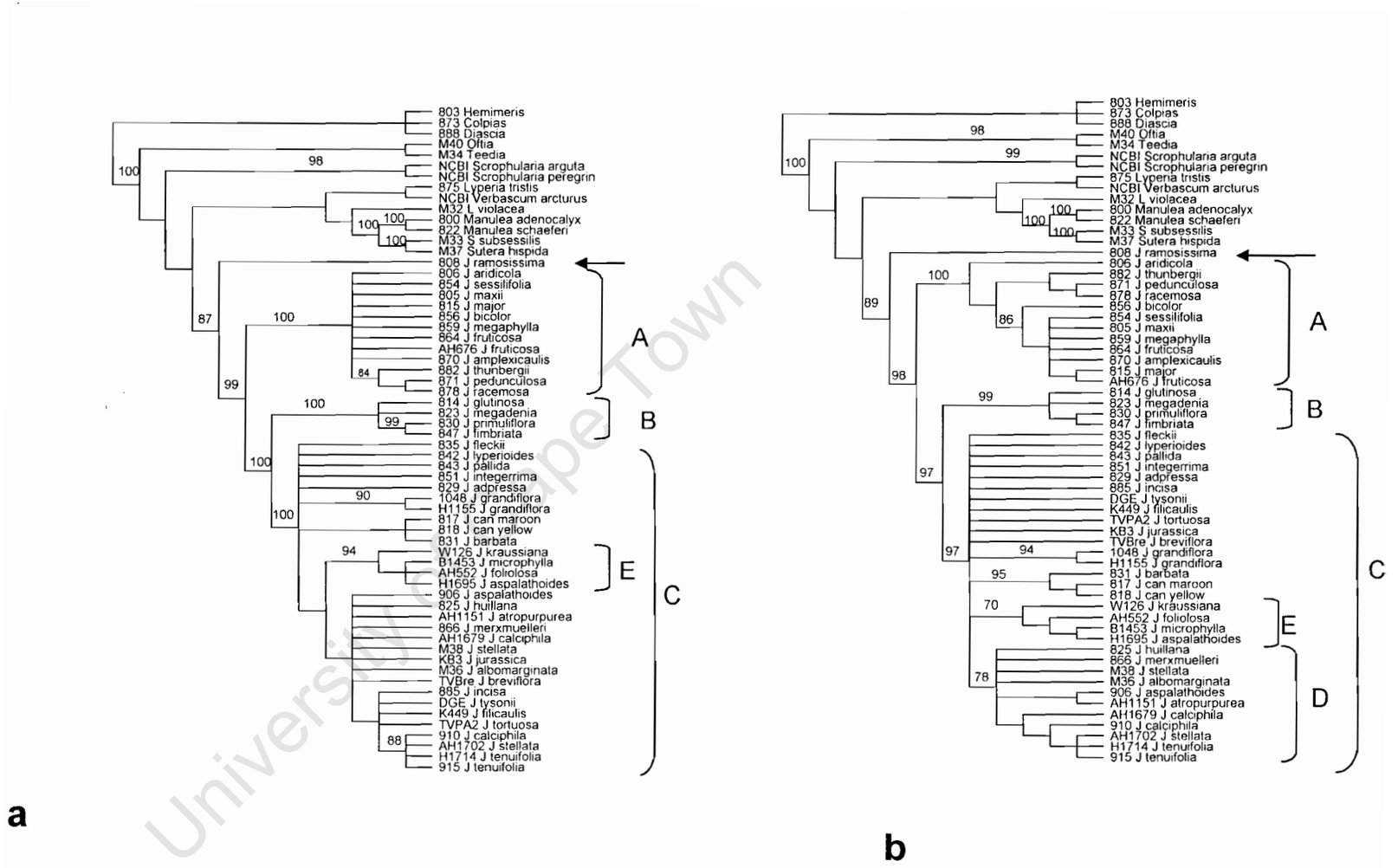

Figure 7. Strict consensus topologies obtained from analyses based on **(a)** the combined plastid and nuclear markers (32,790 trees) and **(b)** the morphological and molecular data (9,310 trees). Marked clades are referred to in the text. Note also that *J. pristisepala* is excluded and that numbers above the nodes reflect bootstrap support >80% (support for clades D and E referred to in the text).

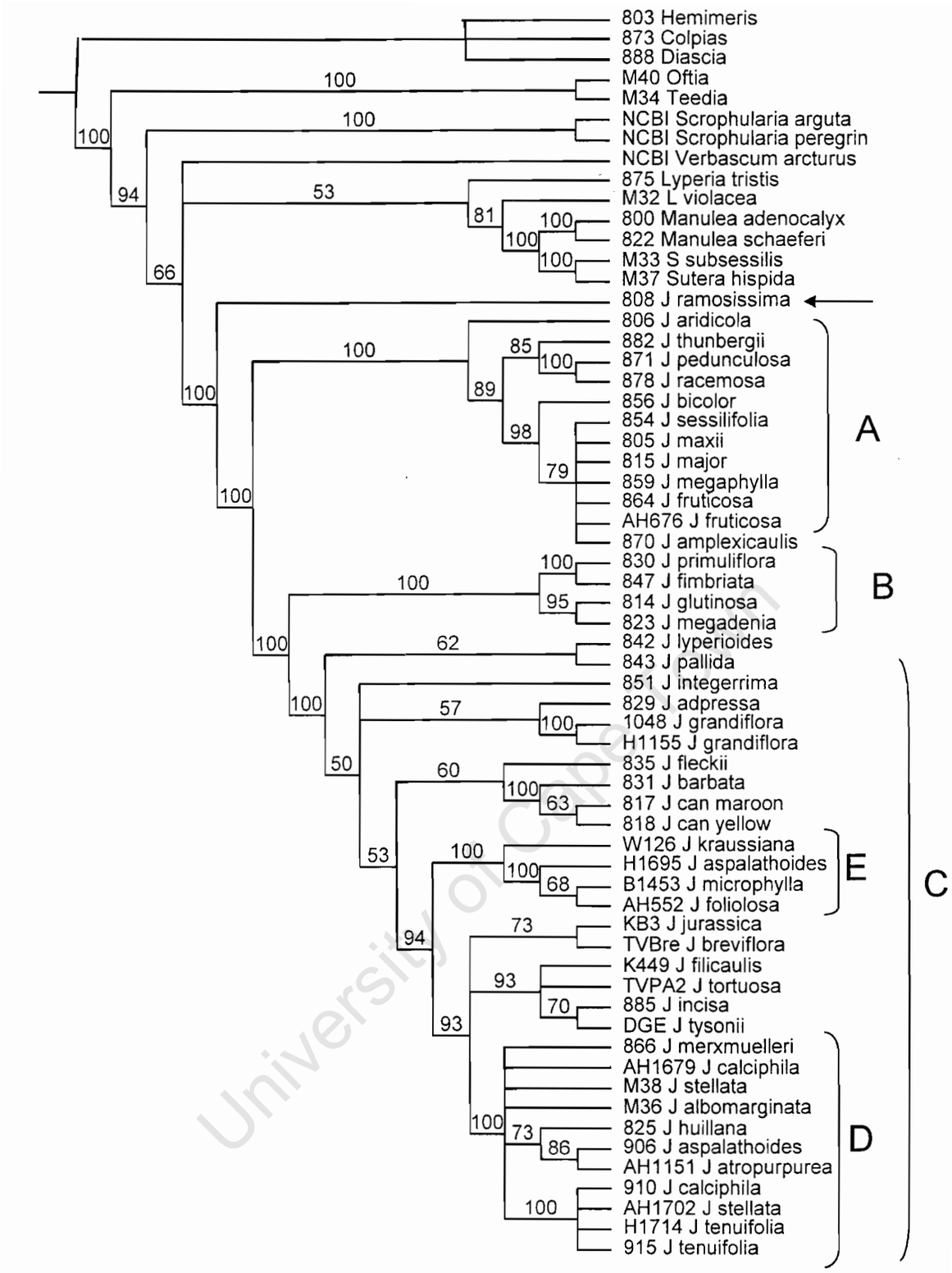

Figure 8. Bayesian majority rule consensus based on 22,500 trees sampled from three separate MCMC runs of one million generations each, using total molecular and morphological datasets, excluding *J.pristisepala*. Numbers above nodes are posterior probabilities represented as percentages. Each accession is indicated by a voucher number and species name.

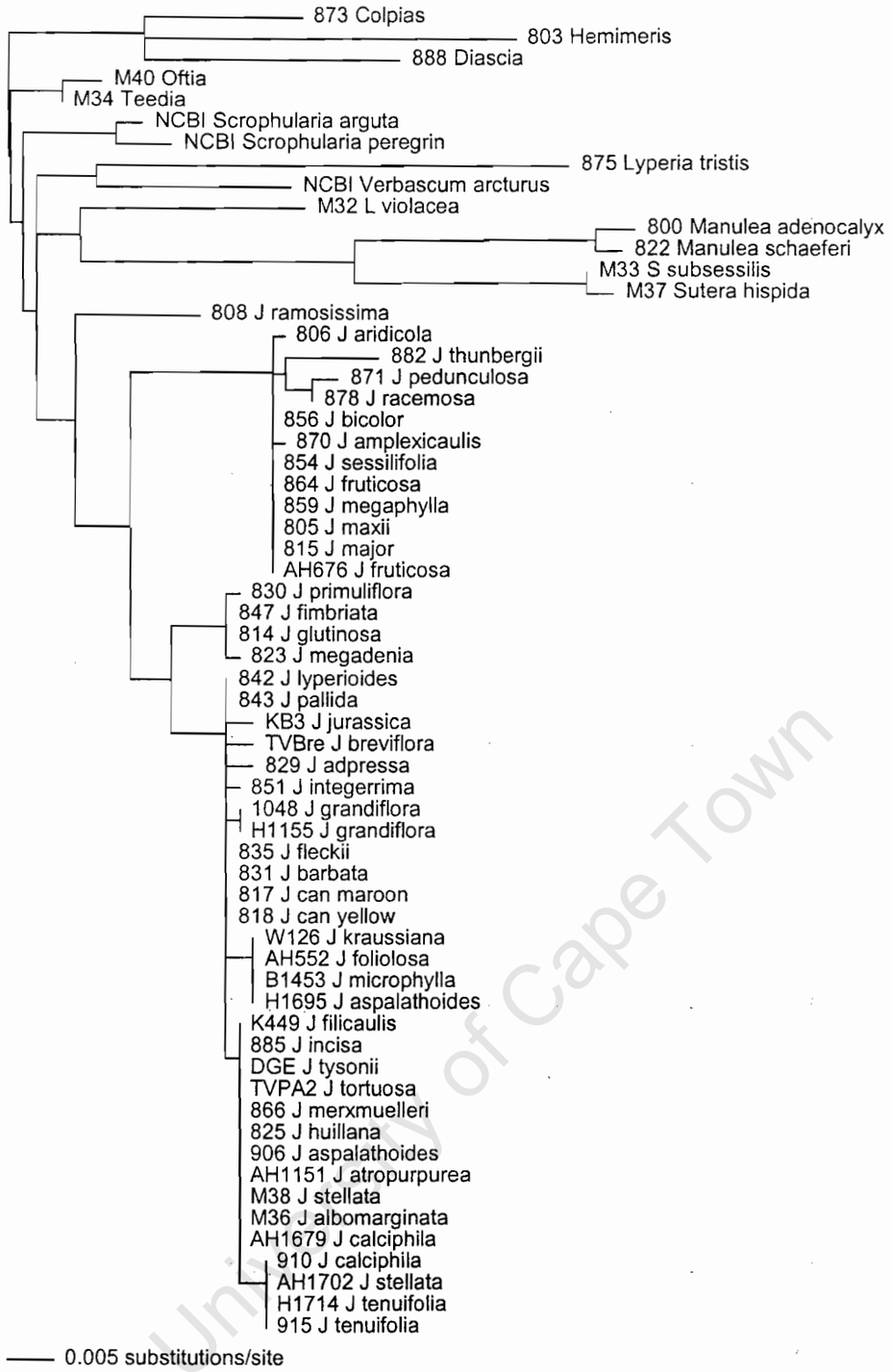

Figure 9. Phylogram showing one of the trees obtained from parsimony analysis of the combined data, with branch lengths calculated using the *rps*16 data under maximum likelihood (GTR+G model).

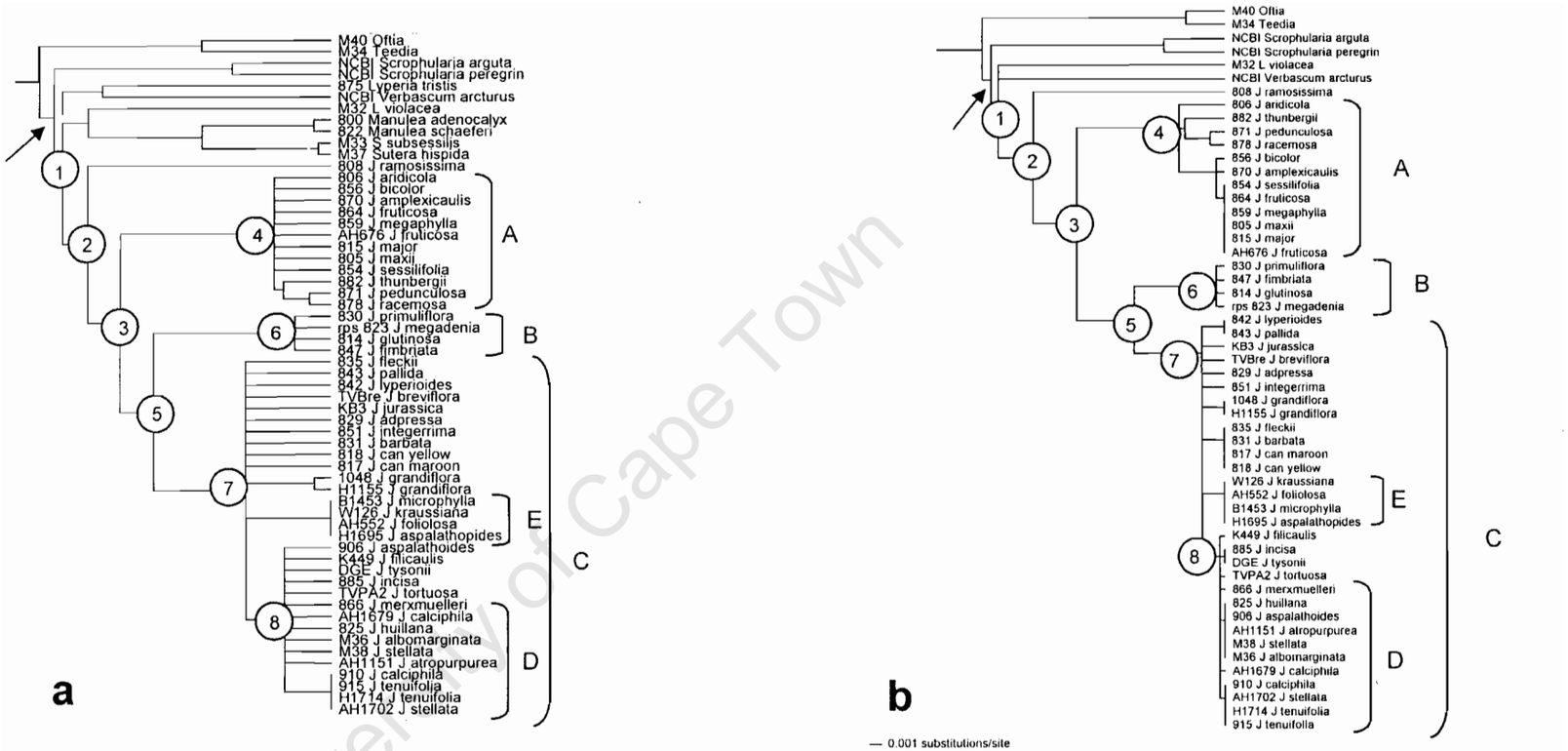

Figure 10. Chronograms generated from the tree shown in Figure 7 by **(a**) using Non Parametric Rate Smoothing in "r8s" and **(b)** enforcing a molecular clock after 6 taxa with long branches were pruned removed. Arrows indicates the calibration node. Node numbers correspond with Table 7.

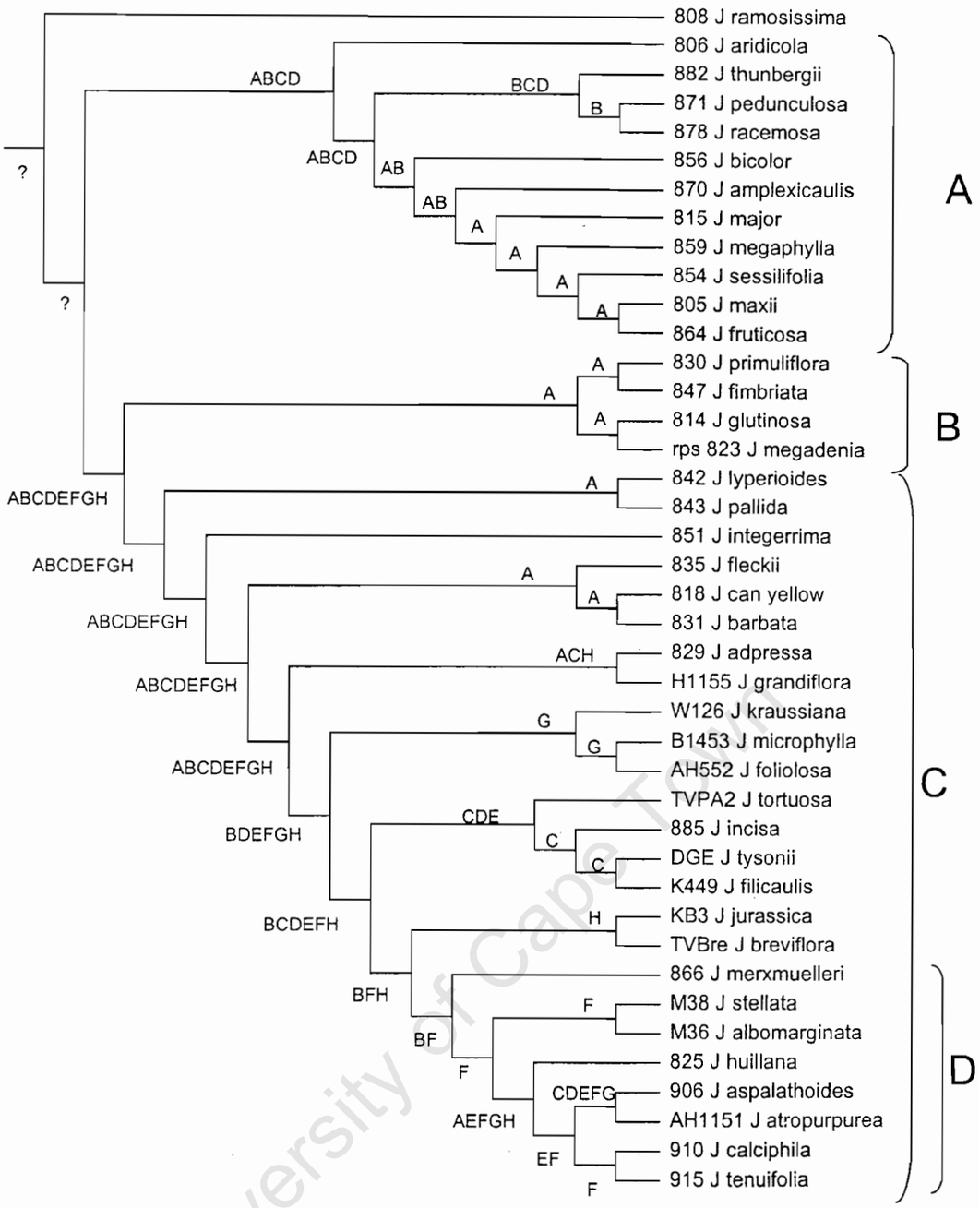

Figure 11. Results of a DIVA analysis to infer ancestral areas in *Jamesbrittenia*. The topology used is the consensus obtained from Bayesian analysis with replicate accessions removed and polytomies arbitrarily resolved. Areas are as indicated in Figure 2. Reconstructions shown on each node represent the union of all alternative reconstructions at that node. The two basal nodes could not be inferred, as explained in the text.

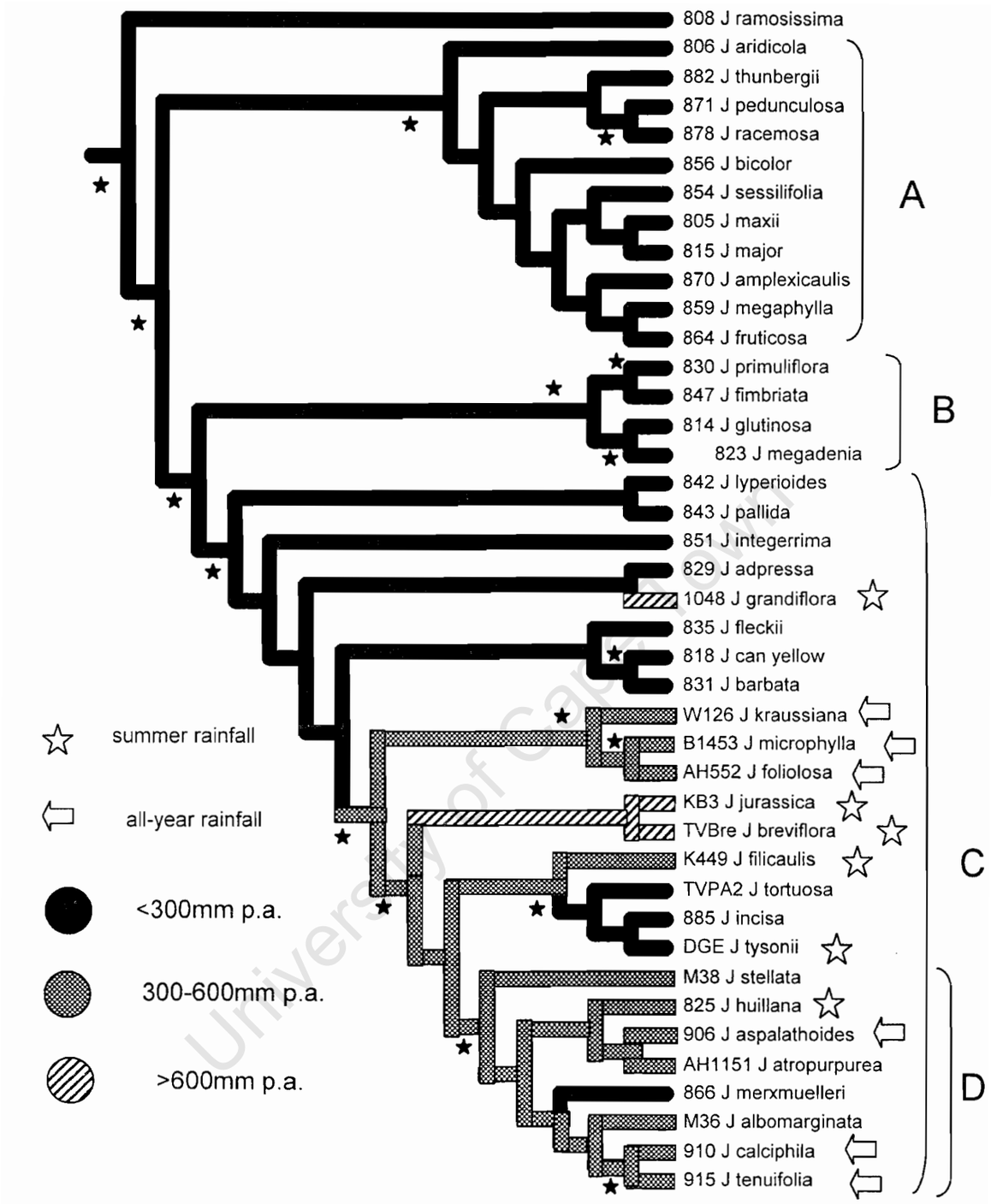

Figure 12. Rainfall distribution in relation to *Jamesbrittenia* species. Annual rainfall volume is optimized onto the tree, while the season in which it occurs is shown on the right. Where no symbols are shown, the rain falls in winter. Small black stars indicate nodes with posterior probability >90%. The topology is the consensus obtained from the Bayesian analysis with replicate accessions removed and polytomies arbitrarily resolved.

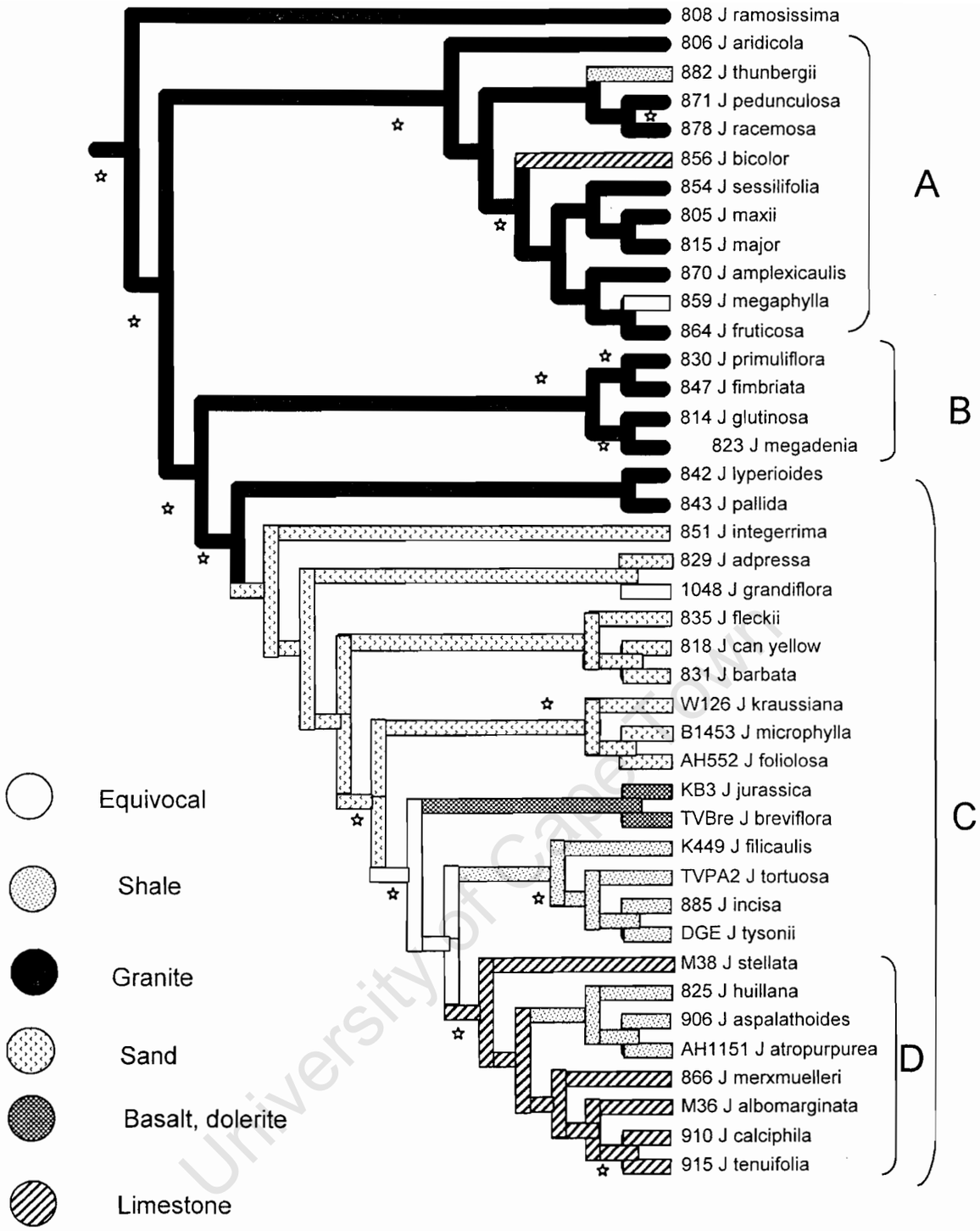

Figure 13. Preferred soil type of *Jamesbrittenia* species optimized onto the phylogeny using ACCTRAN. Small stars indicate nodes with posterior probability >90%. The topology is the consensus obtained from the Bayesian analysis with replicate accessions removed and polytomies arbitrarily resolved.

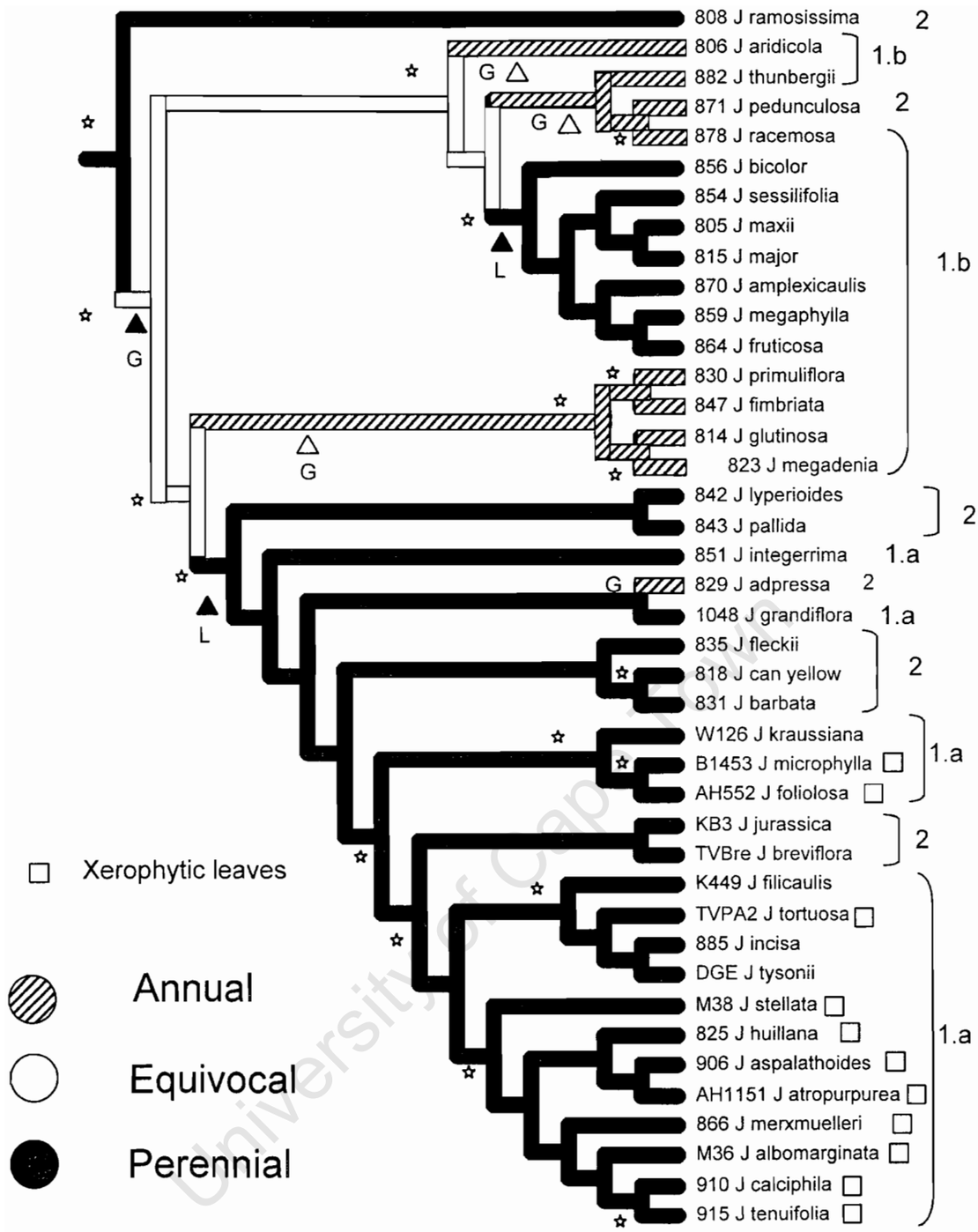

Figure 14. Annual and perennial life histories of *Jamesbrittenia* species optimized onto the phylogeny. Stars indicate nodes with posterior probability > 90%. With ACCTRAN, the equivocal branches become annual as indicated by solid triangles. DELTRAN optimization is shown by open triangles (G=gain, L=loss). Hilliard's groups are indicated on the right, as are species with xerophytic leaves. The topology is the consensus obtained from the Bayesian analysis with replicate accessions removed and polytomies arbitrarily resolved.