The role of pollinators as ecological drivers of diversification in the Drakensberg Mountain Centre endemic genus *Glumicalyx* (Scrophulariaceae, Limoselleae)

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Declaration

i. I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Abstract

The role of pollinators as ecological drivers of diversification in the Drakensberg Mountain Centre (DMC) endemic genus *Glumicalyx* was investigated here. The DMC comprises the Drakensberg Mountains of eastern South Africa and the Maloti Mountains of Lesotho. It forms a biodiversity hotspot as a result of its high species richness and endemism. Speciation in the DMC is predicted to be due to ecological factors, due to the topographic and edaphic variation resulting in a multitude of microhabitats available to both plants and their pollinators in the region. Vicariance due to tectonic uplift and subsequent large-scale erosion creating novel high altitude habitats and fragmenting species ranges is likely to have also played a role (Bentley *et al.* 2014). Diversification of the DMC endemic lineages is predicted to be relatively young, within the last 5 My, and was likely initiated by tectonic uplift creating novel habitats, including the modern alpine zone. *Glumicalyx* (Limoselleae, Scrophulariaceae) is the largest of the DMC endemic genera, with six closely-allied species. *Glumicalyx* is distributed across the DMC with its centre in the Lesotho Maloti Mountains. The aim of this study was to investigate the evolutionary trends and speciation in *Glumicalyx*, specifically exploring the role of pollinators and habitat as likely ecological drivers. With further investigation of the comparative reproductive biology of three co-occurring species of *Glumicalyx*.

To investigate the speciation and evolution of the genus a molecular phylogeny was produced using both nuclear (ETS and ITS) and plastid (*mat*K and *psb*A-*trn*H) DNA regions. The phylogeny supports the monophyly of the genus, although there was supported incongruence between the nuclear and plastid phylogenies. The phylogenies (nuclear and plastid) require better resolution, as there was insufficient variation to fully resolve the species relationships in *Glumicalyx*. The widely distributed species *G. nutans* is paraphyletic, with the Lesotho endemic *G. lesuticus* nested within *G. nutans*. *Glumicalyx lesuticus* most likely diverged from *G. nutans* in sympatry, as the distribution of *G. nutans* overlaps with *G. lesuticus* and the species often co-occur. The incongruence between the phylogenies makes conclusions about the modes of speciation between sister taxa challenging. The genus appears to have speciated ecologically both in sympatry and allopatry, as the species exhibit variation in morphological traits and habitat preference. Differences in floral traits among closely related species often reflects a difference in pollinators. Pollinators play an important role in promoting reproductive isolation and thereby speciation. The reproductive biology of three co-occurring species of *Glumicalyx*, representative of the floral morphology in the genus, are

investigated. This study provides the first account of the breeding system and pollination in *Glumicalyx*. All three species are dependent on pollinators for successful reproduction, although *G. montanus* is partially self-compatible. *Glumicalyx goseloides* was the only species found to be pollen limited, a finding that was supported by the lack of effective pollinators observed. *Glumicalyx nutans* was pollinated by noctuid moths based on the analysis of pollen loads and plant-pollinator fit. *Glumicalyx montanus* was mostly likely pollinated by short-tongued flies and noctuid moths, based on visitation and analysis of pollen loads. Among the three species there is substantial variation in floral morphology (corolla tube length and width) and floral traits that act to attract (floral colour and scent) and reward (nectar) pollinators. Floral scent composition varied between species and between day and night sampling, with significant increases in scent emission rate at night. Although effective pollinators were not observed for *G. goseloides*, the long, narrow corolla tube, floral scent and nectar production indicate specialized pollination. To further explore the role of pollinators as ecological drivers of diversification in *Glumicalyx*, a more resolved phylogeny in combination with a more extensive study of the reproductive biology of the genus is needed.

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List of abbreviations

a.s.l.	Above sea level
ANOSIM	Analysis of Similarity
ANOVA	Analysis of variance
CAS#	Unique numerical identifiers of compounds assigned by the Chemical Abstracts Service
CFR	Cape Floristic Region
DAC	Drakensberg Alpine Centre
DMC	Drakensberg Mountain Centre
EMR	Eastern Mountain Range
ETS	External Transcribed Spacer
GCFR	Greater Cape Floristic Region
GC-MS	Gas chromatography-mass spectrometry
GEE	Generalized Estimating Equation
IAS	Index of autonomous self-pollination
ISI	Index of self-incompatibility
ITS	Internal Transcribed Spacer
KRI	Kovats retention index
matK	maturase K
NDMS	Non-metric Multidimensional Scaling
PCA	Principal Component Anaysis
PCR	Polymerase Chain Reaction
PLI	Pollen limitation index
PPR	Pentatricopeptide repeat
SIMPER	Similarity Percentage analysis
UV	Ultraviolet

Chapter 1

General introduction

Within sub-Saharan Africa, there are some 45 000 species of plants and these species have been classified into six floras, with distinct geographical centres (Linder 2014). These floras differ in several ways, including radiation speed and of these six floras, the most rapidly radiating is the Austro-Temperate flora (Linder 2014). The Austro-Temperate flora includes the floras of the Cape (Marloth 1908; Goldblatt 1978; Linder 2003); the Drakensberg Alpine Centre (Hilliard and Burtt 1987; Carbutt and Edwards 2006); the Namaqualand semi-desert region (Cowling *et al*. 1999; Snijman 2013); and outliers in the Zimbabwean Chimanimani (Phipps and Goodier 1962). The Drakensberg Alpine Centre (DAC) is centred within the Greater Drakensberg Range. The Drakensberg Region forms the southern mountain system of the seven mountain systems forming the Afromontane and Afroalpine phytochoria (White 1978; Carbutt and Edwards 2004) and forms part of one of three centres of Afrotemperate endemism (Galley *et al*. 2007).

The Greater Drakensberg Range extends along the eastern margin of the southern African escarpment (Partridge and Maud 1987), extending from Elliot in the Eastern Cape to Tzaneen in Limpopo along a north-south axis (Carbutt and Edwards 2004). The DAC is centred in Lesotho and include the highest peaks of the Drakensberg Range (Killick 1994; Carbutt and Edwards 2001; Carbutt and Edwards 2004). The delimitation of the DAC has been controversial, van Wyk and Smith (2001) defined it as the high-lying central portion of the Drakensberg range above 1800 m above sea level (a.s.l.), which is centred in Lesotho, and is an update of Killick's (1994) Drakensberg Alpine Region. The DAC is synonymous with Eastern Mountain Region of Phillips (1917). There are inconsistencies in the definition of the DAC, as it forms a floristic centre of 'alpine' plants, but the geographical boundary was originally set at 1800 m a.s.l. (Killick 1963), rather than being restricted to the alpine belt (above 2800 m a.s.l.). It has recently been suggested that the extent of the DAC should be delimited to only include the alpine belt of the Drakensberg (Carbutt and Edwards 2015), which would be consistent with the classification of Körner *et al*. (2011). For the purpose of this study, the Drakensberg Alpine Centre as defined by van Wyk and Smith (2001) will be

referred to as the Drakensberg Mountain Centre (DMC) in order to accommodate the subalpine (upper montane) and alpine belts above 1800 m a.s.l.

The DMC is nested within the Grassland Biome (Rutherford and Westerfall 1986; Low and Rebelo 1996) and covers an area of an estimated 40 000 km^2 (Killick 1994; Carbutt and Edwards 2004). The DMC is comprised by the Maloti Mountains of Lesotho, the eastern Free State Drakensberg, the KwaZulu-Natal Drakensberg and the Eastern Cape Drakensberg (Hilliard and Burtt 1987; Carbutt and Edwards 2004; Mucina and Rutherford 2006). The DMC contains the only true alpine region in southern Africa (Linder 1990). The alpine vegetation consists of climax heath vegetation, mostly species of *Erica* and *Helichrysum* interspersed with alpine grasses. The subalpine vegetation consists of tussock grasses, scrub-type woody vegetation and herbaceous vegetation (Killick 1963). Montane forests occupy south and south-east facing slopes in sheltered sites on the northern Drakensberg (Hilliard and Burtt 1987), supporting trees, ferns and mosses (Carbutt and Edwards 2004). The geology forms part of the Karoo Supergroup (Whitmore *et al*. 1999), made up of horizontal strata of sedimentary and igneous deposits (van Zinderen Bakker 1981). The escarpment and plateaus are predominantly igneous (≈basalt) of the Drakensberg Group and sandstones of the Clarens Formation underly the basalt formation (Carbutt and Edwards 2004). The basalt deposits are a relic of lava flows resulting from the Gondwana breakup approximately 160 to 180 Mya (King 1982; Whitmore *et al*. 1999; Carbutt and Edwards 2004). Prior to the separation of Gondwana the Lesotho highlands had an estimated elevation of 2400 m due to uplift during the rifting (Partridge and Maud 2000). Since Africa's separation from Gondwana, the eastern side of southern Africa has experienced cycles of uplift that stimulate erosion. The most recent being in the early Miocene and the in the early Pliocene, this uplift is thought to have raised the Great Escarpment by between 150–300 m and 600–900 m respectively (Partridge and Maud 1987; Bentley *et al*. 2014). It also increased stream incision and produced the dissected topography of the Drakensberg foothills (Grab 2010). Upwarping during the late Miocene contributed to gorge incision and gully development (King and King 1959; Grab 2010).

The Cape Floristic Region (CFR) and the DMC form the southern source of temperate flora in Africa (Hilliard and Burtt 1987; Linder 1990, 1994; Carbutt and Edwards 2004). The flora of the Drakensberg has a close alliance with the Cape Flora (Weimarck 1941; Killick 1978; Hilliard and Burtt 1987; Bainbridge *et al*. 1991; Carbutt and Edwards 2004). Examples of such alliances include the *Disinae* (Orchidaceae) (Linder 1983; Carbutt and Edwards

2004), Manuleeae (≈Limoselleae, Scrophulariaceae) (Hilliard 1994; Carbutt and Edwards 2004) and the families Asteraceae (Hilliard 1978; Carbutt and Edwards 2004) and Iridaceae (Goldblatt 1983; Carbutt and Edwards 2004). This strong floristic affinity between the Cape and Drakensberg regions (Killick 1963; Hilliard and Burtt 1987; Carbutt and Edwards 2002) may be a result of the multiple migration events over a long span of time from the Cape to the Drakensberg (Galley *et al*. 2007). For four Cape-centred lineages that are widespread in the Afrotemperate flora (*Disa*, *Pentaschistis*, *Moraea* (Irideae) and the Restionaceae), Galley *et al*. (2007) found that in all four clades most dispersals from the Cape were to the Drakensberg and from the Drakensberg northwards to tropical Africa. The Drakensberg therefore forms an important link for the dispersal of plants between the Cape and the tropical Afrotemperate region and may have facilitated floristic radiations to tropical Africa north of the Limpopo River (Galley *et al*. 2007). The Drakensberg may also the source of *Aloe* (Weimark 1941) and *Kniphofia* to tropical Africa (Galley *et al*. 2007), whilst migrations of the orchids *Pterygodium* and *Satyrium* are hypothesised from the Drakensberg to the Cape (Kurzweil *et al*. 1991). The Cape mega-genus *Erica* is hypothesised to have had a northern hemisphere origin (Pirie *et al*. 2011; McGuire and Kron 2015), and is an important component of the Drakensberg flora. McGuire and Kron (2015) proposed a north to south migration of the ancestor of *Erica* from Europe to Africa during the mid-Miocene after the establishment of a land bridge.

Migrations into the Drakensberg from the Cape in the groups studied by Galley *et al*. (2007) date as early as 25.65 My ago, with an increase in migrations in the last 9 My. Tectonic uplift during the early Miocene and the Pliocene has been suggested to have further uplifted the Great Escarpment, including the Drakensberg (Partridge and Maud 1987; Galley *et al*. 2007). The modern alpine zone of the Drakensberg was likely formed by significant uplift along the south-eastern margin of the subcontinent around the Miocene-Pliocene boundary, 3–5 Mya (Partridge and Maud 2000; Linder 2014; Linder and Verboom 2015; Neumann and Bamford 2015). Tectonic uplift may stimulate speciation in two ways, the first in the creation of novel, high-altitude habitats that allow for adaptive radiation (Simpson 1975), and the second is the subsequent erosion. Erosive forces result in habitats that vary in altitude, aspect, slope, geology, soil type, microclimate and moisture regime, allowing for ecological specialisation. Large-scale erosion may result in increased river gradients and drainage potentially separating populations and limiting gene flow, allowing for geographical speciation (Lande 1976; Bentley *et al*. 2014). It has been suggested that the speciation events that resulted in Drakensberg endemics commenced within the last five Ma and are therefore

relatively young (Galley *et al*. 2007). Studies of Drakensberg-endemic lineages of large genera (e.g. *Helichrysum* and *Moraea*) show the DMC endemic clades/subclades are a result of repeated independent dispersals into the region, rather than large-scale *in situ* diversification (Galley *et al*. 2007; Cron *et al.* in prep.).

The DMC forms both a source and a sink for species dispersal, whilst also promoting *in situ* speciation. The high elevation of the DMC relative to the surrounding grassland means it may be likened to an "island", much like the "sky islands" of tropical Africa (Gehrke and Linder 2011). Speciation within the DMC is likely to be driven by ecological factors, including adaptations to the abiotic and biotic environment. The DMC varies topologically and edaphically both at a broad scale, including altitude, climate, topography and edaphic gradients (Bainbridge *et al*. 1991), and at a finer scale with relief, aspect, exposure and slope angle, resulting in a multitude of potential microhabitats (Hilliard & Burtt 1987; Carbutt and Edwards 2004). Pollinators are predicted to also play a role in the floral diversification in the eastern regions of southern Africa (including the Drakensberg), as there is a wide range of flower shape, size, and colour within genera (Pooley 2003, Johnson *et al*. 2009). Specialized pollination by long-proboscis flies, the oil-collecting bee *Rediviva* and the mountain pride butterfly, has been identified within the Drakensberg (Johnson 2010). Tectonic uplift that created novel habitats in the subalpine and alpine zones of the DMC and post-upliftment vicariance resulting from erosion may also play a role in speciation in the DMC, as evident in *Arrowsmithia* (=*Macowania*; Bentley *et al*. 2014, 2017). Of the two Drakensberg clades of *Arrowsmithia*, one clade shows little habitat and morphological variation but are geographically isolated, whilst in the second clade variation in vegetative and floral morphology, as well as microhabitats, indicate a role for adaptive divergence in the sympatric species.

The DMC forms a centre of plant endemism as identified by van Wyk and Smith (2001). There are an estimated 2818 species of vascular plants from 630 genera and 134 families present, ranking it the fourth richest regional flora (Carbutt and Edwards 2004, 2006). Of the flora, 13% is endemic and a further 24% is near-endemic, with only five endemic genera, three of which are monotypic (Carbutt and Edwards 2004). Of the endemic and near-endemic taxa, most belong to the Asteraceae, Scrophulariaceae and Iridaceae (Carbutt and Edwards 2006). The number of species/10^3 km^2 is 65 (Carbutt and Edwards 2004), for reference it is equivalent to the flora of KwaZulu-Natal (Scott-Shaw 1999), compared to 100 species/10^3 km^2 for the CFR (Goldblatt and Manning 2002). The high plant

diversity is evident in its abundant array of life-forms and is influenced by the more mesic conditions of the KwaZulu-Natal escarpment and the drier conditions of the Eastern Cape Drakensberg and Lesotho interior (Hilliard and Burtt 1987).

Unlike in the Cape Floristic Region (CFR), the drivers of diversification of plant lineages in the Drakensberg are not well understood. Drivers of diversification in the Cape are hypothesised to be a result of geological and edaphic variation (Goldblatt and Manning 2002), geomorphic evolution including landscape rejuvenation in the late Cenozoic (Cowling *et al*. 2009), climate variability including cooling and drying events (Goldblatt and Manning 2000), altitudinal variation in precipitation (Linder 1991) and pollinator specificity (Goldblatt and Manning 2006; Johnson 1996; van der Niet and Johnson 2012). Overall the combination of complex environmental conditions and the relative stable climate lead to the present day diversity (Schnizler *et al.* 2011). Linder *et al*. (2006) suggested that understanding the evolution of the southern African flora, beyond the CFR, may be achieved by studying the individual genera that make up the flora of southern Africa. Such may be applied to the Drakensberg Mountain Centre. Due to the topographic variation in the DMC that has resulted in many microhabitats being available for plants and their pollinators, the drivers of speciation are predicted to be as a result of ecological speciation. Ecological speciation focuses on speciation, a macroevolutionary process, as the result of the microevolutionary process of ecologically-based divergent selection (Funk 1998; Schluter 2001). Ecological speciation occurs when ecologically-based divergent selection results in reproductive isolation between populations (Schluter 2001; Rundle and Nosil 2005). Drivers of divergent selection on phenotypic traits include differences in the ecological niche, the ecological interaction of populations and forms of sexual selection, including adaptation to specific pollinators (Muller 1942; Schluter and Nagel 1995; Schluter 2001; Rundle and Nosil 2005). Ecological speciation may occur in sympatry (overlapping geographic distribution) or allopatry (separated geographically; Rundle and Nosil 2005). The evolution of reproductive isolation is determined by the evolution of barriers to gene flow between populations. There are many forms of reproductive isolation, including pre-zygotic and post-zygotic isolation (Coyne and Orr 2004; Rundle and Nosil 2005), for example habitat and temporal (phenological differences) isolation (Dres and Mallet 2002; Funk *et al.* 2002) and sexual isolation (including pollinator isolation) (Rundle and Nosil 2005). Adaptations to pollinators in plants may occur when populations adapt to different environments or habitat-specific adaptations may act on traits that affect pollinator preference (Rundle and Nosil 2005; Nosil 2012). Pollinator-mediated reproductive isolation results if divergence in floral traits as a

result of adaptations to a new pollinator/pollination system causes an exclusionary shift from one pollinator to another (van der Niet *et al*. 2014).

Pollinators have the potential to drive diversification at the microevolutionary (cf. van der Niet *et al*. 2014) and the macroevolutionary (Johnson 1996, Johnson 2010, van der Niet *et al*. 2012) scales. Macroevolutionary evidence of pollinator-driven divergence is from species-level angiosperm phylogenies whereby sister taxa are associated with shifts in pollination system and associated floral traits (for example: Whittall and Hodges 2007, Valente *et al*. 2012, van der Niet and Johnson 2012, Forest *et al*. 2014). The theory of ecological speciation provides a framework to link microevolutionary (adaptation) and macroevolutionary (speciation) processes (van der Niet *et al*. 2014). A signature of pollinator-driven adaptation is the divergence of floral features (Johnson 1996), whereas adaptations to the physical environment are reflected predominantly in vegetative characteristics that depict the high functional and morphological diversity (Verboom *et al*. 2004). This approach is useful in determining the drivers of ecological speciation, by exploring the link between phenotypic divergence and the selective agent (Verboom *et al.* 2004), as shown by Johnson (1996) for the flora of the Cape Floristic Region, South Africa.

The diversification of many plant families have been attributed to the adaptive radiation of breeding and pollination systems, accompanied by changes in ecology and life history (Barrett *et al*. 1996). The majority of plant species produce flowers that are hermaphroditic and therefore have the potential for self-pollination (Hörandl 2010). Self-compatibility may be beneficial in order to avoid pollen limitation (Larson and Barrett 2000). Pollen limitation can occur in habitats where pollinator availability is low (García-Camacho and Totland 2009), such as would apply to alpine habitats (Totland 1994; Körner 1999). It may result in increased incidence of self-compatibility and self-pollination (Medan *et al*. 2002) and increased generalist pollination (Totland 1993; Larson *et al*. 2001). If shifts to self-pollination are associated with a shift in pollinator mediated floral traits, such shifts may lead to reproductive isolation (van der Niet *et al*. 2014). Johnson (2006) suggests that the most common outcome of pollen limitation may be a shift between pollinators.

To study the drivers of diversification in the Drakensberg Mountain Centre, ideally the endemic and near-endemic genera should be studied, as endemic genera are less likely to have experienced extinctions and post-speciation range expansion and as a result provide better insights into the process of divergence (Herrera *et al*. 2006; Johnson 2010). *Glumicalyx* Hiern as the largest of the six genera endemic to the region, may be used as a model to study

DMC diversification. The use of species-level phylogenies to infer speciation and biogeography in the DMC is still in its early stages. Species-level phylogenies may be used to investigate geographical modes of speciation, based on the distribution of sister species (Brooks and McLennan 1991, Berlocher 1998, Chesser and Zink 1994, Barraclough and Nee 2001), and the frequency of ecological shifts within a group may be assessed (Barraclough and Nee 2001; van der Niet and Johnson 2012). Studies conducted on speciation in the DMC include the Drakensberg clade of *Arrowsmithia* (=*Macowania* Asteraceae; Bentley *et al.* 2014, 2017), *Galtonia* (Hyacinthaceae; Minaar 2018) and *Killickia* (Lamiaceae; Kgaboesele 2019), *Helichrysum* (Asteraceae; Cron *et al*. in prep), *Rhodohypoxis* (Hypoxidaeceae; Uys unpublished data) and *Craterocapsa* (Campanulaceae; Uys and Cron 2013). In the studies on *Craterocapsa*, *Killickia* and *Galtonia* – all of which are DMC near-endemic genera – ecological speciation through adaptations to micro-habitats and altitude, in both allopatry and sympatry, has been associated with their diversification. In *Killickia* the pollinators are likely to be different based on floral morphology. However, in none of the other studies above has the role of pollinators been identified as important in their speciation, even though pollinators have been implicated as one of the most important drivers of diversification in the neighbouring CFR (Schnitzler *et al.* 2011).

Understanding the drivers of speciation in the DMC provides further understanding of the diversification of southern African flora. The DMC serves as a stepping stone for dispersal from the Cape northwards along the afromontane archipelago of White (1983) and into adjacent grassland biomes (Galley *et al*. 2007). It has also experienced *in situ* radiations in some lineages – serving as both a sink and a source of speciation. Understanding drivers of diversification is particularly important when considering that all biodiversity is under threat (Sakar *et al*. 2006). The DMC is under threat by invasive plants, soil erosion, over-grazing, unsustainable cropping practices and water extraction (Hall *et al*. 1984; Killick 1994; Talukdar 1994; Carbutt and Edwards 2004, 2006). Despite the high species endemism and species richness of the DMC, only 5.5% of the land is currently conserved (Cowling and Hilton-Taylor 1994; Killick 1994; Carbutt and Edwards 2006).

Glumicalyx, in the family Scrophulariaceae, tribe Limoselleae, is endemic to the DMC (Hilliard and Burtt 1987; Carbutt and Edwards 2004, 2006; Figure 1.1). The type species of *Glumicalyx* is *G. montanus* as described by Hiern (1903) and *Glumicalyx* was a monotypic genus for some 70 years. The genus was expanded by Hilliard and Burtt (1977) to include the following six species: *G. apiculatus* (E.Mey.) Hilliard and Burtt, *G. flanaganii*

(Hiern) Hilliard & Burtt, *G. goseloides* (Diels) Hilliard & Burtt, *G. lesuticus* Hilliard & Burtt, *G. montanus* Hiern and *G. nutans* (Rolfe) Hilliard & Burtt. The remaining five species were previously placed in other genera, including *Zaluzianskya* Schmidt, *Selago* (L.) Hilliard & Burtt and *Walafrida* E.Mey. (Hilliard and Burtt 1977).

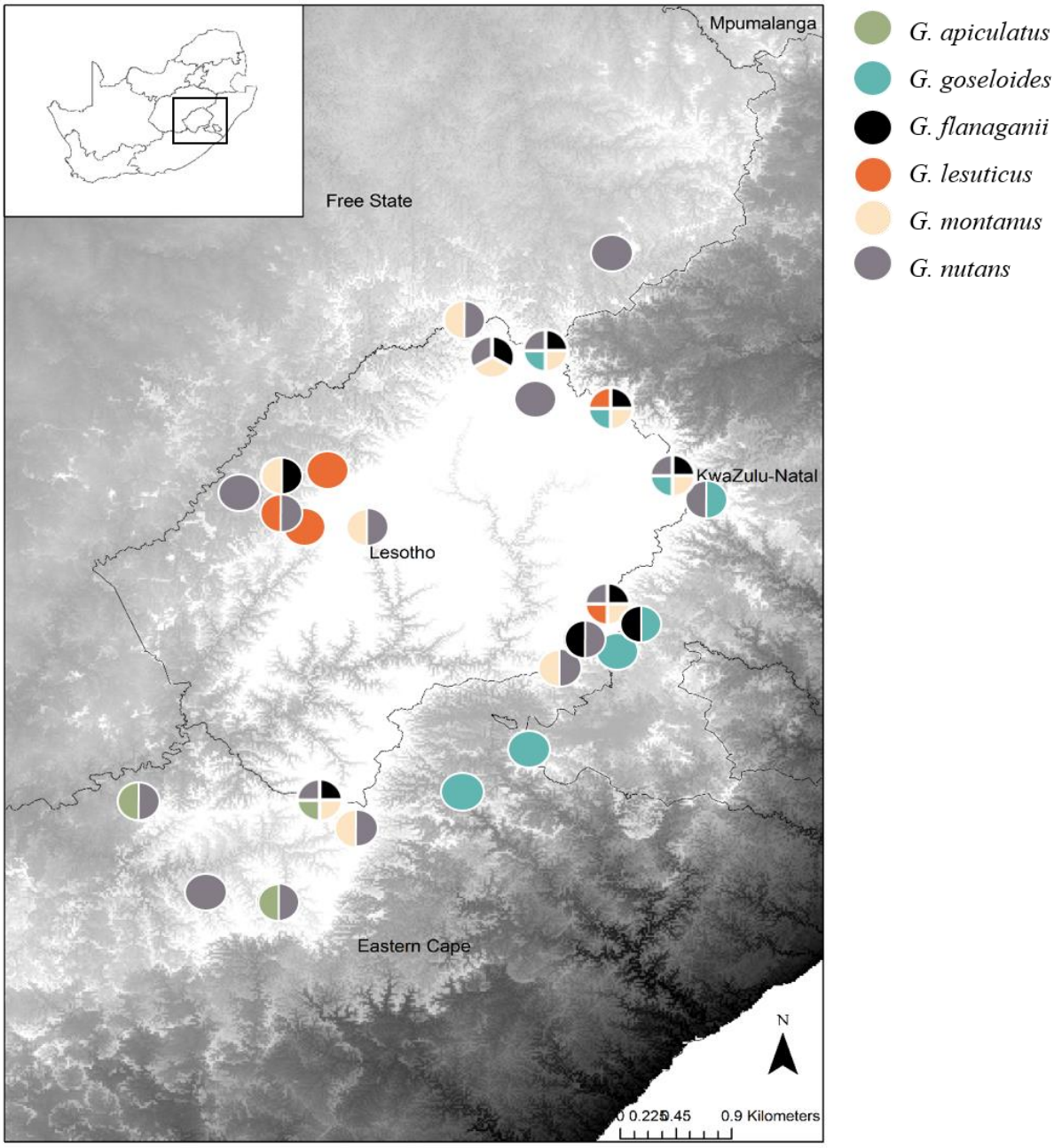

Figure 1.1. Distribution map of *Glumicalyx* across the Drakensberg Mountain Centre of South Africa and Lesotho based on herbarium specimens, comprising of the eastern Free State, KwaZulu-Natal Drakensberg, Lesotho Maloti Mts. and Eastern Cape Drakensberg/Witteberg.

The genus *Glumicalyx* is distinguished by a racemose inflorescence with a nodding orientation in flower that becomes erect and elongated in fruit (Hilliard and Burtt 1977; Figure 1.2). The bracts are broad, adnate to the pedicel and free from the calyx, with the calyx unequally five-lobed. All species of *Glumicalyx* are perennial herbs or dwarf subshrubs, woody at the base, and range from 6–60 cm tall (Figure 1.3). Breeding systems in the Scrophulariaceae appear mixed, with reports of self-compatibility in many species (Kampny 1995). Self-pollination in the *Parahebe* (Scrophulariaceae) may be dependent on variability of pollinator availability as a result of climate, with a higher prevalence of self-pollination in subalpine and alpine species. Within the Limoselleae little is known about the breeding system – two perennial species of *Zaluzianskya* were found to be self-incompatible (Johnson *et al*. 2002), however this contradicts Hilliard's (1994) suggestion that *Zaluzianskya* is self-compatible. The only published data on pollinators in the Limoselleae is that of Johnson *et al*. (2002) on the specialized hawkmoth and long-proboscid fly pollination in *Zaluzianskya* section *Nycterinia*. Prior to this study no information was available on the breeding system or pollination of *Glumicalyx*.

The species of *Glumicalyx* are distributed across the DMC occurring at different altitudes and habitats, primarily on basaltic rock substrate. Of the six species, *Glumicalyx nutans* is the most widely distributed species, as it occurs in KwaZulu-Natal, the Free State, Eastern Cape and Lesotho (Figure 1.1) over a wide altitudinal range (1800–3350 m a.s.l.) in bare gravel or silt and around rock sheets (Hilliard and Burtt 1977). In contrast, *Glumicalyx lesuticus* is restricted to Lesotho at altitudes of 2550–3180 m a.s.l., where it grows in gravel on sparsely grassed slopes and in grasslands (Figure 1.2 d, e). Its distribution overlaps most often with *G. nutans* (Figure 1.1). *Glumicalyx nutans* is morphologically very similar to *G. lesuticus* — both are perennial herbs with woody taproots, but *G. nutans* also becomes woody at the base and *G. lesuticus* has fewer leaves present on the stems. The species differ in their inflorescence — in *G. nutans* the inflorescence is turbinate, whereas in *G. lesuticus* it is globose (Figure 1.3). An important distinguishing feature between these two species is that in *G. nutans* the anthers are of unequal length, with two exserted and two included in the mouth of the corolla (Figure 1.2 f), whereas and in *G. lesuticus* the stamens are all exserted from the mouth and the anthers are of equal length (Figure 1.2 d; Table 1.1).

Glumicalyx goseloides (45 cm tall) and *G. flanaganii* (15–60 cm tall) are the largest members of *Glumicalyx* and are perennial herbs to subshrubs, with *G. goseloides* being similar to *G. nutans* in form but much larger in size. *Glumicalyx goseloides* occurs in the Free

State, KwaZulu-Natal and the Eastern Cape, with most localities occurring around the KwaZulu-Natal border with Lesotho (Figure 1.3). It occurs over a wide altitudinal range (1600–2800 m a.s.l.) near streams in boulder beds and in bare gravel (Figure 1.2 b). *Glumicalyx flanaganii* occurs in KwaZulu-Natal, the Eastern Cape and Lesotho at altitudes of 2400–3350 m a.s.l. in damp rocky areas, such as in stream gullies and at the foot of cliffs (Figure 1.2 c; Hilliard and Burtt 1977). *Glumicalyx flanaganii* is the most distinctive species as the stems are leafier than the other species of *Glumicalyx* (Figure 1.3 c) and their shape is quite distinctive (Table 1.1).

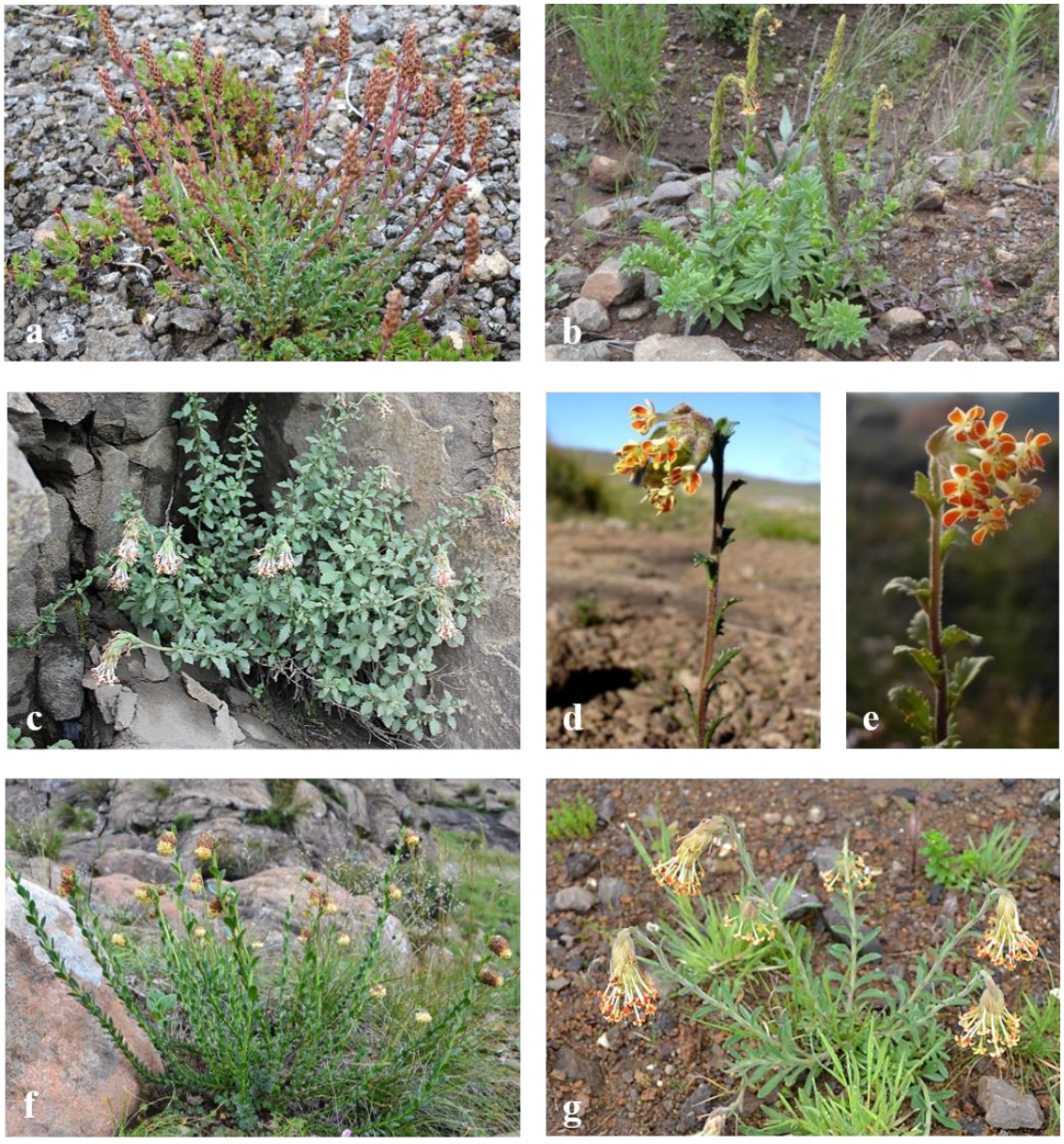

Figure 1.2. The growth form and habitats of species of *Glumicalyx*. (a) *G. apiculatus*, (b) *G. goseloides*, (c) *G. flanaganii*, (d) *G. lesuticus*, (e) *G. lesuticus*, (f) *G. montanus* and (g) *G. nutans*.

Glumicalyx apiculatus is the rarest of the six species, as it is only known from five localities in the Eastern Cape (Hilliard and Burtt 1977, 1987, Carbutt and Edwards 2006). Its distribution overlaps mostly with *G. nutans*, and it co-occurs with *G. montanus* and *G. nutans* at localities in the Eastern Cape Drakensberg (Figure 1.1). It occurs within subalpine grasslands on wet basalt gravel beds and rocks sheets (Figure 1.2 a), at altitudes of 2200–2560 m a.s.l. (Hilliard 1994, Carbutt and Edwards 2006). *Glumicalyx montanus* occurs in KwaZulu-Natal, the Free State, Eastern Cape and Lesotho (Figure 1.1) in stony and rocky habitats and on sparsely grassed slopes (Figure 1.2 f) at altitudes of 2340–3050 m a.s.l. Its distribution overlaps with *G. nutans* and *G. goseloides* in the Free State, and with *G. apiculatus* in the Eastern Cape (Figure 1.1).

Figure 1.3. The floral diversity in *Glumicalyx*. (a) *G. apiculatus*, (b) *G. goseloides*, (c) *G. flanaganii*, (d) *G. lesuticus*, (e) *G. montanus* and (f) *G. nutans*.

Glumicalyx apiculatus is superficially very similar to *G. montanus*; both are small perennial herbs that become woody at the base and even experienced collectors (e.g. Galpin) have mistaken the two (Hilliard and Burtt 1977). *Glumicalyx apiculatus* and *G. montanus* both have yellow upper corolla lobes, whereas the remaining species have orange to orange-red upper corolla lobes. The inflorescences in both *G. montanus* and *G. apiculatus* are globose in flower and elongate in fruit, although less so in *G. apiculatus*. The corolla tube is similar between them and the upper corolla lobe is yellow-orange in *G. apiculatus* and pale yellow in *G. montanus*. The stamens are all exserted and the anthers are of equal length in both species (Figure 1.3 a, d; Table 1.1). The six species flower in the austral summer months, with the flowering time overlapping between the species. Further details of the vegetative and floral morphology are given in Table 1.1 and details of the calyx is shown in Appendix 1.1.

Aims

This study aims to elucidate the phylogenetic relationships and confirm the monophyly of the genus *Glumicalyx*, and infer possible drivers and modes of speciation and evolution in *Glumicalyx*. Specifically, I aim to investigate the role of pollinators and habitat as ecological drivers of speciation in *Glumicalyx*. This study forms part of a more comprehensive study that aims to investigate the drivers of diversification in the Drakensberg Mountain Centre.

Objectives

1) To investigate phylogenetic relationships and evolution within *Glumicalyx* and infer drivers of diversification (biotic/abiotic) using the pattern of morphological variation (vegetative and floral) between sister species.
2) To investigate the comparative reproductive biology of three co-occurring species of *Glumicalyx*, including breeding system, pollination and floral traits.

Table 1.1. Descriptions of the habitat and morphology of the six *Glumicalyx* species, based on Hilliard and Burtt (1977).

	G. apiculatus	*G. flanaganii*	*G. goseloides*	*G. lesuticus*	*G. montanus*	*G. nutans*
Habitat	Basalt gravel beds	Damp rocky places, cliffs and stream gullies	Near streams in boulder beds and in bare gravel	Sparsely grassed slopes	Stony and rocky habitats, sparsely grassed slopes	Sparsely grassed slopes, bare gravel and around rock sheets
Altitudinal range (m above sea level)	2200–2560	2400–3350	1600–2800	2550–3180	2340–3050	1800–3350
Altitudinal zone	Subalpine	Subalpine and alpine	Montane and subalpine	Subalpine and alpine	Subalpine and alpine	Subalpine and alpine
Growth form	Tufted perennial herb, woody at the base	Tufted perennial herb or subshrub	Perennial herb	Perennial herb, woody taproot and crown	Tufted perennial, herb woody at the base	Perennial herb, woody at the base
Stem length (cm)	6–25	15–60	45	10–35	15–35	10–45
Leaf size (L × W mm)	5–13 × 3–4	15–37 × 5–16	20–65 × 4–15	25 × 4	5–20 × 2–8	12–30 × 4–11
Leaf shape (radical leaves)	Elliptic	Ovate, obtuse to subacute	Elliptic, obtuse	Elliptic	Oblong-elliptic to obovate	Oblanceolate, obtuse
Upper leaf margin	Crenate-serrate	Crenate-serrate to lobulate	Entire to obscurely toothed	Crenate-serrate	Crenate-serrate	Crenate-serrate
Bract size (L × W mm)	3–5 × 2–4	8–15 × 2.5–5	12–19 × 7–17	6–9.5 × 2.5–6	5–6 × 4.5–10	6–12 × 3–6
Bract shape	Elliptic to ovate	Oblong	Ovate		Suborbicular	Elliptic to ovate, obtuse to subacute
Calyx length (mm)	2.5–3.5	4.5–6.5	5–7	3–3.5	3.5–4.5	4–7
Calyx shape	Unevenly bilabiate	Unevenly bilabiate	Unevenly bilabiate	Bilabiate	Spathulate	Linear-oblong
Connation	3 segments fused for 2/3 length	Connate only at the base	Free nearly to base or fused 1/3-1/2 length	Connate only at the base	Free or connate only at the very base	Connate only at the base or free anticiously
Inflorescence size (L × W mm)	10 × 10 mm	25–30	30–60	20 × 20	15 × 15 mm	20–30
Inflorescence shape	Globose	Turbinate	Turbinate	Globose	Globose	Turbinate
Corolla tube size (L × W mm)	3–4 × 1.5	13–17.5 × 2	20–29 × 1.25	7–11.5 × 1.5	4–6 × 1.5	12–16 × 1
Corolla limb diameter	4	6–8	7–9	6–8	4–5	4–7
Corolla lobe colour	Yellow-orange	Orange-red	Orange to orange-red	Orange	Pale yellow	Orange-red
Corolla lobe shape	Elliptic	Oblong to suborbicular	Suborbicular	Elliptic to suborbicular	Oblong to elliptic	Narrow, oblong and strongly reflexed
Number of stamens exerted from tube	All four	All four	Two	All four	All four	Two
Capsule size (L × W mm)	3 × 2.5	6 × 3	7–8 × 3–4	5 × 2	4.5 × 3	5–6.5 × 2.5
Flowering time	January–March	December–March	October–July	December–February	December–March	December–March

Dissertation outline

This dissertation comprises four chapters. The first chapter consists of a general introduction, including a review of the literature pertaining to the diversification of the southern African flora and the Drakensberg Mountain Centre, models of speciation with particular focus on ecological speciation, the rationale of this study, the study genus, *Glumicalyx* Hiern and the aims of objectives. Chapter Two addresses the first objective, to elucidate phylogenetic relationships and evolution within *Glumicalyx* and infer drivers of diversification (biotic/abiotic) using the pattern of morphological variation (vegetative and floral) between sister species. Chapter Three addresses the second objective, to investigate the comparative reproductive biology of three co-occurring species of *Glumicalyx*, including the breeding system, pollinators and floral traits. The fourth and final chapter is an overview of the diversification of the *Glumicalyx* with consideration of its phylogenetic relationships, morphological variation and reproductive biology, conclusions and future prospects. This dissertation has been prepared and written in the format of scientific papers, and as a result some repetition of information given is unavoidable.

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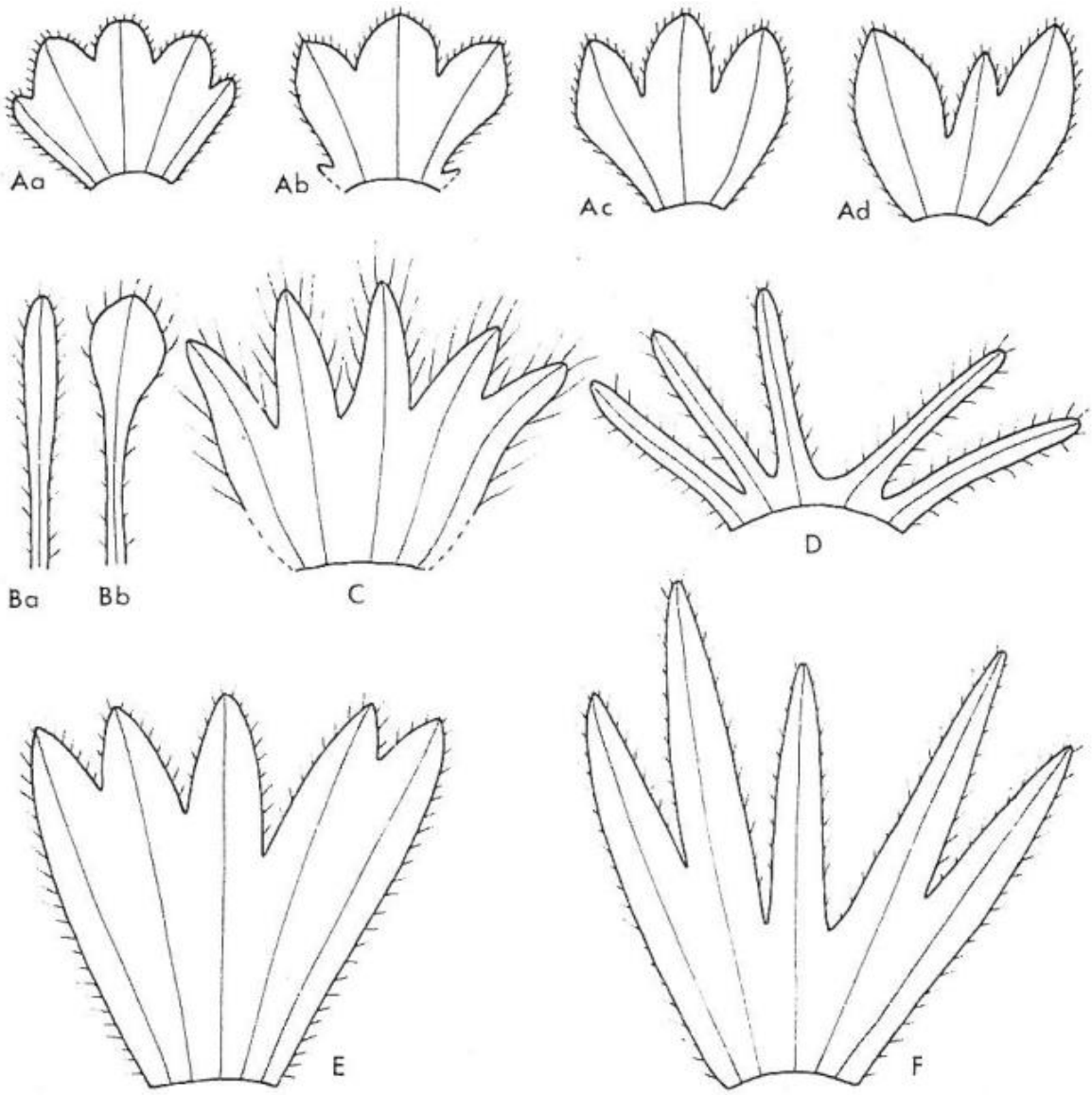
Appendix



Appendix 1.1. Calyx form in *Glumicalyx* (Hilliard and Burtt 1977, pp. 165). A) *G. apiculatus*, B) *G. montanus*, C) *G. flanaganii*, D) *G. lesuticus*, E) *G. nutans* and F) *G. goseloides*.

Chapter 2

Drivers of speciation inferred from comparative analysis of phylogenetics and morphological variation in *Glumicalyx*

Abstract

Despite the high species richness and endemism in the Drakensberg Mountain Centre (DMC), relatively little research has been conducted on the drivers of diversification in the region. This study aims to determine the phylogenetic relationships in the largest DMC endemic genus *Glumicalyx* Hiern (Scrophulariaceae *s.s.*, tribe Limoselleae) and compare selected morphological traits between sister species to infer potential drivers and modes of speciation. *Glumicalyx* comprises six species and is distinguished by its nodding, racemose inflorescence that becomes elongated and erect in fruit. Phylogenetic analyses based on nuclear (ITS and ETS) and plastid (*mat*K) genomes support the monophyly of *Glumicalyx*, although they also reveal well supported incongruence within the genus. This suggests possible past hybridization and/or introgression. The genus most likely radiated in the Drakensberg Mountain Centre during the last 5 Mya, coinciding with the formation of the modern alpine zone. The species exhibit variation in vegetative and reproductive traits, suggesting ecological speciation through adaptations to different microhabitats and pollinators.

Introduction

The flora of southern Africa is ranked among the richest globally and this high diversity has been generated by evolutionary radiations (Linder and Verboom 2015). All biological diversity is the result of radiations, as a radiation is the increase in diversity of a lineage over a relatively short period of time (Linder 2008). Southern Africa's Greater Cape Floristic Region (GCFR) angiosperm richness may be product of distinct radiation scenarios, both recent and mature radiations (Linder 2008; Verboom *et al*. 2014), and this pattern may extend to the rest of southern Africa's flora (Linder and Verboom 2015). Radiations of a flora constitute a combination of nested radiations where the geographically and ecologically separated radiations seed one another. Radiations are more likely to occur in regions with environmental complexity, general climatic stability and gradual change that may stimulate complex radiations (Linder and Verboom 2015). Linder (2014) has grouped Africa's flora into four biogeographical regions, the most rapidly radiating of these is the Austro-temperate Flora. The Austro-temperate Flora includes the GCFR (Marloth 1908; Goldblatt 1978; Cowling *et al*. 1999; Linder 2003; Snijman 2013), the Drakensberg Mountain Centre (≈ Drakensberg Alpine Centre; Hilliard and Burtt 1987; Carbutt and Edwards 2006) and outliers in the Chimanimani Mountains of Zimbabwe (Phipps and Goodier 1962). The most recent radiation within the Austro-temperate Flora was initiated tectonic uplift, 3–5 Mya, during the Miocene-Pliocene boundary (Partridge and Maud 2005) that resulted in diversification along the scarp edge of the great escarpment and the formation of the modern alpine zone (Linder 2014; Linder and Verboom 2015; Neumann and Bamford 2015). Uplift during the late Miocene gave rise to gullies and promoted gorge incision in the Drakensberg (King and King 1959; Grab 2010), thus creating a diversity of habitats along the escarpment (Hilliard and Burtt 1987; Uys and Cron 2013). Although the diversity of the GCFR greatly exceeds that of the Drakensberg Mountain Centre (DMC), the DMC forms a centre of southern African endemism and ranks fourth richest in plant diversity (van Wyk and Smith 2001). The diversity in floral and vegetative traits may be attributed to variations on a broad scale, including altitude, climate, topography and edaphic gradients (Bainbridge *et al.* 1991), as well as on a finer scale, including relief, exposure, aspect and slope angle (Körner 1999), that result in a multitude of microhabitats (Hilliard and Burtt 1987; Carbutt and Edwards 2004).

The Greater Drakensberg Range forms part of the Great Escarpment on the eastern side of the southern African plateau extending for nearly 1000 km along a north-south axis (Partridge and Maud 1987; Carbutt and Edwards 2004). Nested within it is the Drakensberg

Alpine Centre, defined as the high-lying central portion of the Drakensberg range above 1800 m, which is centred in Lesotho (van Wyk and Smith 2001). There are inconsistencies in the definition of the DAC, as it forms a floristic centre of 'alpine' plants, but the geographical boundary was originally set at 1800 m a.s.l. (Killick 1994), rather than being restricted to the alpine belt (above 2800 m a.s.l.). As a result Carbutt and Edwards (2015) suggested that the extent of the DAC should be limited to only the alpine belt of the Drakensberg, this would conform to the ecothermal belts of Körner *et al*. (2011) above the tree line. For the purpose of this study, however, the Drakensberg Alpine Centre as defined by van Wyk and Smith (2001) will be referred to as the Drakensberg Mountain Centre (DMC) in order to accommodate the subalpine (or upper montane ecothermal belt; Körner *et al*. 2011) and alpine belts above 1800 m. Speciation within the DMC is predicted to be ecological, due to the topographic and edaphic variation in the DMC that has resulted in many microhabitats being available for plants and their pollinators. The role of vicariance due to tectonic uplift and subsequent erosion in the region may also have played a role in speciation, as evident in *Arrowsmithia* (=*Macowania*; Bentley *et al*. 2014, 2017).

Speciation events may be indirectly inferred through species-level phylogenetic studies of extant taxa (Barraclough and Nee 2001). Specifically, phylogenies may be used to investigate geographical modes of speciation, including allopatric and sympatric speciation, based on the current geographical distributions of the sister species (Brooks and McLennan 1991, Berlocher 1998, Chesser and Zink, Barraclough and Nee 2001). Furthermore, the frequency of ecological shifts may be assessed, whereby ecological characters are mapped onto a tree (Barraclough and Nee 2001). Ecological characters refer to both biotic (resource competition, predation and pollinator interactions) and abiotic habitat features (climate, topography, altitude; Schluter 2001). Thus, phylogenies may elucidate ecological speciation, which is defined as the evolution of reproductive isolation through divergent selection in populations in contrasting environments (Schluter 2001) and which may occur in allopatry or sympatry.

There are many mechanisms that may lead to ecological speciation; an approach proposed by Stebbins (1970, 1974) suggests that adaptations to pollinators are reflected predominantly in the divergence of floral traits, include flowering time, nectar chemistry, flower shape, size and colour (Johnson 1996). Whereas adaptations to the physical environment are reflected predominantly in vegetative traits, as well as variation in growth form and life history strategy (Verboom *et al*. 2004). Inferring the drivers of a radiation from

the traits that vary within a group was suggested and implemented by Johnson (1996) for the flora of the Cape Floristic Region, South Africa, and may also be inferred for the flora of the Drakensberg Mountain Centre. Reproductive isolation (or partial isolation) may result from strong divergence in floral morphology between recently diverged sister taxa (Ramsey *et al*. 2003; Kay 2006), although the role of reproductive isolation as a result of floral divergence in speciation is more likely to act synergistically with other isolating mechanisms rather than result in speciation itself (Kay and Sargent 2009).

As noted, the Drakensberg Mountain Centre forms a hotspot of plant diversity and endemism in southern Africa (Carbutt and Edwards 2006). Little is known about the drivers and modes of speciation in the region (Hilliard and Burtt 1973, 1978, 1987, Carbutt and Edwards 2001, 2006, Uys and Cron 2013), and by studying the largest of the endemic genera, *Glumicalyx*, we may gain novel insights into speciation in the DMC. By comparing distribution, micro-habitats, vegetative and floral variation in the genus, modes of speciation (allopatry, sympatry), and likely drivers of speciation (abiotic and biotic) may be inferred. To determine sister species' relationships to enable inference of likely drivers of speciation events, a phylogeny for *Glumicalyx* was needed.

Glumicalyx Hiern is a genus of perennial herbs or dwarf subshrubs in the tribe Limoselleae of the family Scrophulariaceae *sensu stricto*. The Scrophulariaceae is a large family with a challenging taxonomy, Scrophulariaceae *s.s.* forms a distinct clade in the polyphyletic Scrophulariaceae *s.l.*, shown using molecular phylogenies (Olmstead and Reeves 1995; Oxelman *et al.* 2005). Scrophulariaceae *s.s.* is composed mostly of taxa distributed in the southern hemisphere (Olmstead *et al*. 2001), within which there are eight tribes (Kornhall *et al.* 2001, Kornhall and Bremer 2004, Oxelman *et al.* 2005). Within the Scrophulariaceae *s.s.*, Kornhall *et al*. (2001) used phylogenies derived from molecular data to show that the tribe Selagineae arose from within Manuleeae. The tribes were previously separated by having either multiple or single ovules, however single ovules are seen to have arisen more than once in the Manuleeae and Selagineae. Kornhall and Bremer (2004) later proposed that the genus *Limosella* L., together with the tribes Manuleeae and Selagineae, be combined into the tribe Limoselleae, as the genus *Limosella* also arose within Manuleeae. *Limosella* is the only genus in the Manuleeae with a cosmopolitan distribution, the remaining genera are largely restricted to southern Africa. The tribe Limoselleae *sensu* Kornhall and Bremer (2004) thus includes *Limosella* and 25 genera that were previously placed in the Manuleeae and Selagineae (Hilliard 1994, Hilliard 1999, Kornhall and Bremer 2004). The

Manuleeae was comprehensively reviewed by Hilliard (1994), and included the genera *Jamesbrittenia* O.Kuntze, *Sutera* Roth, *Zaluzianskya* F.W.Schmidt and *Glumicalyx* Hiern.

Figure 2.1. Variation in the inflorescences and flower colour of the six species of *Glumicalyx*: (a) *G. apiculatus*, (b) *G. goseloides*, (c) *G. flanaganii*, (d) *G. lesuticus*, (e) *G. montanus* and (f) *G. nutans*.

Glumicalyx, a genus endemic to the Drakensberg Mountains of South Africa, comprises the following six species: *G. apiculatus* (E.Mey.) Hilliard and Burtt, *G. flanaganii* (Hiern) Hilliard & Burtt, *G. goseloides* (Diels) Hilliard & Burtt, *G. lesuticus* Hilliard & Burtt, *G. montanus* Hiern and *G. nutans* (Rolfe) Hilliard & Burtt (Figure 2.1 a–f). The genus was monotypic until it was revised by Hilliard and Burtt (1977), to include four species that were previously paced in *Zaluzianskya, Selago* (L.) Hilliard & Burtt and *Walafrida* E.Mey and the newly described species *G. lesuticus*. The genus is distinguished by a racemose inflorescence

that is nodding while flowering, and elongates as the fruits mature (Hilliard and Burtt 1977). The bracts are broad, adnate to the pedicel and free from the calyx, which is unequally five-lobed. The known distribution of *Glumicalyx* extends from the eastern Free State, across Lesotho's Maloti Mountains, the KwaZulu-Natal Drakensberg escarpment and the Eastern Cape Drakensberg. The genus occurs at altitudes ranging from 1600 to 3550 m above sea level (a.s.l.) and is thus present in the alpine, subalpine and montane altitudinal bands (Hilliard and Burtt 1977). *Glumicalyx* is the largest of five genera endemic to the DMC, and shows both morphological and ecological diversity.

The aim of this study was to elucidate the likely drivers of speciation in *Glumicalyx*, a Drakensberg endemic genus. The objectives were to 1) investigate phylogenetic relationships within the genus, 2) hypothesise evolutionary trends based on species relationships and morphological variation, 3) compare sister species to infer whether biotic or abiotic features predominantly influenced their speciation.

Methods and materials

Taxon sampling

Leaf material from all six species of *Glumicalyx* was collected from across the Drakensberg region. Where possible, multiple exemplars for each species were collected. Specimens of *G. apiculatus*, *G. goseloides*, *G. montanus* and *G. nutans* were collected during January and April 2017 (Table 2.1). Existing collections and DNA material of *Glumicalyx* collected by G.V. Cron, S.P. Bester, O.M. Hilliard and B.L. Burtt were also sourced (Table 2.1). Two specimens of putative hybrids were collected, one from Witsieshoek, Free State — possibly *G. goseloides* × *G. nutans* [and previously reported by Hilliard and Burtt (1977) to occur at this locality], and the second from Tenahead Lodge, Eastern Cape — possibly *G. montanus* × *G. nutans*. These two putative hybrids were excluded from the primary phylogenetic analyses, and were subsequently included in separate analyses of each dataset.

In the field, healthy, young leaves were collected and placed in silica gel to dry rapidly and then stored in a freezer at −20°C. The collected leaf tissue samples were used for DNA extraction. Voucher specimens are deposited in the C.E. Moss Herbarium, University of the Witwatersrand (J), Bews Herbarium, University of KwaZulu-Natal (NU) and the National Herbarium, Pretoria (PRE).

The outgroup taxa for the phylogenetic analyses were chosen based on their putative relationship to *Glumicalyx* as seen in phylogenies of the Scrophulariaceae *s.s.* (Kornhall *et al*. 2001; Oxelman *et al*. 2005), viz. species from the genera *Zaluzianskya, Selago* (L.) Hilliard & Burtt, *Hebenstretia* L. and *Jamesbrittenia* (Table 2.2). Scrophularieae is sister to the Limoselleae (Oxelman *et al*. 2005). Therefore, *Scrophularia* L., a member of Scrophulariaceae *s.s.*, in the tribe Scrophularieae was used to root the phylogeny. Sequences of *Scrophularia* and *Selago* were obtained from GenBank (Table 2.2). The sister group to *Glumicalyx* is contentious and depends on the taxon sampling: in the phylogeny of *Zaluzianskya* which includes all the major clades of the Limoselleae (Archibald *et al*. 2017) and that of Scrophulariaceae *sensu* APG II (Oxelman *et al*. 2005, *Glumicalyx* is sister to a clade composed of *Selago, Tetraselago, Melanospermum, Microdon* and *Cromidon*; in the phylogeny of Scrophulariaceae *s.s*. focusing on the former tribes Manuleeae and Selagineae, *Glumicalyx* is sister to the monotypic genus *Strobolopsis* Hilliard & Burtt (Kornhall *et al*. 2001).

DNA extraction, amplification and sequencing

DNA was extracted from silica gel-preserved leaf tissue of specimens from plants collected in the field or from fragments of herbarium specimens obtained from the National Herbarium, Pretoria (PRE, with permission). The GeneJET^{TM} Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts USA) was used to extract DNA from the leaf tissue samples using the protocol outlined in Appendix 2.1.

The nuclear ribosomal internal transcribed spacer (ITS) region, comprising ITS1, the 5.8S gene and ITS2, and the external transcribed spacer (ETS) region were used to reconstruct the bi-parentally inherited phylogenetic history. The plastid maturase K (*mat*K) and *psb*A-*trn*H regions were selected to reveal the maternally inherited phylogenetic history. The *trn*L-*trn*F region was investigated but was not variable enough to be informative. The ETS region (together with other regions) was used in the phylogenetic reconstruction of *Nemesia* (Scrophulariaceae) in the Greater Cape Floristic Region (Datson *et al*. 2008) and for the tribe Buddlejeae, Scrophulariaceae (Chau *et al*. 2017). The coding region of the plastid *mat*K gene was used in the phylogenetic reconstruction of the relationships in the tribe Cheloneae, Scrophulariaceae (Wolfe *et al*. 2002). The *psb*A-*trn*H intergenic spacer was used in the reconstruction of the New World *Scrophularia* (Scheunert and Heubl 2011) and for the southern African genus *Jamesbrittenia* (Verboom *et al*. 2016).

Table 2.1. Specimens of *Glumicalyx* and outgroups used for DNA extraction and phylogenetic analyses. Voucher collector and number, with herbarium acronym (Theirs [continuously updated]) and locality of collection indicated. Herbarium specimens used for DNA extraction indicated with an asterisk.

Species	Voucher ID	Herbarium	Locality	Date collected
G. apiculatus	Hilliard 16666*	PRE	EC, Bastervoetpad, Elliot-Maclear	1983
G. apiculatus	Springer 26	J, NU, PRE	EC, Joubert's Pass, Lady Grey	2017
G. flanaganii	Cron & Cingo 1119	J, NU, PRE	L, Tlaeng Pass, Lesotho	2013
G. flanaganii	Cron & Cingo 1120	J, NU, PRE	L, Tlaeng Pass, Lesotho	2013
G. flanaganii	Cron & Cingo 1126	J, NU, PRE	L, Sani Pass, Lesotho	2012
G. goseloides	Cron 1128	J, NU, PRE	KZN, Sani Pass	2012
G. goseloides	Cron 1128b	J, NU, PRE	KZN, Sani Pass, KwaZulu-Natal	2012
G. goseloides	Bester 10992*	PRE, J	FS, base of Sentinel Peak, Witsieshoek	2012
G. goseloides	Springer 9	J, NU, PRE	FS, road to Sentinel Peak, Witsieshoek	2017
G. goseloides	Springer 18	J, NU, PRE	FS, base of Sentinel Peak, Witsieshoek	2017
G. lesuticus	Hilliard & Burtt 8813*	PRE	L, Sani Top	1976
G. lesuticus	Cron & Cingo C1117b	J, NU, PRE	L, AfriSki Lodge	2013
G. montanus	Bester 10986*	PRE	FS, base of Sentinel Peak, Witsieshoek	2012
G. montanus	Bester 11580	PRE, J	FS, road to Sentinel Peak, Witsieshoek	2013
G. montanus	Springer 17	J, NU, PRE	FS, base of Sentinel Peak, Witsieshoek	2017
G. montanus	Springer 38	J, NU, PRE	EC, Naudes Nek Pass	2017
G. montanus	Springer 45	J, NU, PRE	EC, Past Tenahead Lodge	2017
G. nutans	Bester 10995*	PRE, J	FS, road to Sentinel Peak, Witsieshoek	2012
G. nutans	Bester 11575*	PRE, J	FS, road to Sentinel Peak, Witsieshoek	2013
G. nutans	Cron & Cingo C1115	J, NU, PRE	L, Oxbow Lodge	2013
G. nutans	Cron & Cingo C1116	J, NU, PRE	L, AfriSki Lodge	2013
G. nutans	Springer 1	J, NU, PRE	FS, Platberg summit, Harrismith	2017
G. nutans	Springer 10	J, NU, PRE	FS, road to Sentinel Peak, Witsieshoek	2017
G. nutans	Springer 27	J, NU, PRE	EC, Joubert's Pass, Lady Grey	2017
G. nutans	Springer 35	J, NU, PRE	EC, Summit Naudes Nek Pass	2017
G. nutans	Springer 46	J, NU, PRE	EC, Past Tenahead Lodge	2017

Table 2.2. Taxon table for outgroup taxa, sequences obtained from GenBank indicated with an asterisk.

Species	Voucher ID	Herbarium	Locality	Date collected	GenBank accession number			
					ETS	ITS	*mat*K	*psb*A-*trn*H
Zaluzianskya sp. 1	Springer 15	J	Witsieshoek Pass, Free State	2017				
Zaluzianskya microsiphon	Springer 34	J	Joubert's Pass, Lady Grey, Eastern Cape	2017				
Selago monticola	Springer 55	J	Monk's Cowl, KwaZulu-Natal	2017				
Hebenstretia dura	Springer 53	J	Monk's Cowl, KwaZulu-Natal	2017				
Jamesbrittenia aurantiaca	Springer 56	J	Heidelberg, Gauteng	2017				
Selago thomsonii *	Bremer 3095	ITS: Kornhall and Bremer unpublished data, *mat*K: Bremer *et al*. 2002				AJ584795.1	AJ429348.1	
Scrophularia kakudensis *	ETS: Jang SO02 ITS: Jang SK03-110831	Chung and Oh unpublished data			JN984907.1	JQ065669.1		
Scrophularia koraiensis *	ETS: Jang Jang SO02, ITS: Jang SO02-110723	Jang and Oh unpublished data			JN984906.1	JQ065679.1		HQ130099.1
Scrophularia peregrina *	Wolfe s.n.	Wolfe *et al*. 2002				AF375146.1	AF375185.1	

The ITS region was amplified using ITS-Leu (forward; Andreasen *et al*. 1990) and ITS 4 (reverse; White *et al*. 1990) primers, and the ETS region was amplified using ETS B (forward, Beardsley and Olmstead 2002) and 18s (reverse, Baldwin and Markos 1998) primers. The coding region of the *mat*K gene was amplified using the two primers *mat*K 1f (forward) and *mat*K 1r (reverse) (Sang *et al.* 1997). The *psb*A-*trn*H intergenic spacer was amplified and sequenced using the primers psbA (forward) and trnH2R (GUG) (reverse) (Shaw and Small 2004).

All of the regions (ITS, ETS, *mat*K and *psb*A-*trn*H) were amplified using the Phusion Fast Green Hot Start II enzyme, with the master mix consisting of 1 µl 10 % DMSO (dimethyl sulfoxide), 13.4 µl H_2O, 4 µl PCR buffer, 0.4 µl dNTPs (deoxyribonucleotide triphosphate), 0.25 µl of each of the forward and reverse primers (10 mM) and 0.2 µl of the enzyme. The master mix was combined with 0.5 µl of DNA template resulting in a total reaction volume of 20 µl. Cycling conditions for the polymerase chain reaction (PCR) for each region are summarised in Table 2.3.

Table 2.3. Polymerase chain reaction (PCR) protocols, using Phusion Fast Green Hot Start II, for selected nuclear and plastid DNA regions in molecular phylogenetic analyses of *Glumicalyx*.

DNA region	Primer	Cycling conditions					
		Premelt	Denaturation	Annealing	Extension	Final extension	Number of cycles
ITS	ITS-Leu; ITS 4	98°C	98°C	52°C	72°C	72°C	30
ETS	ETS B; 18s	98°C	98°C	53°C	72°C	72°C	30
*mat*K	matK 1f; matK 1r	94°C	94°C	54°C	72°C	72°C	35
*psb*A-*trn*H	psbA; trnH2R	80°C	94°C	52°C	72°C	72°C	30–42

Diagnostic gels using electrophoresis were run on the PCR products, to check for the presence of bands sufficiently large DNA fragments. Only PCR products that yielded a bright, clear band were sequenced. The protocol for the gel electrophoresis is outlined in Appendix 2.2. The PCR products were purified using the Exo/SAP method (Werle *et al*. 1994; Bell 2008), in which 0.9 µl Exonuclease I and 1.8 µl of FastAP^{TM} Thermosensitive Alkaline Phosphatase were added to 18 µl of PCR product. The mixture was incubated in a thermal cycler at 37 °C for 15 min, with a stop reaction at 85 °C for 15 min. The purified PCR products were sequenced using Sanger sequencing (Sanger *et al.* 1977) using the same primers as in the PCR protocols at the Central Analytical Facility, Stellenbosch University.

Phylogenetic analyses

The forward and reverse sequences were edited by checking the sequences against the electropherograms and consensus sequences were created in Sequencher® v. 5.0 (Gene Code Corporation, Ann Arbor, Michigan, USA). The consensus sequences were exported in Fasta format and aligned in BioEdit v. 7.0.9.0 (Hall 1999) using the ClustalW Multiple alignment option (Thompson *et al*. 1994). The alignment was refined manually in BioEdit and apomorphies were confirmed by reference to electropherograms in Sequencher. Insertions and deletions (indels) were coded as binary characters using FastGap v. 1.2 (Borchsenius 2009), which applies the method of 'simple indel coding' of Simmons and Ochoterena (2000). Phylogenetic analyses were performed including and excluding indels and resultant topologies and support values were compared visually. The phylogenetic analyses including indels were implemented using Bayesian inference and maximum parsimony, as indels as binary states could not be analysed using maximum likelihood (PhyML).

Maximum parsimony, maximum likelihood and Bayesian Inference analyses were done for all regions sequenced. Maximum parsimony analysis was performed using TNT (Tree Analysis Using New Technology) v. 1.5 (Goloboff *et al*. 2003; Goloboff *et al*. 2008; Goloboff and Catalano 2016). The memory capacity set at 30 000 trees, followed by a traditional heuristic search with all characters equally weighted and unordered to obtain the most equally parsimonious tree. The parameters were set to Wagner trees, using tree bisection reconnection (TBR) with 1000 replications. A 50% majority rule consensus tree was constructed if multiple equally most parsimonious (EMP) trees were obtained for the molecular data. The branch support was estimated by conducting standard bootstrap analyses with 1000 repetitions (sample with replacement), and a 50% majority rule consensus tree was produced (Felsenstein 1985). The consistency index (CI) excluding uninformative characters and retention index (RI) of the EMP trees were calculated in Mesquite v. 3.2 (Maddison and Maddison 2011) and tree length was calculated in TNT.

Maximum likelihood was performed using PhyML version 3.0 (Guindon *et al*. 2010; Guindon and Gascuel 2003) and the Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist *et al*. 2012) run through the CIPRES Science Gateway version 3.3 (Miller *et al*. 2010). First, the appropriate model of evolution was determined in jModelTest v. 2.1.3 (Guindon and Gascuel 2003; Darriba *et al*. 2012) and using Smart Model Selection (SMS) in PhyML (Lefort *et al*. 2017). The substitution model for each region was chosen according to the Akaike information criterion (AIC) and AICc (corrected for small data sets). The models of evolution were compared between the different methods to ensure they were the same. The model of evolution for the ETS region was GTR + G, and the results were similar for the data set that included the two putative hybrids; for the ITS region it was GTR + G, but was GTR + G + I for the data set including putative hybrids; for the *mat*K region it was GTR + I (and also when including the putative hybrids); for the *psb*A-*trn*H region it was TN93 + G for both datasets. Indels were coded as a modified F81 model (Ronquist and Huelsenbeck 2003). For the maximum likelihood analyses, the branch support was estimated by conducting standard bootstrap analyses with 1000 repetitions, and a 50% majority rule consensus tree was produced. For the Bayesian analyses, phylogenetic inference was done using the Markov Chain Monte Carlo (MCMC) method (Ronquist *et al*. 2012; Huelsenbeck *et al*. 2004). In each analysis, two analyses of 10 000 000 generations sampled every 1 000 generations were run, each used four linked chains (three heated and one cold). Convergence of the analyses was assessed by checking that the average standard deviation of split frequencies was < 0.05.

Of the sampled trees, 25% were discarded as burn-in and a 50% majority rule tree was constructed from the remaining trees.

The phylogenies generated via separate analyses of the nuclear and plastid datasets were evaluated for congruence and if they were congruent combined (via concatenation) and Parsimony, Bayesian and Likelihood analyses were then implemented according to the search criteria described above, with different models assigned to the different partitions. An Incongruence Length Difference (ILD) test (Farris *et al*. 1994, 1995) executed as a Partition Homogeneity test with 1000 replications as implemented by PAUP* was run to test the incongruence between the various data sets, and we also compared the various topologies. No substantive incongruence between the ETS and ITS datasets was found (P = 0.923) and the datasets were combined. The two plastid regions, *mat*K and *psb*A-*trn*H were also combined. Supported incongruence between the nuclear and plastid DNA phylogeny was found, however the partition homogeneity test did not indicate significant incongruence (P = 0.082). I chose to present separate phylogenies for the nuclear and plastid gene regions.

Morphological variation

Vegetative and reproductive morphological characters were measured for all *Glumicalyx* species using herbarium specimens on loan from the Bews Herbarium (NU) and accessed at the National Herbarium (PRE) and J, as well as specimens collected during 2017 fieldwork. Numbers of specimens measured ranged from 8 (*G. apiculatus*) to 34 (*G. nutans*), using all available specimens (Appendix 2.3). Three replicates of each measurement were done and the data was averaged per specimen. The following vegetative features were measured: maximum leaf length and width using digital calipers and length of the longest branch (plant height) measured using a tape measure or ruler. The following floral features were measured using digital callipers: length of corolla tube, diameter of the opening to the corolla tube and the dimensions of the corolla lobes. The length of the inflorescence was also measured (from the tip of the inflorescence to the last bract) using digital callipers or a ruler.

To investigate phenotypic variation among the species, plots of the various means ± SE and a principal component analysis (PCA) were used to visualize the data. The PCA was implemented in PAST (Hammer *et al*. 2001) and used as a multivariate tool to visualize differences in floral traits between species. The mean ± SE plots were generated in SigmaPlot v. 8 (Systat Software Inc.). To investigate if there was a statistical difference in the vegetative and morphological traits between the six species of *Glumicalyx*, an analysis of variance

(ANOVA) and Tukey post-hoc analysis were used analyze the data (data were transformed if necessary to ensure normally distributions). The ANOVA and Tukey post-hoc test were implemented in RStudio version 3.3.3 (R Core Team 2018), using the base package.

Morphological and ecological character mapping

Characters were optimized onto the phylogeny of *Glumicalyx* to investigate character evolution within the genus. Characters were optimized onto the most parsimonious combined nuclear phylogeny generated using a single accession for each *Glumicalyx* species (randomly selected), and *Selago monticola* as outgroup. Characters that were optimized included the morphological measurements done on the herbarium specimens, additional categorical vegetative and floral features of *Glumicalyx* obtained from Hilliard and Burtt's (1977) review of the genus, as well as altitudinal range and habitat (according to field observations and the available literature) (Table 2.4). All taxa were coded as having a single state for each trait; character states for each species are given in Appendix 2.4–2.5. Using Mesquite v. 3.2 (Maddison and Maddison 2011), the morphological and ecological characters were mapped onto the phylogeny using the parsimony ancestral state reconstruction method.

Table 2.4. Characters and character states used in the morphological and ecological character optimizations in *Glumicalyx*.

Plant height: >280 cm (0), 220–270 cm (1), 150–200 cm (2).
Leaf length: 8–14 mm (0), 17–30 mm (1), >35 mm (2).
Leaf width: 2.2–2.8 mm (0), 3–4 mm (1), >6 mm (2).
Leaf indumenta: pubescent (0), glabrous (1).
Leaf shape: oblong-elliptic (0), elliptic (1), oblong-elliptic to obovate (2), ovate (3).
Upper leaf margin: entire to obscurely toothed (0), crenate-serrate (1).
Altitudinal zone: montane and subalpine (0), subalpine (1), subalpine and alpine (2).
Habitat type: grassy slopes near streams(0), cliffs, gullies and damp places (1), gravelly basalt rock sheets (2), boulder beds and rocky grass slopes (3)
Inflorescence length: 5–15 mm (0), 17–30 mm (1), >35 mm (2).
Bract length: 3–5 mm (0), 7–9 mm (1), >9 mm (2).
Bract width: 2–3.6 mm (0), 4.2–6 mm (1), >7 mm (2).
Calyx length: 2.6–3.5 mm (0), 4.8–5.2 mm (1), >7.5 mm (2).
Tube length: 1.8–4.4 mm (0), 8.8–10 mm (1), 10.6–12 mm (2), >22 mm (3).
Limb diameter: 3–4.5 mm (0), 4.6–5.8 mm (1)
Throat diameter: 1.1–1.34 mm (0), 1.4–1.64 mm (1), >1.95 (2)
Corolla lobe colour: white (0), pale-yellow (1), orange to red (2).
Inflorescence shape: globose (0), turbinate (1).
Corolla lobe shape: elliptic (0), elliptic to oblong to suborbicular (1), suborbicular (2), narrowly oblong (3).
Number of stamens exerted: 4 (0), 2 (1).
Anther length: equal in length (0), unequal in length (1).

Results

Phylogenetic analysis

To test the monophyly of *Glumicalyx* and to hypothesise species relationships molecular phylogenetic analyses were conducted on six species of *Glumicalyx*, with up to 25 accessions, and up to nine outgroup species from six genera. Topologies generally differed only slightly between the maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) reconstructions, with generally better support for the latter two reconstructions than the MP reconstructions.

Nodes were considered to be strongly supported if Bootstrap support (BS) values were equal to or greater than 80% and posterior probabilities (PP) were equal to or greater than 0.95. Nodes were moderately supported if the BS values were between 70% and 80% and if the PP were between 0.90 and 0.95. Characteristics for the four datasets analysed are described in Table 2.5. Support values are presented as bootstrap values (%) = maximum likelihood/maximum parsimony, posterior probabilities = Bayesian inferences (BS = (ML/MP) %, PP = BI).

Table 2.5. Characteristics of the individual locus datasets sequenced for *Glumicalyx* and the outgroup taxa.

Locus	ETS	ITS	Combined nuclear	*mat*K	*psb*A-*trn*H	Combined plastid
Genome	Nuclear	Nuclear	Nuclear	Plastid	Plastid	Plastid
Sequenced length range (bp)	500–506	677–691	NA	1255–1270	367–442	NA
Aligned length (bp)	520	717	1237	1853	608	2461
Number of indels	30	37	67	39	88	129
Parsimony-informative characters	126	97	223	60	58	118
Number of equally most parsimonous trees (with coded indels, without coded indels)	3, 3	123, 11	213, 20	6, 1	3, 4	15, 89
Tree length (with coded indels, without indels)	286, 248	293, 217	470, 589	1173, 1037	874, 354	1176, 545
Retention index (with coded indels, without indels)	0.857, 0.857	0.840, 0.845	0.842, 0842	0.838, 0.843	0.616, 0.744	0.828, 0.831
Consistency index (with coded indels, without indels)	0.827, 0827	0.807, 0.811	0.81, 0.811	0.973, 0.974	0.87, 0.909	0.908, 0.91
% Taxa with sequence data [% of all *Glumicalyx* taxa (n = 25)]	100	96	100	60	60	60

Phylogenies based on the nuclear regions: ETS and ITS

Topologies of the ETS and ITS phylogenies were similar in many ways, but were not identical. The MP, ML and BI reconstructions were largely congruent for the ETS region. In the ITS analyses the topology of the MP and BI reconstructions were largely congruent, but the maximum likelihood reconstruction differed. For both the ITS and ETS regions, the indels were informative and resulted in a better supported phylogeny for the BI and MP analyses, and did not affect the topology in the BI reconstruction. The BI reconstructions with for the nuclear regions with indels coded are presented.

Selago was sister to *Glumicalyx* in the ITS reconstruction (BS = 24%, PP = 0.93), and in the ETS reconstruction, where *Selago* was not included, *Glumicalyx* was sister *Zaluzianskya* (BS = 100, PP = 0.95). For both the ETS and ITS regions *Glumicalyx* formed a monophyletic group (ETS: BS = 81/99%, PP = 0.94; ITS: BS = 57/81%, PP = 0.98; Figures 2.2 and 2.3). One of the main differences between the ETS and ITS topologies is the position of *G. montanus*; in the ITS reconstruction the accessions of *G. montanus* form an unresolved clade sister to the rest of the *Glumicalyx* species (Figure 2.3). In the ETS reconstruction, *G. montanus* forms a moderately-supported clade with *G. nutans* and *G. lesuticus* (BS = 79/64%, PP = 0.96), and *G. apiculatus* is sister to the rest of the *Glumicalyx* species (Figure 2.2). In both the ETS and ITS reconstructions *G. nutans* is paraphyletic, with *G. lesuticus* nested inside (ETS: BS = 89/90%, PP = 0.99; ITS: PP = 0.73). In the ITS reconstruction, *G. flanaganii* is sister to *G. apiculatus* (PP = 0.98), however this clade collapses in the MP reconstruction (Figure 2.3). In the ETS reconstruction, *G. apiculatus* and *G. flanaganii* together with *G. goseloides* form an unresolved clade (Figure 2.2). In ITS reconstructions *G. apiculatus* and *G. flanaganii* form a clade, but there is a lack of resolution between these two species in the ETS reconstructions. In both the ETS and ITS reconstructions a second clade by *G. lesuticus* and *G. nutans*. Overall the ITS reconstruction is less resolved than the ETS, especially with regards to providing synapomorphies for the species. There were very few informative point mutations to support monophyly of the species. The individual ETS and ITS reconstructions for each analysis are presented in the Appendix (Appendix 2.8–2.13).

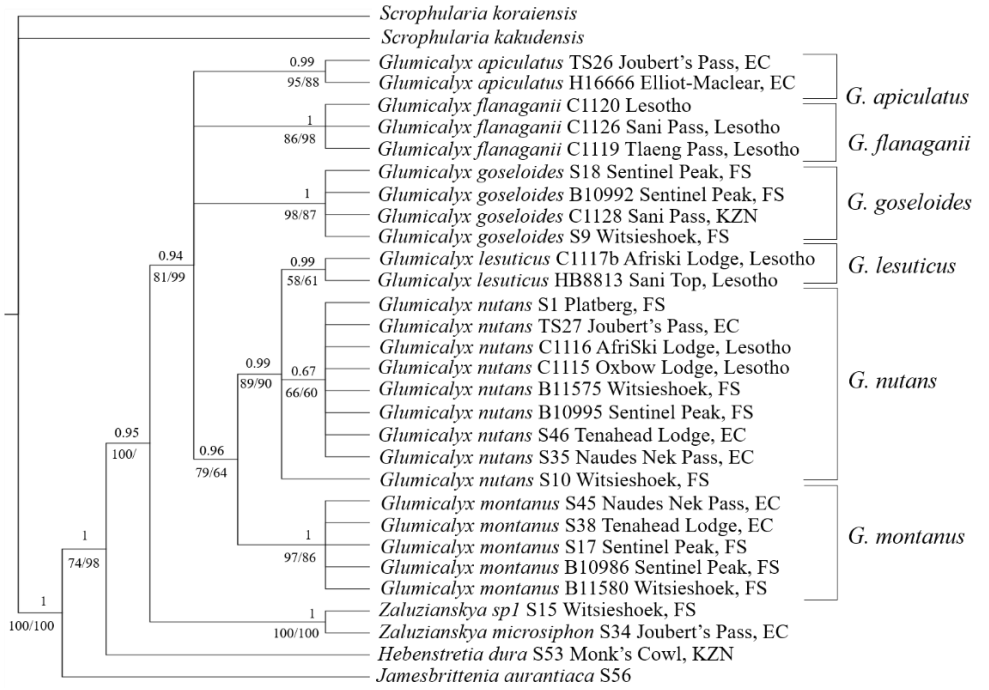


Figure 2.2. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the ETS region with indels coded. Bootstrap values (%) are presented below the lines for ML/MP, and PP values are presented above the line.

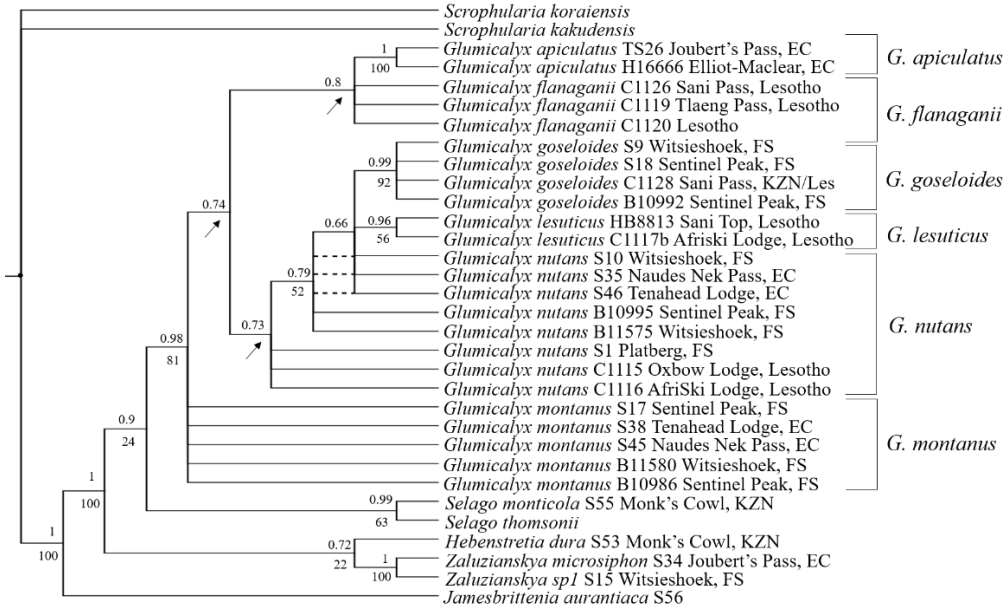


Figure 2.3. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum credibility consensus of the Bayesian inference of the ITS region with indels coded. Posterior probability values are presented above the line and MP bootstrap support below the line. Arrows below the line indicate nodes that collapsed in the MP analysis and dotted lines indicate where the clades differed in the MP analysis.

Although there was topological incongruence between the ETS and ITS reconstructions, the incongruence was not well supported. Many of the clades collapsed in both the ETS and ITS reconstructions. The combined analysis of the ETS and ITS regions resulted in the same topology for all three analyses (MP, ML and BI), and is similar to that of the ETS reconstruction, but with better support. Unlike in the single ETS and ITS reconstructions, the indels for the combined nuclear reconstruction resulted in overall lower support in the MP and BI analyses. (Combined nuclear phylogeny with coded indels presented in Appendix 2.12).

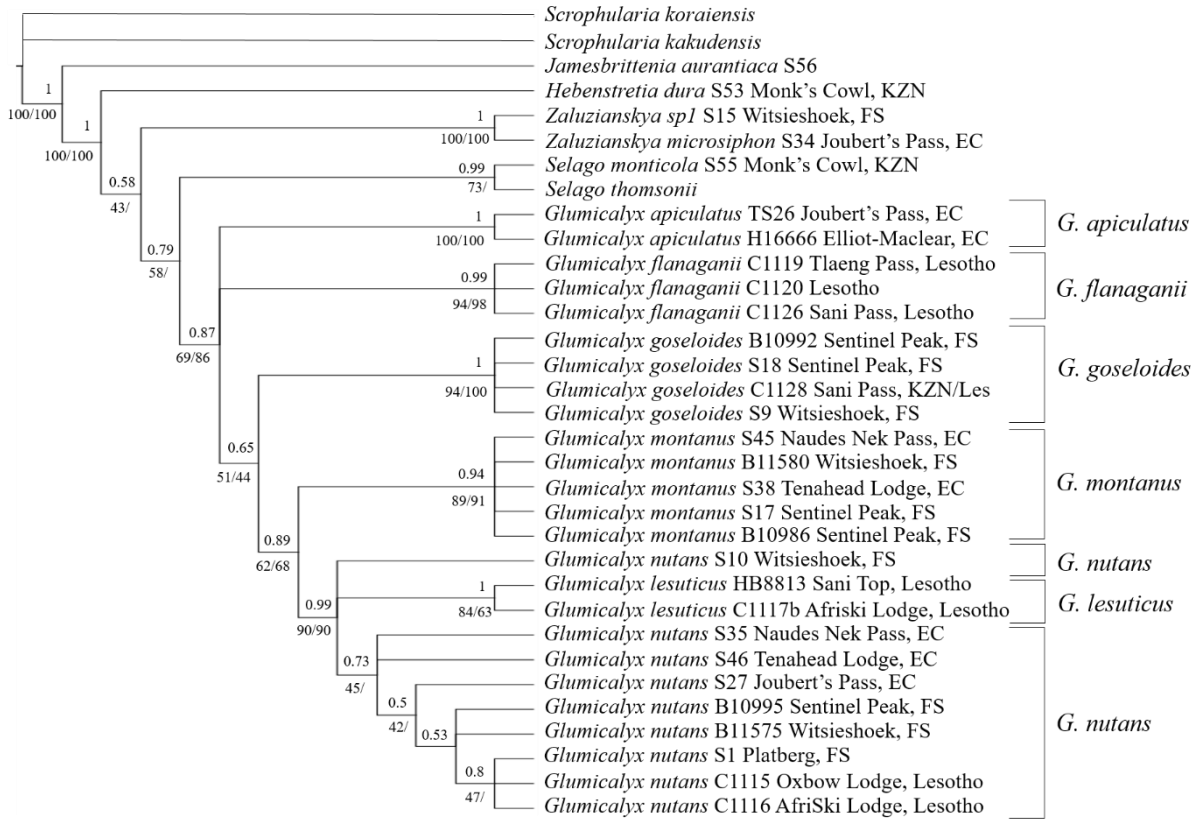


Figure 2.4. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the combined ETS and ITS region (indels not included). Posterior probabilities are presented above the line and bootstrap values (%) are presented below the lines for ML/MP.

Glumicalyx forms a moderately-supported monophyletic group (BS = 69/86%, PP =0.87, Figure 2.4). *Glumicalyx apiculatus* and *G. flanaganii* form a polytomy at the base of the clade that is sister to the remaining species of *Glumicalyx*. *Glumicalyx goseloides* is sister to a clade comprising *G. lesuticus*, *G. montanus* and *G. nutans* (BS = 51/44%, PP = 0.65), with *G. montanus* forming a well-supported clade (BS = 89/91%, PP = 0.94) and is sister to *G. lesuticus* and *G. nutans*. *Glumicalyx nutans* and *G. lesuticus* form a well-supported clade

(BS = 90/90%, PP = 0.99) with *G. lesuticus* well-supported (BS = 84/63%, PP = 1) and nested within *G. nutans*. The *G. nutans* accessions from the Platberg in the eastern Free State and Lesotho (Oxbow and Afriski Lodge) form a moderately-supported clade (BS = 47, PP = 0.8).

Phylogenies based on the plastid regions: matK and psbA-trnH

As in the analyses based on the nuclear DNA regions, *Glumicalyx* forms a monophyletic group in the phylogeny based on the plastid *mat*K region (BS = 99%, PP =1), *psb*A-*trn*H region (PP = 0.93) and the combined plastid (BS = 88%, PP = 0.91). The combined plastid analysis resulted in a phylogeny that was similar to the *mat*K reconstruction (appendix 2.14–2.17) with some clades showing better resolution. The indels were particularly informative for the combined plastid analysis, providing better support and more resolved clades. The BI reconstruction is presented, and the topology was largely congruent with the MP reconstruction, however the BS support for the MP was very low for most clades. *Glumicalyx nutans* is paraphyletic, with two accessions from the Eastern Cape (S35, S46) as sister taxa (BS = 98%, PP = 1) which are sister to rest of the *Glumicalyx* clade (Figure 2.5). A poorly supported clade is formed between *G. apiculatus* and two of the three *G. montanus* accessions – the same clade, but with better support, also formed in the *mat*K reconstruction (Figure 2.5). The third accession of *G. montanus* is sister to the clade of the remaining species (PP = 0.99); the clade contains *G. goseloides* and *G. nutans* forming a polytomy with a subclade comprising *G. lesuticus* and a *G. nutans* accession from Lesotho (PP = 0.55), and second well-supported subclade of *G. flanaganii* (BS = 95%, PP = 0.97).

The *psb*A-*trn*H region resulted in a very poorly resolved phylogeny (Appendix 2.18) there were many insertions and deletions in the alignment and as a result the indels were informative and improved the tree by providing better resolution to *G. lesuticus* and better support overall.

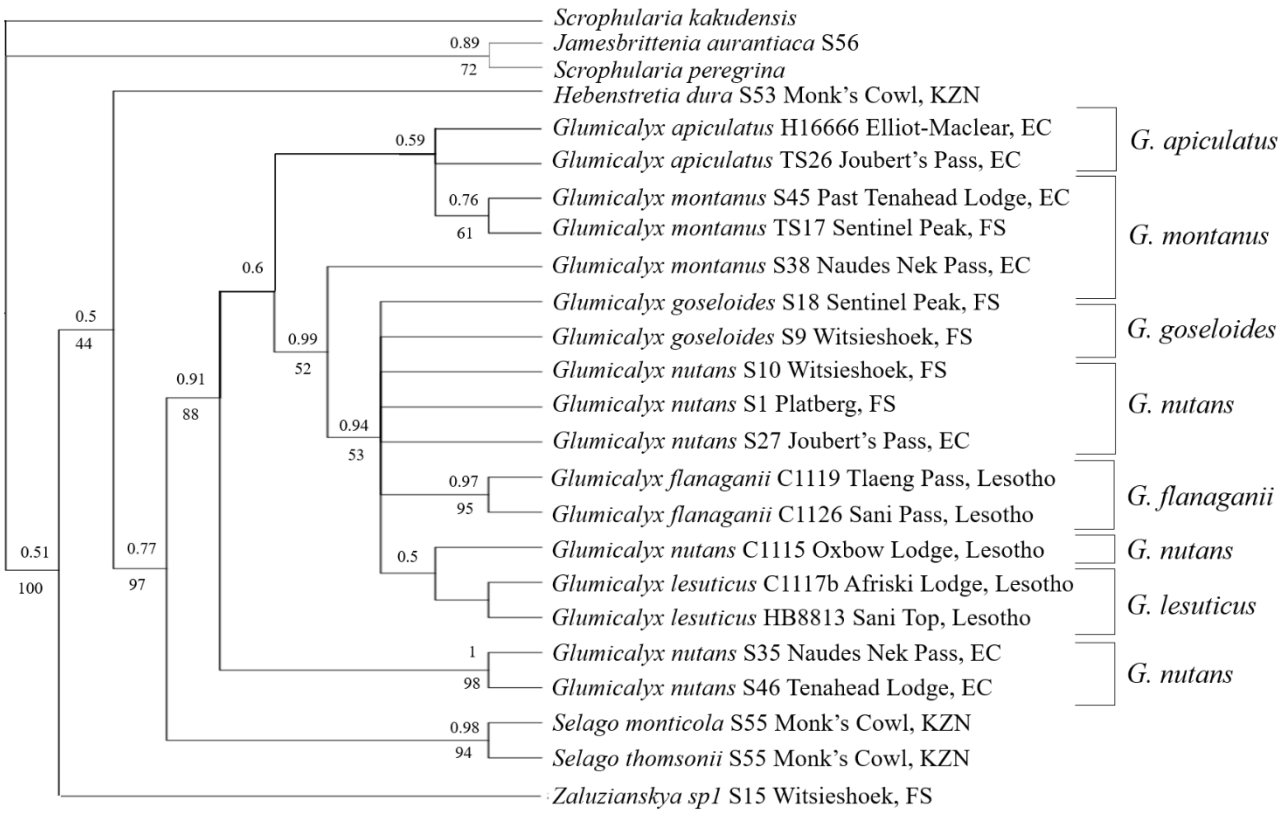


Figure 2.5. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the combined *mat*K and *psb*A-*trn*H with indels coded. Posterior probabilities are presented above the line and bootstrap values (%) are presented below the lines for MP.

Morphological traits

Vegetative and floral morphological traits were measured for all six species of *Glumicalyx* to investigate the morphological variation within the genus and its implications in the evolution of the genus. Three vegetative traits were measured and compared, viz. plant height, leaf length and leaf width, and six floral traits; inflorescence length, bract length, calyx length, corolla tube length, corolla limb diameter, corolla tube diameter (Figure 2.6). Plant height differed significantly between the larger species, *G. goseloides*, *G. flanaganii* and *G. nutans* and the smaller species *G. apiculatus*, *G. lesuticus* and *G. montanus* (Figure 2.6 a). Leaf length differed significantly between the species overall, but was not significant between the three smaller species, *G. apiculatus*, *G. lesuticus* and *G. montanus* (Figure 2.6 b). Leaf width differed significantly between the larger and smaller species, i.e. it was significantly different between *G. apiculatus* and *G. goseloides*, *G. flanaganii*, *G. nutans*, *G. montanus*; between *G. lesuticus* and *G. goseloides*, *G. flanaganii*, *G. montanus*; and between *G. montanus* and *G.*

goseloides, *G. flanaganii* and *G. nutans* (Figure 2.6 c). Inflorescence length differed between the species, except between *G. apiculatus* and *G. montanus*, *G. flanaganii* and *G. nutans*, and *G. montanus* and *G. lesuticus* (Figure 2.6 d). Bract length differed between all of the species, except between *G. flanaganii* and *G. nutans* (Figure 2.6 e). Calyx length was significantly different between the larger species — *G. flanaganii*, *G. goseloides* and *G. nutans*, and the smaller species — *G. apiculatus*, *G. lesuticus* and *G. montanus* (Figure 2.6 f). Corolla tube length differed significantly between all species, except between *G. apiculatus* and *G. montanus*, and *G. flanaganii* and *G. lesuticus* (Figure 2.6 g). The diameter of the corolla limb only differed in *G. apiculatus* compared to the other species, as it had the narrowest corolla limb (Figure 2.6 h). The corolla tube width only differed significantly in *G. montanus* compared to the other species, as it had the widest corolla tube (Figure 2.6 i). For the level of significance for each morphological trait refer to Appendix 2.22.

Based on the multivariate analysis of the vegetative morphological traits, *Glumicalyx* species form two clusters along PC1 and PC2, which explain 73% and 19% of the variation in vegetative traits respectively, i.e. a total of 92% (Figure 2.7). Three vegetative traits were included, plant height, leaf length and leaf width. Variation along PC3 has a positive contributions from plant height and leaf width, and a negative contribution from leaf length. *Glumicalyx goseloides* and *G. flanaganii* cluster together in the right upper quadrant, whereas *G. apiculatus*, *G. lesuticus*, *G. montanus* cluster closely together towards the left hand side, particularly in the left upper quadrant. *G. nutans* is morphologically very variable, with some overlap with the *G. apiculatus*, *G. lesuticus*, *and G. montanus* cluster (Figure 2.7). Variation along PC3 explains 8% of the total variation in vegetative traits in *Glumicalyx*, with leaf width making a positive contribution and plant height and leaf length both making negative contributions (plot not shown).

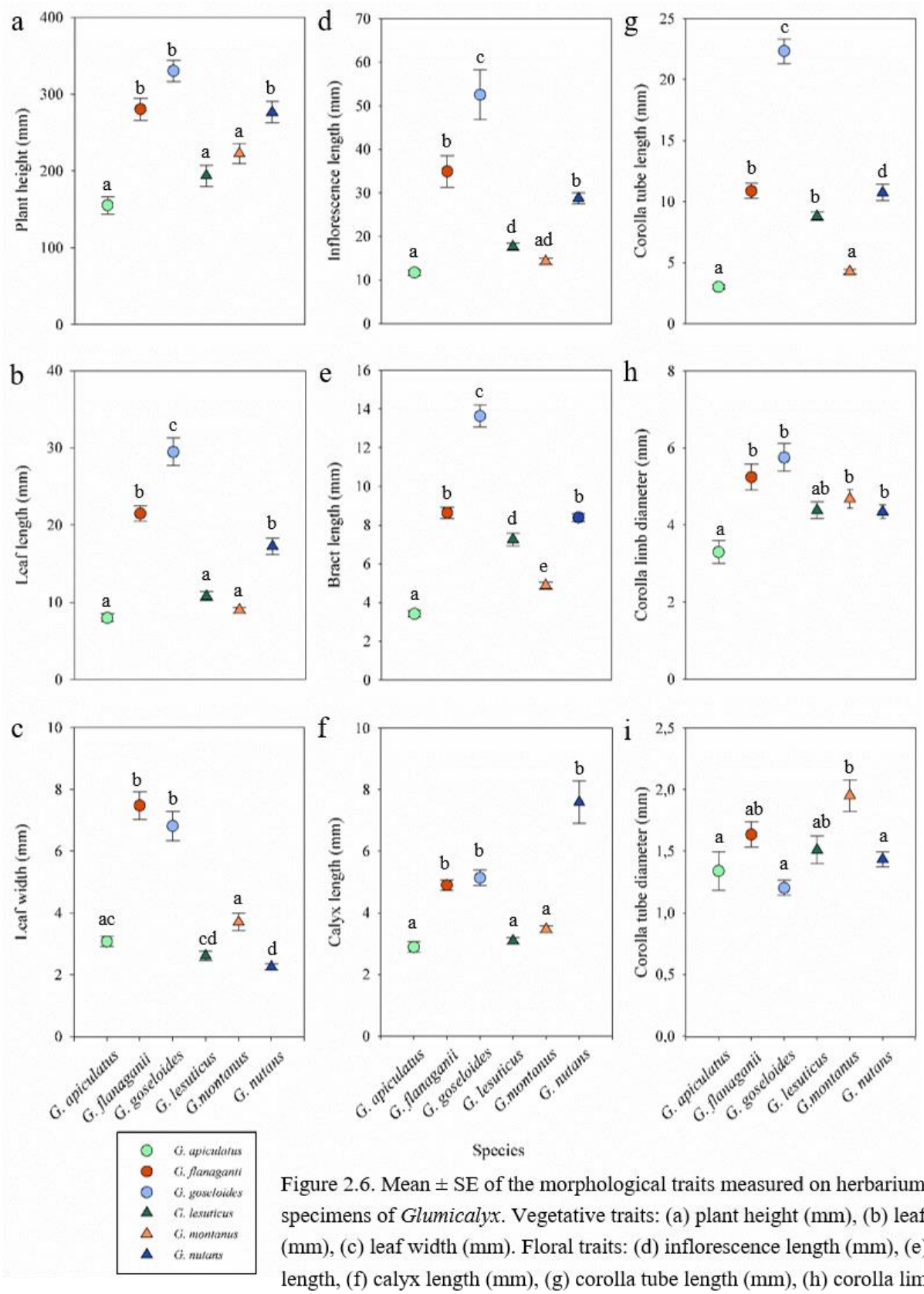


Figure 2.6. Mean ± SE of the morphological traits measured on herbarium specimens of *Glumicalyx*. Vegetative traits: (a) plant height (mm), (b) leaf length (mm), (c) leaf width (mm). Floral traits: (d) inflorescence length (mm), (e) bract length, (f) calyx length (mm), (g) corolla tube length (mm), (h) corolla limb diameter (mm), (i) corolla tube diameter (mm). Means for a particular attribute with different superscript letters are significantly different ($P < 0.05$).

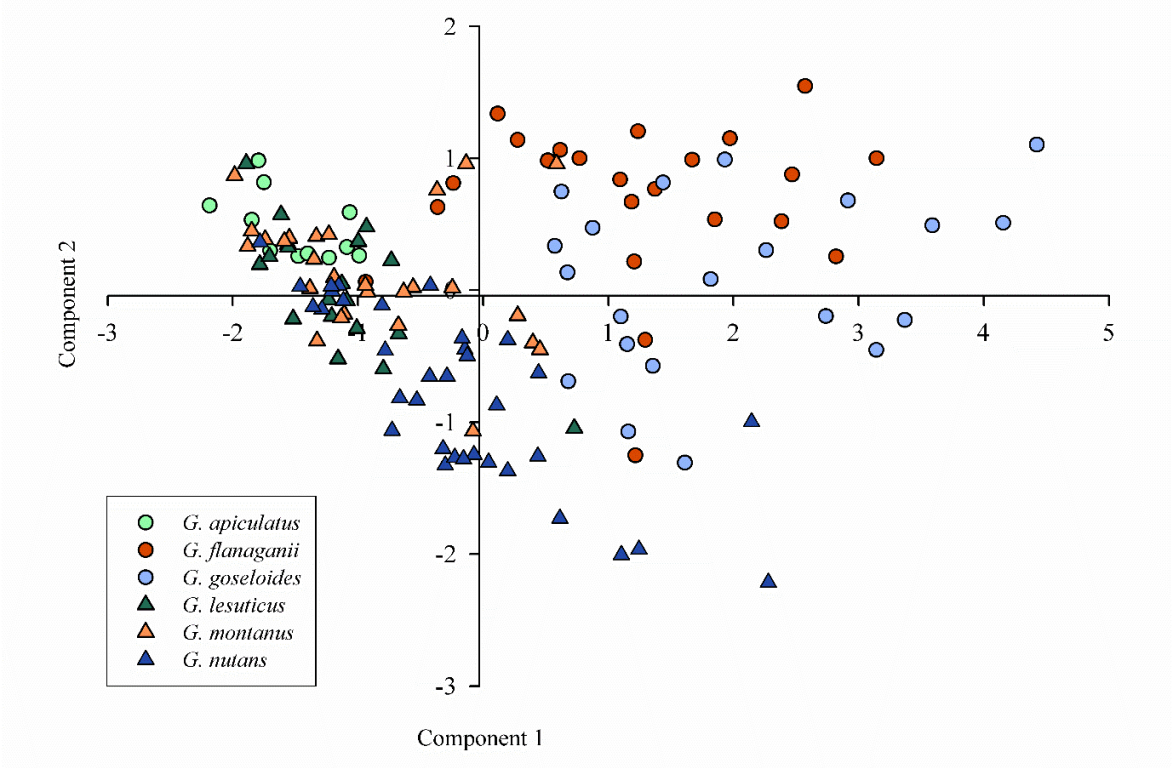


Figure 2.7. Principal component analysis of three vegetative morphological traits (plant height, leaf length and leaf width) measured on herbarium specimens of *Glumicalyx*. Principal component 1 represents 73% and principal component represents 19% of the variance.

Based on the multivariate analysis of the floral morphological traits, *Glumicalyx* species cluster along a continuum, whilst forming discrete groupings along PC1 and PC3, which explain 59% and 12% of the variation in floral traits, respectively, i.e. a total of 71% (Figure 2.8). The species did not however form discrete groupings along PC1 and PC2, with PC2 explaining 17% of the total variation (figure not shown).Seven floral traits were included, including inflorescence, bract, calyx and corolla measurements. Apart from calyx length, all traits contribute positively to PC1. Loadings along PC3 are more variable, with corolla limb diameter and bract width making the largest positive contribution and inflorescence length making the largest negative contribution. The species fall on a continuum going from the upper left quadrant to the upper right quadrant, with *G. apiculatus* first followed by *G. montanus*, *G. lesuticus*, *G. nutans* and *G. flanaganii*. *Glumicalyx goseloides* clusters separately from the other five species on the upper right quadrant (Figure 2.8). The position of the species is linked to size, as *G. apiculatus* furthest to the left is the smallest and *G. goseloides* furthest to the right is the largest. *Glumicalyx nutans* overlaps with *G. lesuticus* and *G. flanaganii*.

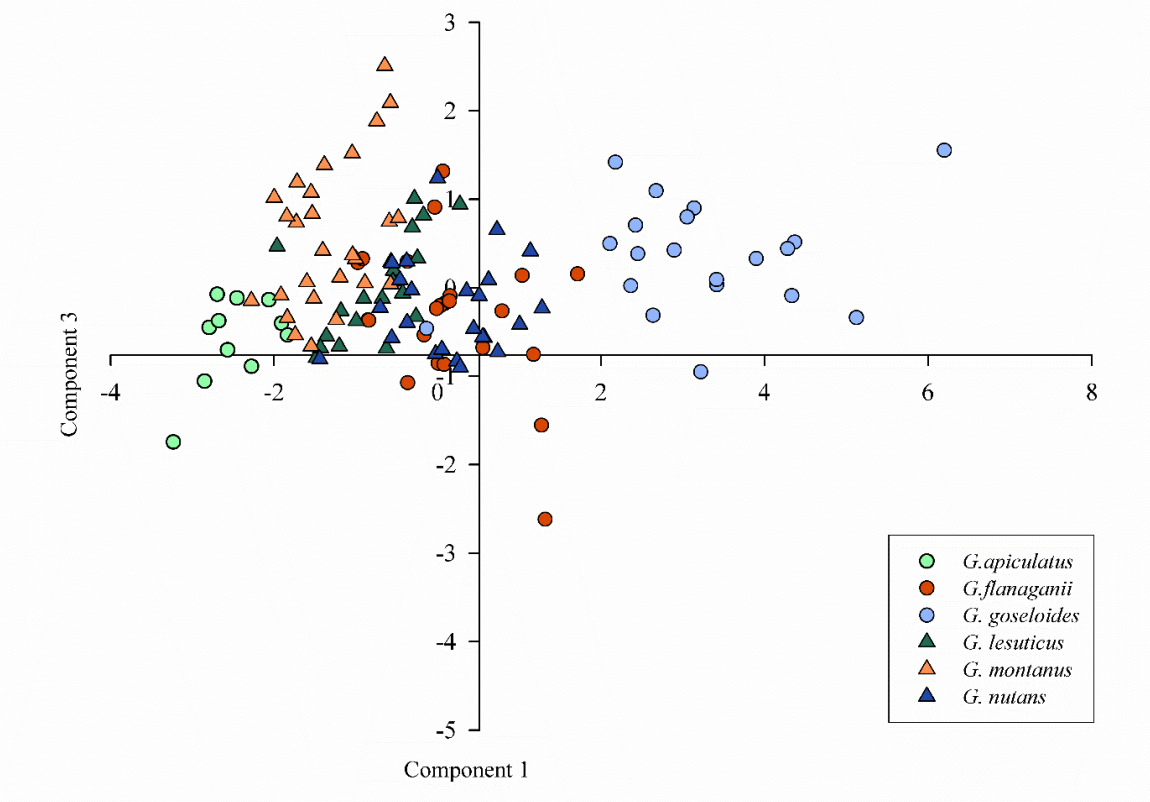


Figure 2.8. Principal component analysis of seven floral morphological traits (inflorescence, bract, calyx and corolla tube length, bract width, limb diameter and corolla tube diameter) measured on herbarium specimens of *Glumicalyx*. Component 1 represents 59% and component 3 represents 12% of the variance.

Morphological and ecological character mapping

Selected morphological traits, habitat type and altitudinal zone were mapped onto the combined nuclear phylogeny with a single accession per species (Figure 2.9). *Glumicalyx apiculatus*, *G. montanus* and *G. lesuticus* all occur in similar habitats, and there are habitat shifts in *G. flanaganii*, *G. goseloides* and *G. nutans*. The morphological traits selected show that *G. goseloides* is larger than the other species, in terms of leaf length, inflorescence length and corolla tube length. Morphological traits including corolla lobe colour, pubescent leaf surface, short inflorescence and corolla tube, and habitat are all shared between *G. montanus* and *G. apiculatus*. A shift to two stamens exerted from the corolla tube and two inserted in the corolla with anthers of unequal length in *Glumicalyx goseloides* and *G. nutans*, from all four stamens exerted and of equal length. *Glumicalyx flanaganii* shows shifts in habitat and altitudinal zone (Figure 2.9 a), leaf length (Figure 2.9 b), inflorescence size and corolla lobe colour (Figure 2.9 c) and the length of the corolla tube (Figure 2.9 d).

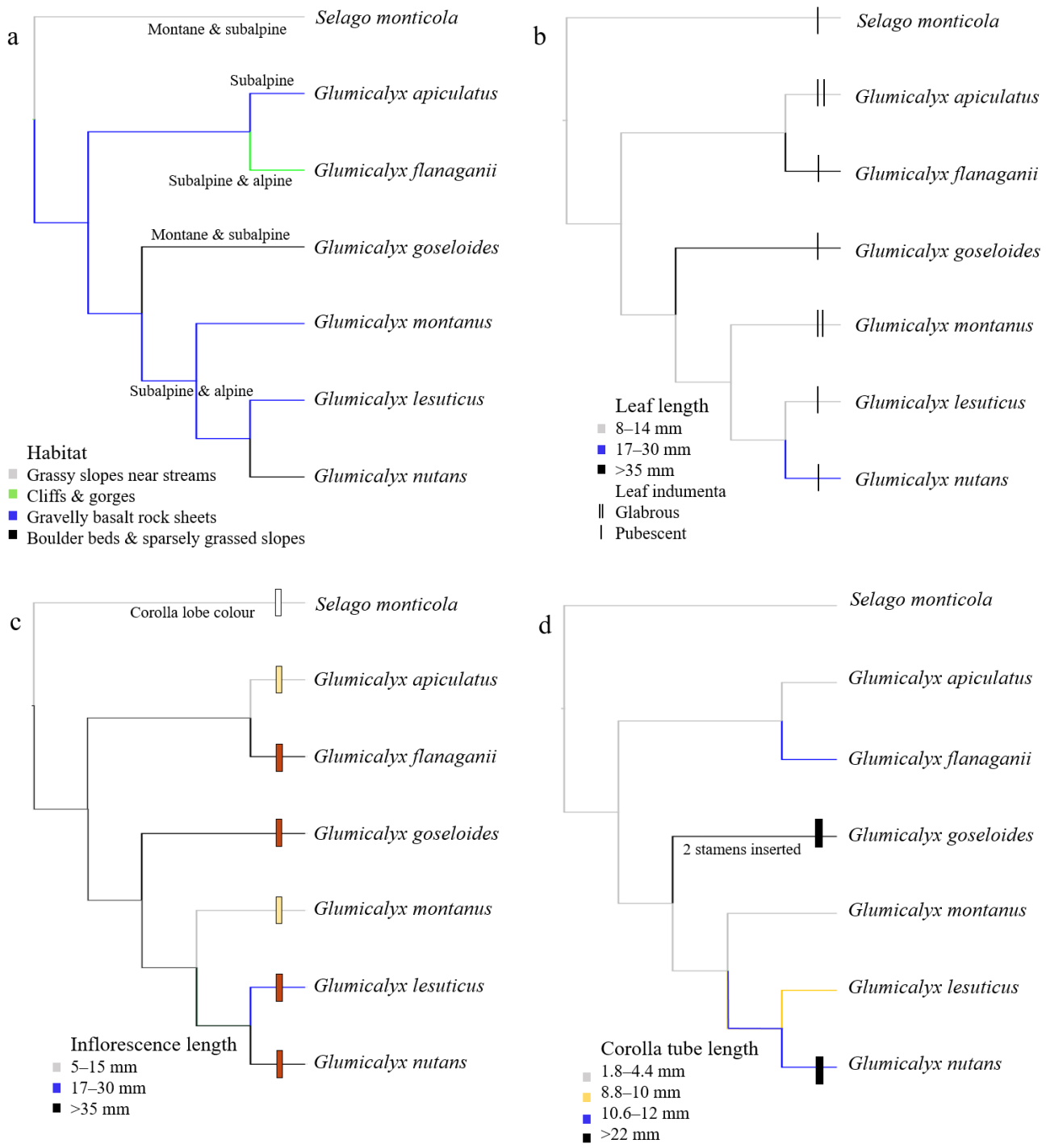


Figure 2.9. Morphological and ecological characters optimized on the combined nuclear phylogeny resulting from the maximum parsimony analysis of *Glumicalyx* (single accession per species) and *Selago monticola* as outgroup, (a) habitat and altitudinal zone, (b) leaf length (mm) and leaf surface, (c) inflorescence length (mm) and corolla lobe coloration, (d) corolla tube length (mm) and number of stamens inserted in the corolla tube.

Discussion

Phylogenetic relationships within the genus

Within the sampling of this study, *Glumicalyx* is clearly a monophyletic genus in both the nuclear and plastid molecular phylogenetic analyses. *Selago* is the sister genus to *Glumicalyx* when it was included, in three out of four analyses. Investigating the sister relationships of *Glumicalyx* within the Limoselleae was not an aim of this study and the sampling within the tribe was not comprehensive enough to be able to unambiguously establish sister genera, however our results do correspond to other phylogenies. Hilliard (1994) noted that although a number of *Glumicalyx* species were previously placed in *Zaluzianskya*, they were not particularly closely related to one another. This was later confirmed by Kornhall *et al*. (2001) in their phylogeny of the Manuleeae and Selagineae based on *ndh*F, *Glumicalyx* was placed in a separate clade to *Zaluzianskya* within the Manuleeae clade. In a separate clade, *Glumicalyx* was sister to a clade comprised of the genera *Tetraselago*, *Melanospermum*, *Selago*, *Microdon* and *Cromidon*. *Strobilopsis* Hilliard and Burtt is a monotypic genus endemic to the DMC and is particularly rare. In the phylogeny of Kornhall *et al*. (2001) *G. flanaganii* was sister to *Strobilopsis wrightii*. The outgroup taxa used in this study formed part of the former tribes Selagineae (*Hebenstretia* and *Selago*) and Manuleeae (*Jamesbrittenia* and *Zaluzianskya*). *Glumicalyx*, *Selago* and *Tetraselago* Junell were suggested by Hilliard and Burtt (1977) to be the link between the Manuleeae and Selagineae.

The nuclear reconstructions were overall more resolved than the plastid reconstructions, although the support for the monophyly of the genus was strongest in the *mat*K phylogeny. There was incongruence between all the DNA regions, most of the incongruence was in poorly resolved clades. However, there was supported incongruence between the combined nuclear phylogeny and the *mat*K phylogeny. In the nuclear phylogeny *G. montanus* is sister to *G. nutans* and *G. lesuticus*, and in the *mat*K phylogeny *G. montanus* is sister to *G. apiculatus*. The plastid phylogeny shows a closer relationship between morphologically similar species, ie. *G. apiculatus* and *G. montanus*, and *G. flanaganii* and *G. goseloides*, than is evident in the nuclear phylogeny. In both the nuclear and plastid phylogenies *G. nutans* is paraphyletic and *G. lesuticus* is nested within *G. nutans*. *Glumicalyx lesuticus* diverged from *G. nutans*, and forms a well-supported species clade. There is insufficient variation in both the nuclear and plastid regions to resolve species relationships in *Glumicalyx.*

Of the six *Glumicalyx* species, only *G. nutans* was not monophyletic. A single point mutation separated the *G. nutans* accessions in the *mat*K analysis, i.e. the two accessions from the Eastern Cape and the remainder of the accessions. The low number of mutations supporting the species possibly results in there being no buffer from homoplasious characters within a species. *Glumicalyx nutans* is the most widely distributed species of *Glumicalyx*, occurring across the Drakensberg Mountain Centre (eastern Free State, KwaZulu-Natal Drakensberg, Eastern Cape Drakensberg and Lesotho Maloti's) over a wide altitudinal range (1800–3350 m a.s.l). Populations of *G. nutans* from similar localities tended to be placed closer to each other, including the accessions from Lesotho, Eastern Cape and the Free State. *Glumicalyx nutans* is paraphyletic in both the nuclear plastid reconstructions, and in the plastid phylogeny two accessions from the Rhodes district of the Eastern Cape are sister to the result of *Glumicalyx*; this may be as a result of the large distribution of *Glumicalyx*, whereby there is genetic diversity within the species.

Incongruence between datasets and possible hybridization

Poor taxon sampling is suggested to cause incongruence (Wendel and Doyle 1998); this unlikely to explain the incongruence in *Glumicalyx* as there were multiple accessions for the six species of *Glumicalyx*. The incongruence between the nuclear and plastid topologies, and among the nuclear topologies, may be as a result of hybridization, gene duplication, horizontal transfer and lineage sorting, as all these possibilities may result in patterns of incongruence in molecular phylogenies (Maddison 1997; Wendel and Doyle 1998; Andersson 1999; Sang and Zhong 2000; Holder *et al.* 2001; Sota *et al*. 2001; van Oppen *et al*. 2001; Kimball *et al*. 2003; Rokas *et al*. 2003; van der Niet and Linder 2008; Cron *et al*. 2008). Speciation by hybridization is common in plants (Hegarty and Hiscok 2005), and as a result hybrid events are important in the species tree (Linder and Rieseberg 2004; van der Niet and Linder 2008). To further investigate hybrid origins, the use of multiple low-copy nuclear genes is needed (Smedmark *et al*. 2003; Mason-Gamer 2004; van der Niet and Linder 2008).

Within the context of *Glumicalyx*, the reasons for the incongruence are not yet known, however hybridization, both current and historical, appears to occur in *Glumicalyx*. Hilliard and Burtt (1977) reported putative hybridization between *G. goseloides* and *G. nutans* in a large population on the road to Sentinel Peak, Witsieshoek district, Free State. The large population of *G. nutans* thought to have been affected by introgression by *G. goseloides* does not appear to still exist, as the area described has been almost completely encroached by

woody species. A further 2 km along the road to Sentinel, I found a specimen that appeared intermediate in floral morphology between *G. goseloides* and *G. nutans* located between two populations. Another intermediate specimen was found in a co-occurring population of *G. montanus* and *G. nutans* (Tenahead Lodge, Rhodes, Eastern Cape). These specimens were considered putative hybrids and when included in the phylogenetic analyses, they either clustered with *G. goseloides, G. nutans* or *G. lesuticus* depending on the data set. These putative hybrids did not cause the collapse of any clades in the phylogeny. Considering the variability of *G. nutans*, it is unclear if both of these putative hybrids are true hybrids or variable specimens of *G. nutans*. The morphology of these putative hybrids and those collected by Hilliard and Burtt (1977) should be included in the morphological analyses to investigate if they are intermediate in morphology.

Hybridization is a common occurrence in the Manuleeae (≈Limoselleae), particularly in areas of sympatry in species of *Jamesbrittenia*, *Manulea* L., *Polycarena* Benth and *Sutera* Roth as observed by Hilliard (1994). Incongruence in the *Jamesbrittenia* phylogeny spans several well-supported clades, therefore incomplete lineage sorting is unlikely (Rosenburg 2003), and it is more likely to be hybrid-mediated lateral gene transfer (Verboom *et al*. 2016). There is a general lack of post-zygotic isolation in *Jamesbrittenia*, which allows species to produce fertile hybrids. Although there is only one documented case of putative hybridization in *Glumicalyx* (Hilliard and Burtt 1977), the species occur in overlapping altitudinal zones and geographical areas, albeit different microhabitats. Almost all of the species have been recorded to co-occur within a small geographical area, for example *G. goseloides* and *G. montanus* occur less than 5 m apart at the base of Sentinel Peak, Witsieshoek district, Free State. These co-occurring species have different floral morphology, indicating the potential role of pollinators in maintaining reproductive isolation in sympatric species of *Glumicalyx*.

Evolutionary trends and speciation

Based on its morphology and ecology, *Glumicalyx* appears to have evolved in response to both its abiotic environment and to biotic factors (including pollinators). *Glumicalyx flanaganii, G. lesuticus, G. montanus* and *G. nutans* all occur in the subalpine and alpine zones of the Drakensberg. Alpine vegetation is exposed to extreme conditions and this limits the type of plants that can occur in this zone, and in the DMC, the alpine vegetation is dominated by *Helichrysum* and *Erica* heath (Killick 1994). Four vegetation types generally dominate mountain summits – dwarf shrubs, grasses and sedges, rosette plants and cushion plants (Körner 2007). Plants growing in alpine zones usually have adaptive features, for

example they are often evergreen and have woolly trichome covered leaves (van Wyk and Smith 2001). *Glumicalyx* differs from the typical vegetation adapted to withstand the alpine conditions – the genus is perennial, however the plants die back in the winter leaving only the woody rootstock, the flowering stems show annual growth (Hilliard and Burtt 1977).

The six species occur in different habitats, and these differences may correspond to some of their vegetative traits. For example, *Glumicalyx flanaganii* grows in damp habitats, including the edge of cliffs and in gullies, and has lush vegetative growth, including stems with many leaves and larger ovate leaves. *Glumicalyx apiculatus* and *G. montanu*s both occur on gravelly basalt rock sheets and sparsely grassed slopes, and they are smaller herbs that develop thick woody rootstocks, possibly an adaptation to this harsh habitat.

Glumicalyx flanaganii and *G. apiculatus* diverge first in the *Glumicalyx* phylogeny, and in some analyses they were sister taxa (although the clade usually collapsed or was poorly supported). These species are morphologically and ecologically distinct, and do not co-occur. They inhabit different habitats and occur at different altitudinal zones – *G. apiculatus* occurs in the subalpine zone (2200–2560 m a.s.l.) on gravelly basalt rock sheets, whereas *G. flanaganii* occurs in both the subalpine and alpine zones (2400–3350 m a.s.l.). With regards to microhabitat, *G. flanaganii* is distinct from the other five species as it is restricted to damp rocky places, such as stream gullies and the foot of cliffs. The differences in reproductive traits, including floral size (corolla tube length is 3 mm in *G. apiculatus* and 11 mm in *G. flanaganii*) and corolla lobe colour (yellow in *G. apiculatus* and brick-red in *G. flanaganii*) suggest that these species have different pollination systems. *Glumicalyx apiculatus* and *G. flanaganii* likely speciated in allopatry by ecological speciation, through adaptations to different microhabitats and pollinators.

Glumicalyx goseloides, like *G. apiculatus* and *G. flanaganii*, has poor resolution within the *Glumicalyx* phylogeny in the nuclear analysis. In the *plastid phylogeny G. goseloides* is nested within *G. nutans*, forming a polytomy. This relationship is likely due to the lack of resolution in the plastid phylogeny. *Glumicalyx goseloides* co-occurs with other *Glumicalyx* species, including *G. nutans*, *G. flanaganii* and *G. montanus*. In areas where *G. goseloides* co-occurs with other species, it occupies different habitats to *G. flanaganii* and *G. montanus*, as it occurs near streams in boulder beds, bare gravelly areas and sparsely grassed slopes (Hilliard and Burtt 1977). The habitat of *G. goseloides* most closely overlaps with that of *G. nutans*, however *G. goseloides* occurs at lower altitudes (montane to subalpine, 1650–2800 m a.s.l.). *Glumicalyx goseloides* is also the only species of *Glumicalyx* that occurs on an

outlying mountain of the DMC, specifically Mt. Ngeli at 1650 m a.s.l. Both of these species have orange to orange-red corolla lobes and long, narrow corolla tubes, however the flowers are much larger in *G. goseloides* and the corolla lobes are strongly reflexed in *G. nutans* – a characteristic feature of *G. nutans* (Hilliard and Burtt 1977). It is therefore highly unlikely that they share pollinators, based on the length of the corolla tube, however they may fall within the same pollinator syndrome. There are also differences in their flowering phenology, as *Glumicalyx goseloides* flowers from late spring to early summer (October to December; Hilliard and Burtt 1977), although it is very variable, and *G. nutans* flowers in summer (December to January; Hilliard and Burtt 1977) and therefore may be reproductively isolated as a result differences in flowering times. The wide altitudinal range of *G. nutans* results in overlap with *G. goseloides*, and these species co-occur at three localities along the border of KwaZulu-Natal and Lesotho. They are likely to be reproductive isolated as a result of differences in pollinators, based on floral morphology.

In the plastid phylogeny *G. apiculatus* and *G. montanus* are sister taxa, and these species are morphologically very similar based on vegetative and floral traits. They occupy similar habitats – gravelly basalt rock sheets, although *G. montanus* also occurs in sparsely grassed slopes (Hilliard and Burtt 1977). The main difference is their distribution — *G. apiculatus* is restricted to the Eastern Cape Drakensberg in the subalpine zone and *G. montanus* occurs in the eastern Free State, KwaZulu-Natal Drakensberg, Eastern Cape Drakensberg and Lesotho Maloti's in the subalpine and alpine zones (Hilliard and Burtt 1977). *Glumicalyx apiculatus* and *G. montanus* co-occur at the base of 'Doodmans Krans', Barkley East district, Eastern Cape (Hilliard and Burtt 1977). The similarity in their floral traits, including short corolla tubes with pale-yellow corolla lobes suggests a common pollinator guild. Ecological speciation seems unlikely between these species, and their speciation may be as a result of non-adaptive processes. *Arrowsmithia*, previously *Macowania* a DMC near-endemic (Bentley *et al*. 2017), shows distinct range allopatry indicating geographical isolation as a key driver of speciation (Bentley *et al*. 2014). Geographical isolation in *G. apiculatus* and *G. montanus* may have been the driver of speciation in this clade, with its current distribution showing secondary contact post allopatric reproductive isolation.

Like *G. apiculatus* and *G. montanus*, *G. lesuticus* and *G. nutans* are morphologically similar. *Glumicalyx lesuticus*, as its name suggests, is restricted to Lesotho's Maloti Mountains and occurs in gravel patches and thinly grassed slopes in the subalpine and alpine

zones (2550–3180 m a.s.l.; Hilliard and Burtt 1977). *Glumicalyx nutans* is widely distributed across the DMC over a wide altitudinal range and is the most frequently collected species. Whilst *G. lesuticus* is a small herb, the vegetative growth of *G. nutans* is variable and depends on its growing conditions (Hilliard and Burtt 1977). Differences between the species are mostly in the floral traits, including corolla lobe shape, and inflorescence size and shape. All four stamens are exerted from the corolla tube in *G. lesuticus*, and two stamens are inserted and two stamens are exerted from the corolla tube in *G. nutans*. *Glumicalyx lesuticus* is known from six localities, and at four of these six localities *G. nutans* co-occurs with *G. lesuticus*, indicating that *G. lesuticus* most likely diverged from *G. nutans* in sympatry. Further sampling is required, particularly of the co-occurring populations, to investigate if there are reproductive isolating mechanisms between the species.

Diversification in the Drakensberg Mountain Centre

There is a lack of synapomorphies that support relationships between and within the genus *Glumicalyx*, indicating a relatively recent divergence, possibly in the Pliocene or Pleistocene, subsequent to the Pliocene uplift (Partridge and Maud 2000). The alpine radiation of the DMC is characterized by multiple small radiations and no large ones, and this may be because the area above 1800 m a.s.l. is small and the alpine zone is almost continuous (Linder and Verboom 2015). Although fragmentation of the alpine zone through post-uplift erosion may account for some speciation, as seen in *Arrowsmithia* (*Macowania*; Bentley *et al*. 2014, 2017).

Conclusions and future work

Glumicalyx is a monophyletic genus that appears to have speciated ecologically both in sympatry and allopatry, based on the extant species current distribution. The species exhibit variation in the vegetative and reproductive traits, allowing them to adapt to different microhabitats and very likely to pollinators, thus facilitating the species coexistence. The incongruence between the nuclear and plastid phylogenies have made conclusions about the modes of speciation in sister taxa near impossible. Improved resolution of the nuclear and plastid phylogenies, through more DNA regions, particularly multiple low-copy nuclear genes is needed. Whole genome sequencing may be required to determine the source of the tree incongruence and investigate the possibility of historical hybridization within the genus. Investigating if morphological trait differences between the species are genetically determined would be informative. Studies of the reproductive biology of all six species, including the identification of pollinators and establishing if they are specific to the various

species, would allow for further investigation into the role of pollinators in the speciation of *Glumicalyx*.

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Appendices

Appendix 2.1 Protocol for DNA extraction

The GeneJET^{TM} Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) was used to extract DNA from the leaf tissue samples using the protocol outlined in Appendix 1. Ca. 300 mg of frozen leaf tissue was placed into a vial containing one sterilized stainless steel bead and was mechanically disrupted using a grinding mill. The ground tissue was immediately placed into a 1.5 ml microcentrifuge tube containing 350 µl of Lysis Buffer and vortexed for 10–20s to mix thoroughly, after which 50 µl of Lysis Buffer B and 20 µl RNase A were added. The samples were incubated for 10 min at 65°C, after which 130 µl of the Precipitation Solution was added and mixed by inverting the microcentrifuge 2-3 times and then incubated for 5 min on ice. The samples were then centrifuged for 5 min at ≥14 000 rpm. The supernatant (450–550 µl) was collected and transferred to a clean microcentrifuge tube and 400 µl of Plant gDNA Binding Solution and 400 µl of 96 % ethanol were added and the solution was well mixed. The prepared mixture was transferred to a spin column and the spin column was centrifuged for 1 min at 8 000 rpm, the flow-through solution was discarded. This was done for half the mixture at a time. 500 µl of Wash Buffer I (with added ethanol) was added, and the samples were centrifuged for 1 min at 10 000 rpm and the flow-through discarded. 500 µl of Wash Buffer II (with added ethanol) was added to the column and centrifuged for 3 min at 13 000 rpm. The collection tube containing the flow-through solution was discarded and the column was transferred to a 1.5 ml microcentrifuge tube. To elute the genomic DNA 50 µl of Elution Buffer was placed in the centre of the column membrane, and incubated for 5 min at room temperature, and then centrifuged for 1 min at 10 000 rpm. A second elution step was performed by placing 25 µl of Elution Buffer. The final purified DNA was stored at -20 °C until use during DNA amplification.

Appendix 2.2 Protocol for electrophoresis gel preperation

The electrophoresis gel was prepared using the following method: a 1% agarose gel was prepared by mixing 0.3g of agarose powder with 30 ml of 1 × TBE Buffer in a conical flask.

The mixture was microwaved for a total of 1 min at 1000W. The mixture was removed from the microwave and swirled. After the agarose was completely dissolved, 1.5 µl ethidium bromide stain was added and mixed by swirling. Once the solution had cooled, but before solidifying, the solution was poured into a gel rig with a cleaned comb in place. Once the gel had set, 6X loading dye (bromophenol blue) was used to load the product into the gel at the proportions of 2 µl of the loading dye and 2 µl of the PCR product. The DNA bands were visualised in ultra violet lght using a BioRad Gel Doc™ XR+.

Appendix 2.3. Specimens of *Glumicalyx* used for morphological measurements. Specimen vouchers for the molecular work are indicated with an asterisk in front of the species' name. Voucher collector and number, with herbarium acronym and locality of collection indicated. Unless otherwise stated, all locations are within South Africa.

	Species	Voucher ID	Herbarium	Locality		Date collected
	G. apiculatus	Galpin 6812	PRE	EC	Base of Doodman's Krans, Barkley East	1904
*	*G. apiculatus*	Hilliard 16666	PRE	EC	Bastervoetpad, Elliot-Maclear	1983
*	*G. apiculatus*	Springer 26	J	EC	Joubert's Pass, Lady Grey	2017
	G. apiculatus	Hilliard & Burtt 16397	NU, PRE	EC	Joubert's Pass, Lady Grey	1983
	G. apiculatus	Hilliard & Burtt 12199	NU, PRE	EC	Joubert's Pass, Lady Grey	1979
	G. apiculatus	de Jesus 61	PRE	EC	Joubert's Pass, Lady Grey	2007
	G. apiculatus	Rattray 7313	PRE	EC	Kraalberg, Barkley Pass	1983
	G. apiculatus	Drege 13060	PRE	EC	Summit of the Witteberg, Aliwal North	Unknown
	G. flanaganii	Hilliard & Burtt 16450	NU, PRE	EC	Ben McDhui, Barkly East	1983
	G. flanaganii	Hilliard & Burtt 10429	NU	KZN	Garden Castle, Underberg	1977
	G. flanaganii	Hilliard & Burtt 16193	NU	KZN	Giant's Castle, Mpendhle	1983
	G. flanaganii	Hilliard 5418	NU	KZN	Sani Pass, Underberg	1973
	G. flanaganii	Edwards 1774	NU	KZN	Sani Pass, Underberg	2000
	G. flanaganii	Hilliard & Burtt 8909	PRE	KZN	Thamathu Pass, Bushmans Nek	1976
	G. flanaganii	Hilliard & Burtt 18278	NU	KZN	Umkomass, Nhlangeni Valley	1985
	G. flanaganii	Hilliard & Burtt 15773	NU, PRE	KZN	Upper tributaries of Mkomazi River	1982
	G. flanaganii	Hilliard & Burtt 8776	NU, PRE	L	Black Mts	1976
	G. flanaganii	Hilliard & Burtt 12027	NU	L	Blue Mt Pass, Maseru	1979
	G. flanaganii	Bester 3944	PRE	L	New Oxbow Lodge, Butha-Buthe	2003
*	*G. flanaganii*	Cron & Cingo 1126	J	L	Sani Pass, Mokhotlong	2012
	G. flanaganii	Hilliard 5329	NU	L	Sani Pass, south-facing summit cliff	1973
	G. flanaganii	Hilliard & Burtt 8792	NU	L	Sani Top	1976
	G. flanaganii	Flanagan 2036	PRE	L	Summit Mont Aux Sources	1894
	G. flanaganii	Jacot Guillarmod 2369	NU	L	Thabantletyana	1955
*	*G. flanaganii*	Cron & Cingo 1119	J	L	Tlaeng Pass, Mokhotlong	2013
*	*G. flanaganii*	Cron & Cingo 1120	J	L	Tlaeng Pass, Mokhotlong	2013
	G. goseloides	Hilliard & Burtt 7300	NU	EC	Mt Insuguia, Mt Auliff	1973

	G. goseloides	Bester 13250	PRE	EC	Scobell's Kop, Naudes Nek Pass	2016
*	*G. goseloides*	Bester 10992	PRE	FS	Base of Sentinel Peak, Witsieshoek	2012
*	*G. goseloides*	Springer 9	J	FS	Road to Sentinel Peak, Witsieshoek	2017
*	*G. goseloides*	Springer 18	J	FS	Base of Sentinel Peak, Witsieshoek	2017
	G. goseloides	Bester 10992	PRE	FS	Sentinel Peak, Witsieshoek	2012
	G. goseloides	Springer 20	J	FS	Sentinel View Point, Witsieshoek	2017
	G. goseloides	Paton 360	PRE	FS	Witsieshoek	1986
	G. goseloides	Hilliard & Burtt 7741	NU	KZN	Drakensberg Garden Hotel, Underberg	1975
	G. goseloides	Hilliard & Burtt 13685	PRE, NU	KZN	Castle View Farm, Underberg	1980
	G. goseloides	Killick 1593	PRE	KZN	Cathedral Peak, Bergville	1951
	G. goseloides	Hilliard & Burtt 6915	NU	KZN	Cathedral Peak, Bergville	1973
	G. goseloides	Hilliard & Burtt 9259	NU	KZN	Cobham Nature Reserve, Underberg	1976
	G. goseloides	Edwards 1100	NU	KZN	Dedema Gorge, Cathedral Peak, Bergville	1992
	G. goseloides	Stewart 2065	NU	KZN	Giants Castle Game Reserve	1978
	G. goseloides	Balkwill, Manning & Meyer 1101	PRE, NU	KZN	Monks Cowl Forest Reserve, Cathekin Park	1983
	G. goseloides	Hilliard & Burtt 5813	NU	KZN	Ngeli Mt, Kokstad	1969
	G. goseloides	Physick 20	NU	KZN	Royal Natal National Park, Bergville	1976
*	*G. goseloides*	Cron 1128	J	KZN	Sani Pass, Underberg	2012
*	*G. goseloides*	Cron 1128b	J	KZN	Sani Pass, Underberg	2012
	G. goseloides	Balkwill, Manning & Cadman 1132	NU	KZN	Sani Pass, Underberg	1983
	G. goseloides	Edwards 1757	NU	KZN	Sani Pass, Underberg	2000
	G. goseloides	Hilliard 987	NU	KZN	Sani Pass, Underberg	1962
	G. goseloides	Getliffe 1313	NU	KZN	Tseketseke Valley, Cathedral Peak, Bergville	1984
	G. goseloides	Strey 10819	PRE	EC	Kakas Hill, Insizwa	1972
	G. lesuticus	Edwards 1159	PRE, NU	KZN	Cleft Peak, Cathedral Peak, Bergville	1956
*	*G. lesuticus*	Cron and Cingo C1117b	J	L	AfriSki Lodge, Oxbow	2013
	G. lesuticus	Hilliard & Burtt 8764	NU	L	Black Mt	1976
	G. lesuticus	Hilliard & Burtt 12025	NU	L	Blue Mt Pass, Maseru	1979
	G. lesuticus	Hilliard 5451	PRE	L	Across Sani River, Sani Top	1977
	G. lesuticus	Pringle S.N.	PRE, NU	L	Hills between Sani Pass and Thabantletyana	1976
	G. lesuticus	Hilliard 5313	NU	L	Sani Pass, Underberg	1973
	G. lesuticus	Hilliard 5336	NU	L	Sani Pass, Underberg	1973
*	*G. lesuticus*	Hilliard and Burtt 8813	PRE, NU	L	Sani Top	1976
	G. lesuticus	Hilliard and Burtt 8719	PRE, NU	L	Sani Top	1976
	G. lesuticus	Hilliard 5397	PRE	L	Sani Top	1973
	G. lesuticus	Guillarmod 112	PRE	L	Little Bokong	1946
	G. lesuticus	Coetzee 493	PRE	L	Meniaming Pass	1956
	G. lesuticus	Ruch 2407	PRE	L	Sani Pass, Underberg	1962
	G. lesuticus	Jacot-Guillarmod 5900	PRE	L	Khalon-la-Mashalu	Unknown
	G. lesuticus	Jacot-Guillarmod 616	PRE	L	Mamalapi	1948
	G. montanus	Hilliard & Burtt 6707	NU	EC	Naudes Nek, Barkly East	1971

	G. montanus	Stewart 1913	NU	EC	Naudes Nek, below summit, Maclear	1976
*	*G. montanus*	Springer 38	J	EC	Naudes Nek Pass summit	2017
*	*G. montanus*	Springer 45	J	EC	Tenahead Lodge, Naudes Nek Pass, Rhodes	2017
	G. montanus	Acocks 12336	PRE	EC	Summit Naude's Nek Pass	1946
	G. montanus	Brusse 5100	PRE	EC	Summit Naude's Nek Pass	1988
	G. montanus	Puff 790113-5/6	PRE	EC	Summit Naude's Nek Pass	1979
*	*G. montanus*	Bester 10986	PRE	FS	Base of Sentinel Peak, Witsieshoek	2012
	G. montanus	Roberts 3076	PRE	FS	Generaalskop, Golden Gate National Park	1965
*	*G. montanus*	Bester 11580	PRE	FS	Road to Sentinel Peak, Witsieshoek	2013
	G. montanus	Hilliard & Burtt 8638	PRE, NU	FS	Road to Sentinel, Witsieshoek	1975
	G. montanus	Stewart 1959	NU	FS	Base of Sentinel Peak, Witsieshoek	1977
*	*G. montanus*	Springer 17	J	FS	Base of Sentinel Peak,Witsieshoek	2017
	G. montanus	Springer 3	J	FS	Top of Zigzags, Sentinel Peak, Witsieshoek	2017
	G. montanus	Wright 338	NU	KZN	Basutoland-Ridge, Giants Castle	1967
*	*G. montanus*	Wright 923	NU	KZN	Busmans River Pass, Giants Castle	1970
	G. montanus	Edwards 1158	PRE	KZN	Cleft Peak, Cathedral Peak, Bergville district	1956
	G. montanus	Manning, Hilliard & Burtt 16079	NU	KZN	Cobham Forest Reserve, Underberg	1982
	G. montanus	Bruyns-Haylett 30	NU	KZN	Giant's Castle Game Reserve,	1949
	G. montanus	Balkwill, Manning & Meyer 1052	NU	KZN	Ships Prow Pass, Giants castle Game Reserve	1983
	G. montanus	Killick 1826	PRE	KZN	Tseketseke River, Cathedral Peak	1952
	G. montanus	Trauseld 526	PRE	KZN	Upper Injisuti, Giant's Castle	Unknown
	G. montanus	Hilliard & Burtt 12096	PRE	L	Blue Mt Pass, Maseru	1979
	G. montanus	Schmitz 9187	NU	L	Blue Mt Pass, Maseru	1981
	G. montanus	Davis & Davis 174	NU	L	Devil's knuckles, Sehlabethabe National Park	1979
	G. montanus	Hilliard 5392	NU	L	Sani Top	1973
	G. montanus	Davis 205	NU	L	Sehlabethabe National Park	1979
	G. montanus	Guillarmod, Getliffe & Mzamane 110	PRE	L	Sehlabathebe National Park	Unknown
	G. nutans	Springer 28	J	EC	Barkly East	2017
	G. nutans	Hilliard & Burtt 12315	NU	EC	Fetcani Pass, Elliot	1979
	G. nutans	Hilliard & Burtt 12180	NU	EC	Jouberts Pass, Lady Grey	1979
*	*G. nutans*	Springer 46	J	EC	Tenahead Lodge, Naudes Nek, Rhodes	2017
	G. nutans	Hilliard & Burtt 12258	NU	EC	Saalboom Nek, Barkly East	1979
	G. nutans	A20175	PRE	EC	Saalboom Nek, Barkley East	1959
*	*G. nutans*	Springer 35	J	EC	Summit Naudes Nek Pass	2017
	G. nutans	Stewart 1969	NU	FS	Base of Sentinel Peak, Witsieshoek	1977
*	*G. nutans*	Springer 1	J	FS	Platberg summit, Harrismith	2017
*	*G. nutans*	Springer 1	J	FS	Platberg, Harrismith	2017
	G. nutans	Hilliard & Burtt 8703	PRE	FS	Platberg, Harrismith	1975
	G. nutans	Jacobsz 2058	PRE	FS	Platberg, Harrismith	1974
*	*G. nutans*	Bester 10995	PRE	FS	Road to Sentinel Peak, Witsieshoek	2012
*	*G. nutans*	Bester 11575	PRE	FS	Road to Sentinel Peak, Witsieshoek	2013

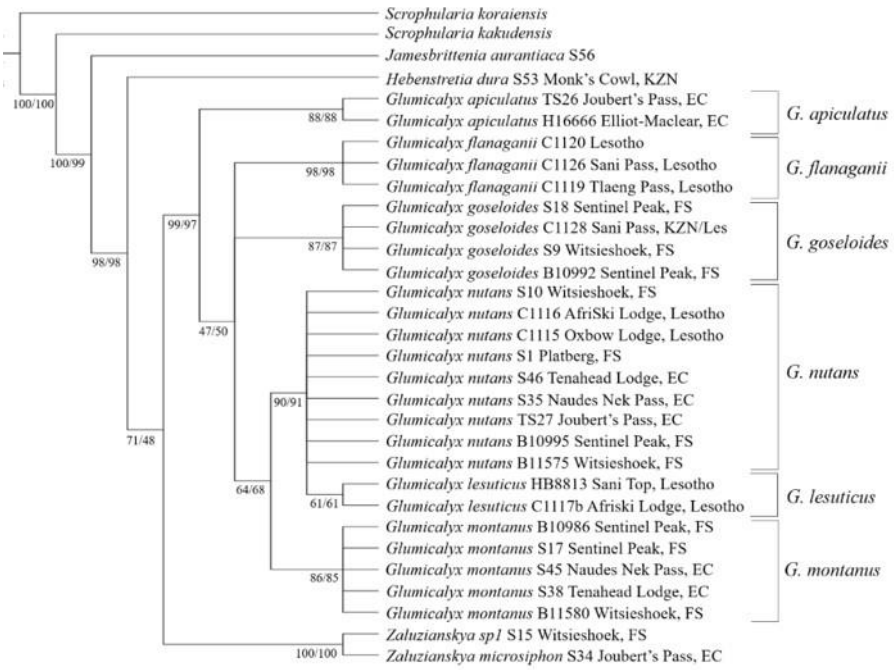
	G. nutans	Springer 4	J	FS	Witsieshoek Pass, Witsieshoek	2017
*	*G. nutans*	Springer 10	J	FS	Witsieshoek Pass, Witsieshoek	2017
	G. nutans	Hilliard & Burtt 8666	NU	FS	Witsieshoek pass, Witsieshoek	1975
	G. nutans	Scholpe 182	NU	KZN	Eastern face Cathedral Peak, Bergville	1943
	G. nutans	Wright 1360	NU	KZN	Giants Castle Pass, Giants Castle	1973
	G. nutans	Wright S.N.	NU	KZN	Kamburg, Escourt	1953
	G. nutans	Trauseld 496	PRE	KZN	Langelebakla Pass, Giant's Castle	1965
	G. nutans	Hilliard 496	NU	KZN	Langelebalela Pass, Giants Castle	1966
	G. nutans	Trauseld 496	PRE	KZN	Langelebalela Pass, Giant's Castle	1965
	G. nutans	Hilliard & Burtt 14880	NU	KZN	Mlambonja Valley, Garden Castle	1982
	G. nutans	Hilliard 5450	NU	L	Across Sani River, Sani Top	1975
*	*G. nutans*	Cron C1116	J	L	AfriSki Lodge, Oxbow	2013
	G. nutans	Schmitz 9173	NU	L	Blue Mt Pass, Maseru	1980
	G. nutans	Hilliard & Burtt 12025	NU	L	Blue Mt, Maseru	1979
	G. nutans	Pringle S.N.	NU	L	Hills between Sani and Thabantletyana	1976
	G. nutans	van Rensburg 51	NU	L	Oxbow area	1975
*	*G. nutans*	Cron and Cingo C1115	J	L	Oxbow Lodge, Oxbow	2013
	G. nutans	Hilliard & Burtt 8797	NU	L	Sani top, east of pass	1976
	G. nutans	Hilliard & Burtt 8815	NU	L	Sani Top, valley west of border post	1976
*	*G. nutans*	Hoener 1724	NU	L	Sehlabethabe National Park	1976
	G. nutans	Richardson 129	NU	L	Thaba Tseka road, Maseru	1980
	G. nutans	Hilliard & Burtt 8814	NU	L	Valley W of border post, Sani Top	1976
	G. nutans	Cron and Cingo 1117a	J	L	AfriSki Lodge, Oxbow	2013
	G. nutans	Cron and Cingo 1121	J	L	Ridge north of Koti, Black Mt Pass	2013

Appendix 2.4. Vegetative and ecological trait character matrix for *Glumicalyx* with *Selago monticola* as outgroup.

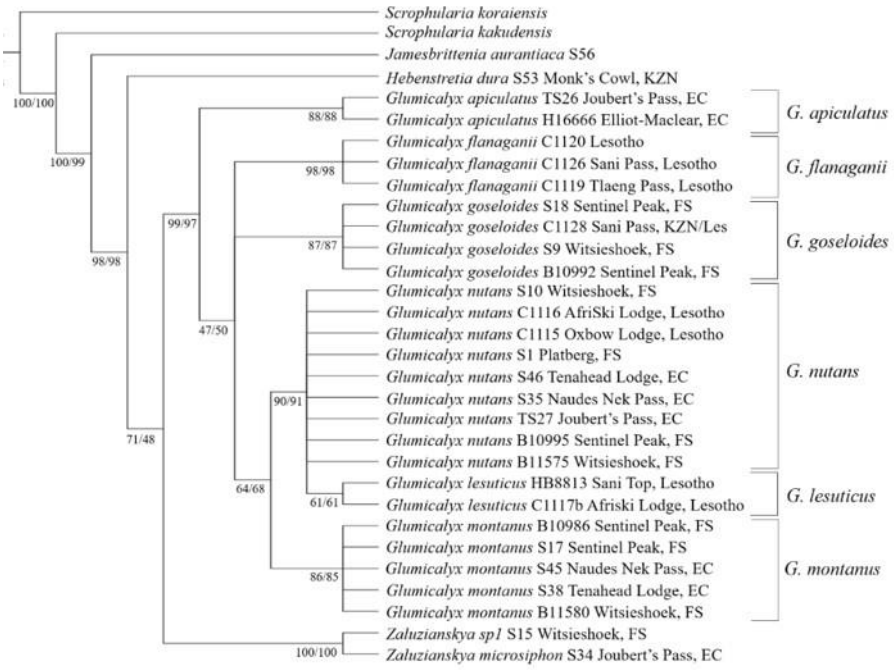
Species	Plant height	Leaf length	Leaf width	Leaf indumenta	Leaf shape	Upper leaf margin	Altitudinal zone	Habitat
G. apiculatus	2	0	1	1	1	1	1	2
G. flanaganii	0	2	2	0	3	1	2	1
G. goseloides	0	2	2	0	0	0	0	3
G. lesuticus	2	0	0	0	1	1	2	2
G. montanus	1	0	1	1	2	1	2	2
G. nutans	1	1	0	0	0	0	2	3
Selago monticola	0	0	0	0	0	0	0	0

Appendix 2.5. Floral trait character matrix for *Glumicalyx* with *Selago monticola* as outgroup.

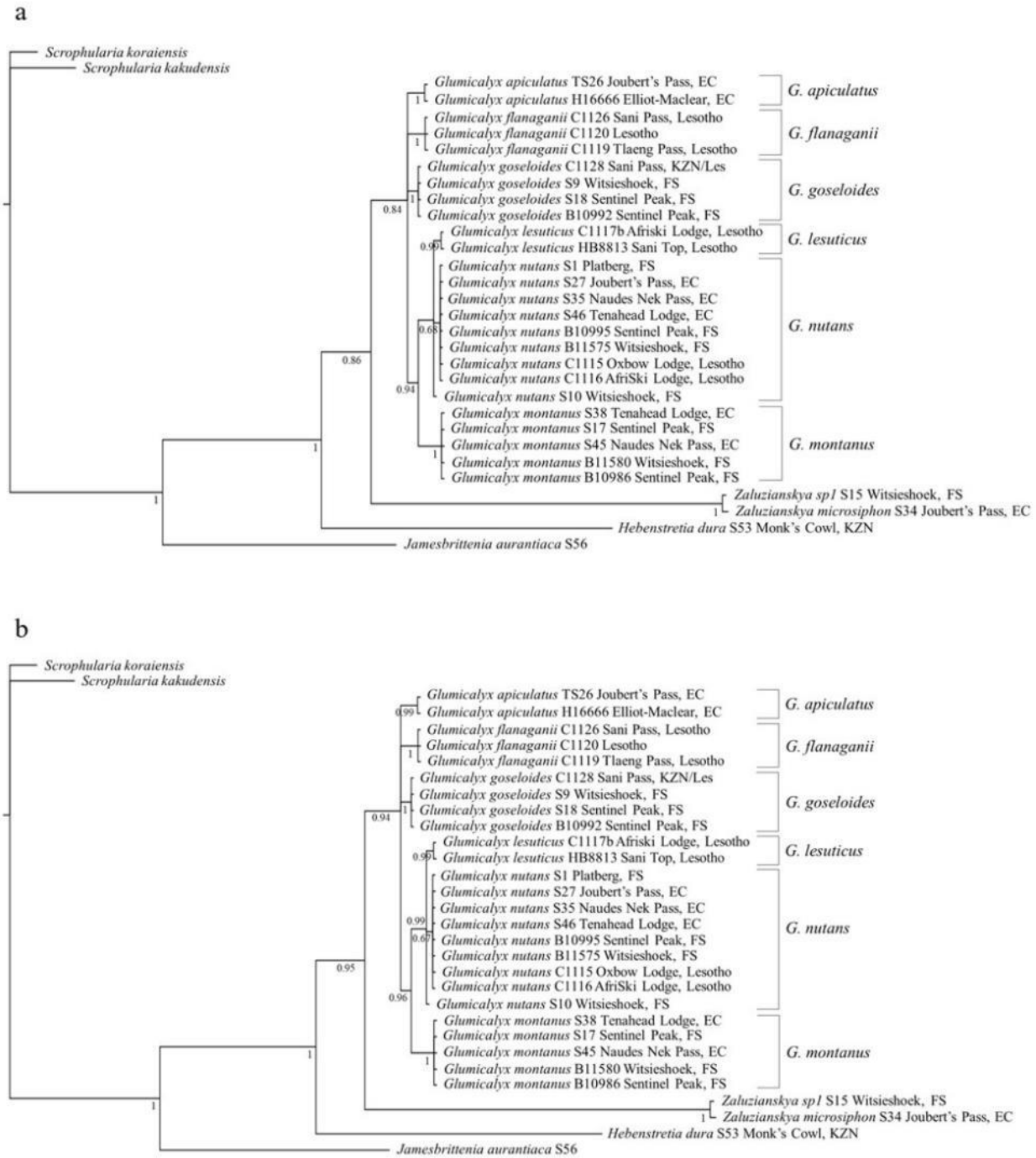
Species	Inflore-scence length	Bract length	Bract width	Calyx length	Tube length	Limb diameter	Throat diameter	Lobe colour	Inflore-scence shape	Lobe shape	Stamen exertion	Anther length
G. apiculatus	0	0	0	0	0	0	0	1	0	1	0	0
G. flanaganii	2	1	1	1	2	1	1	2	1	0	0	0
G. goseloides	2	2	2	1	3	1	1	2	1	2	1	1
G. lesuticus	1	1	1	0	1	0	1	2	0	0	0	0
G. montanus	0	0	2	0	0	1	2	1	0	0	0	0
G. nutans	2	1	1	2	2	0	1	2	1	3	1	1
S. monticola	0	0	0	0	0	0	0	0	0	0	0	0



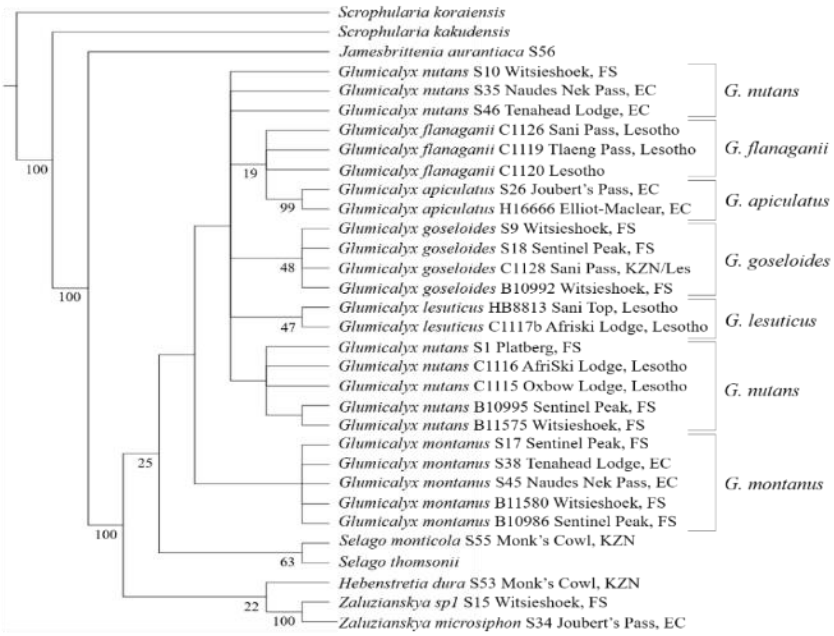
Appendix 2.6. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum parsimony analysis of the ETS region. Bootstrap values are presented below the lines coded indels/without indels.



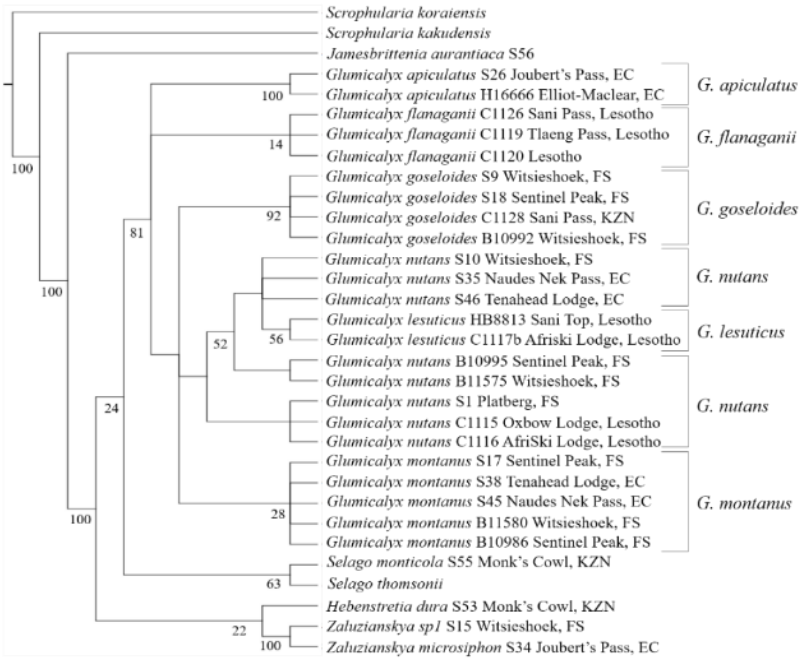
Appendix 2.7. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum likelihood analysis of the ETS region (indels not included). Bootstrap values (%) are presented below the lines.



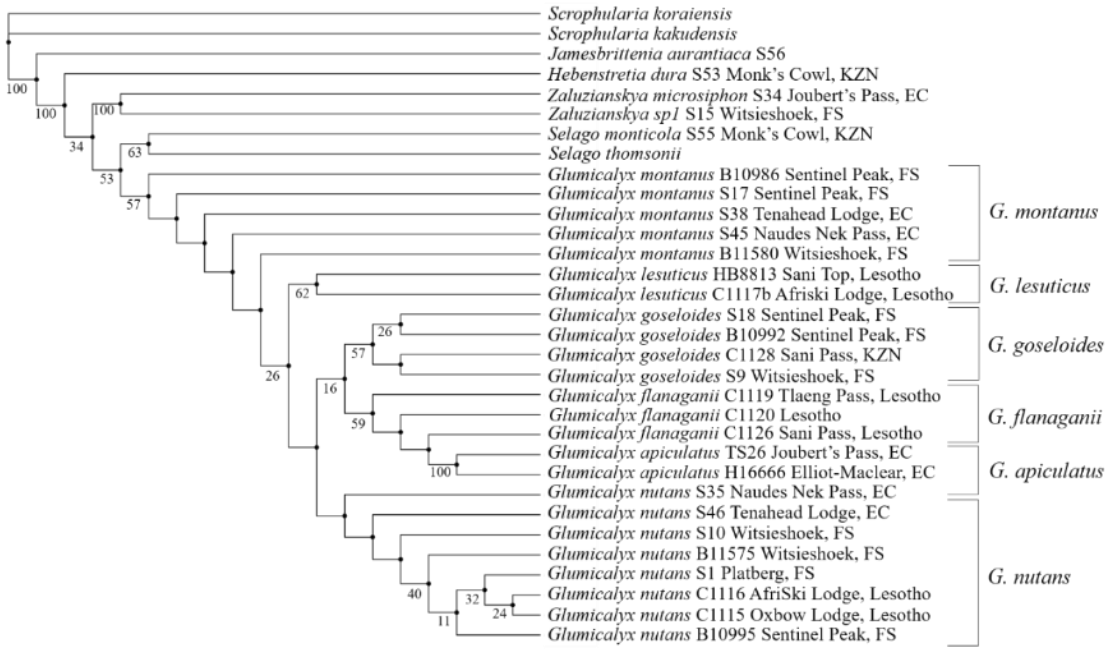
Appendix 2.8. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the ETS region, a) with indels not included, b) with coded indels. Posterior probabilities are presented below the lines.



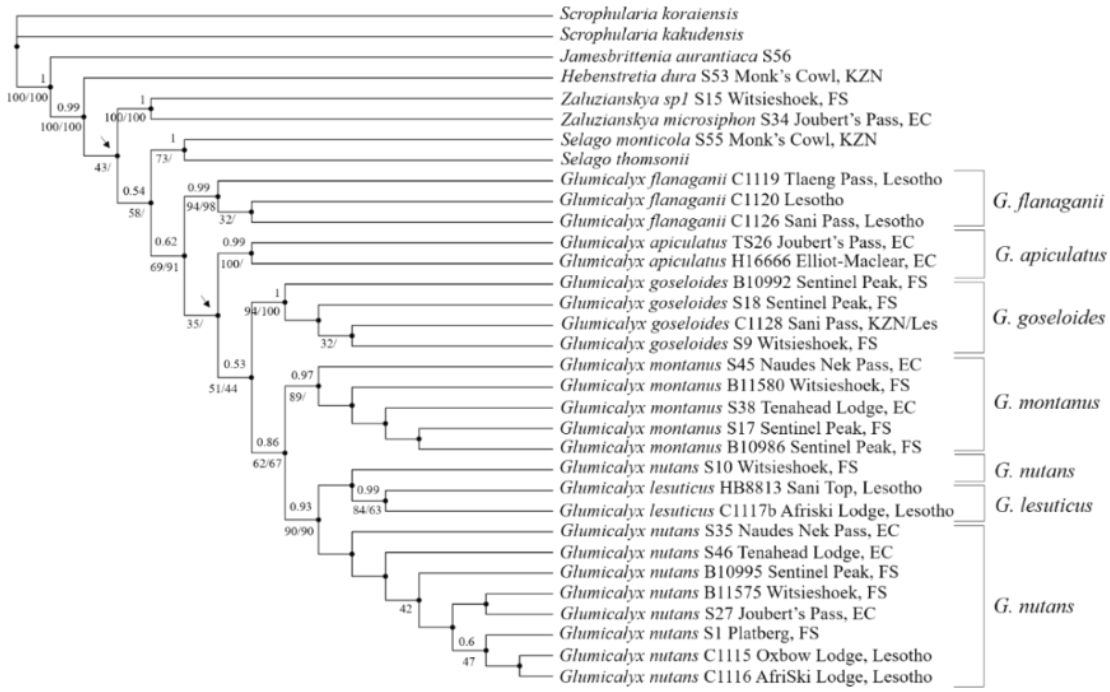
Appendix 2.9. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum parsimony analysis of the ITS region with indels not included. Bootstrap values (%) are presented below the lines.



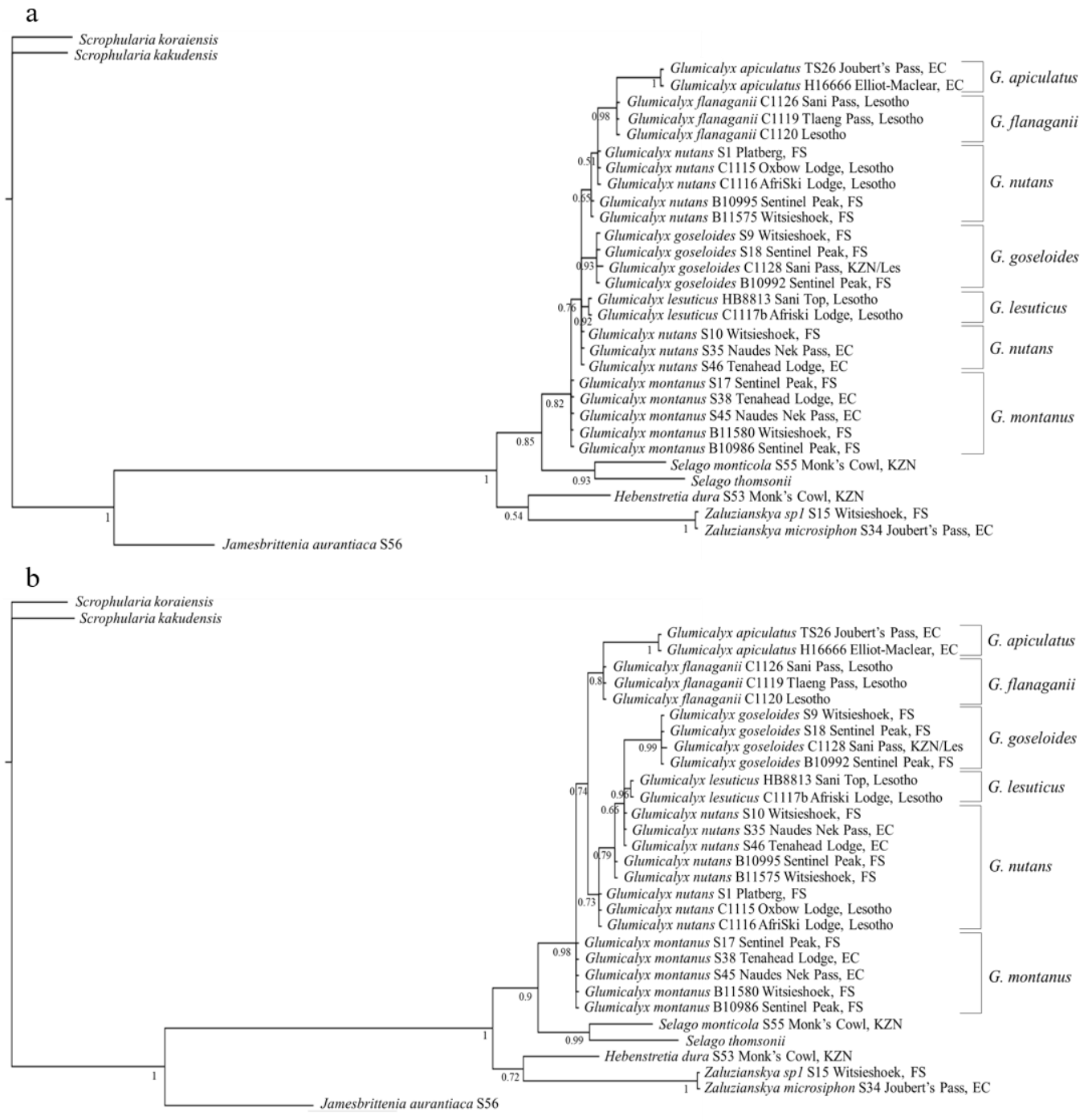
Appendix 2.10. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum parsimony analysis of the ITS region with coded indels. Bootstrap values (%) are presented below the lines.



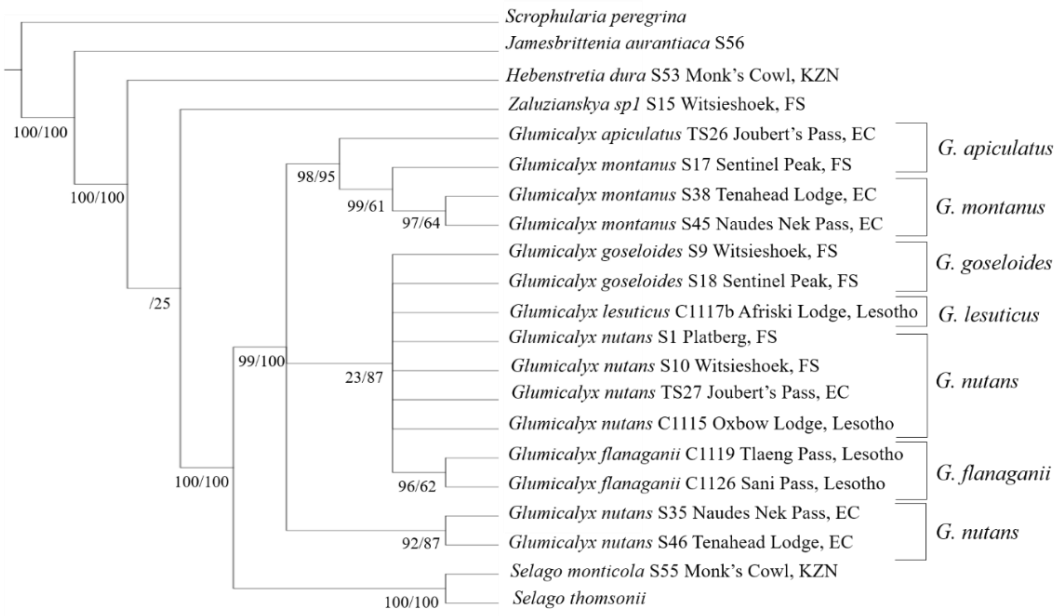
Appendix 2.11. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum likelihood analysis of the ITS region (coded indels not included). Bootstrap values are presented below the lines.



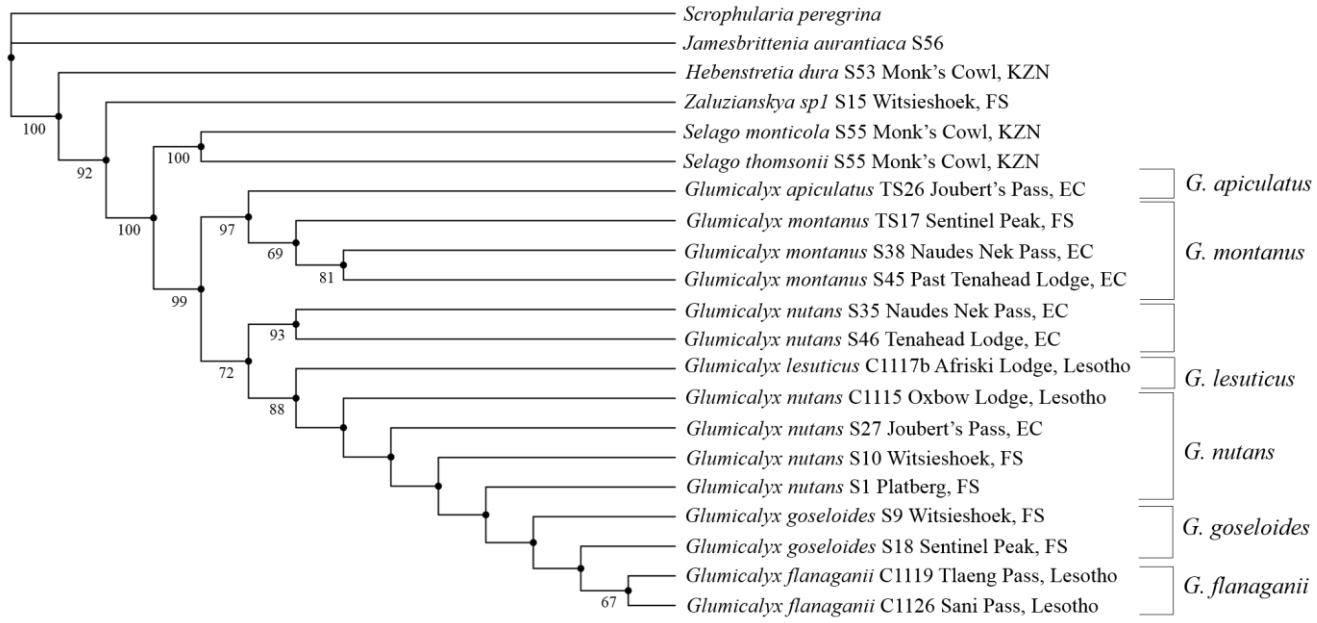
Appendix 2.12. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum likelihood analysis of the combined ETS and ITS region (indels not included). Bootstrap values (%) are presented below the lines for ML/MP, and PP values are presented above the line. Arrows above the line indicate nodes that collapsed or differed in the BI and MP analysis. Support values from the BI and MP analyses are with coded indels.



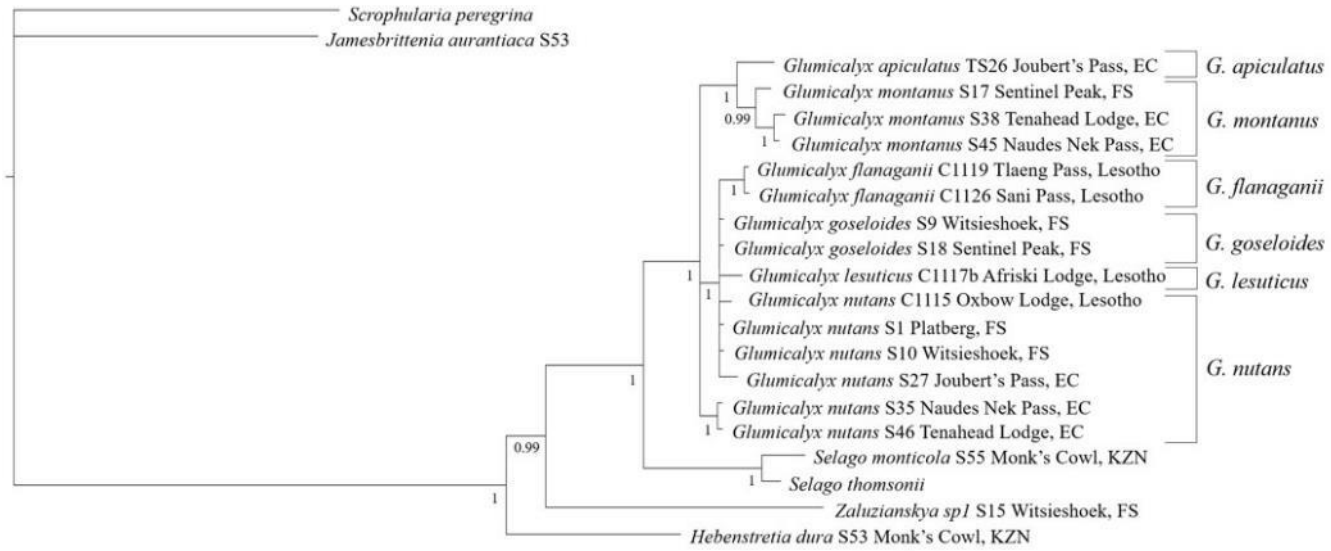
Appendix 2.13. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the ITS region, a) indels not included, b) with coded indels. Posterior probabilities are presented below the lines.



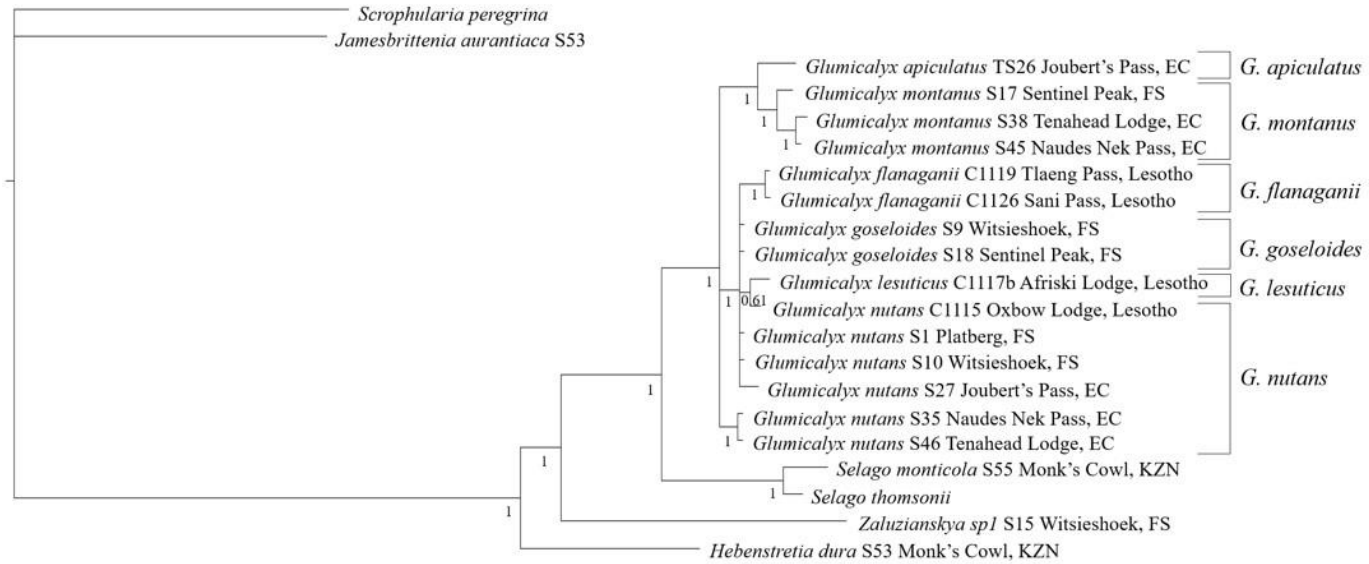
Appendix 2.14. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum parsimony analysis of the *mat*K region. Bootstrap values are presented below the lines with with coded indels/indels not included.



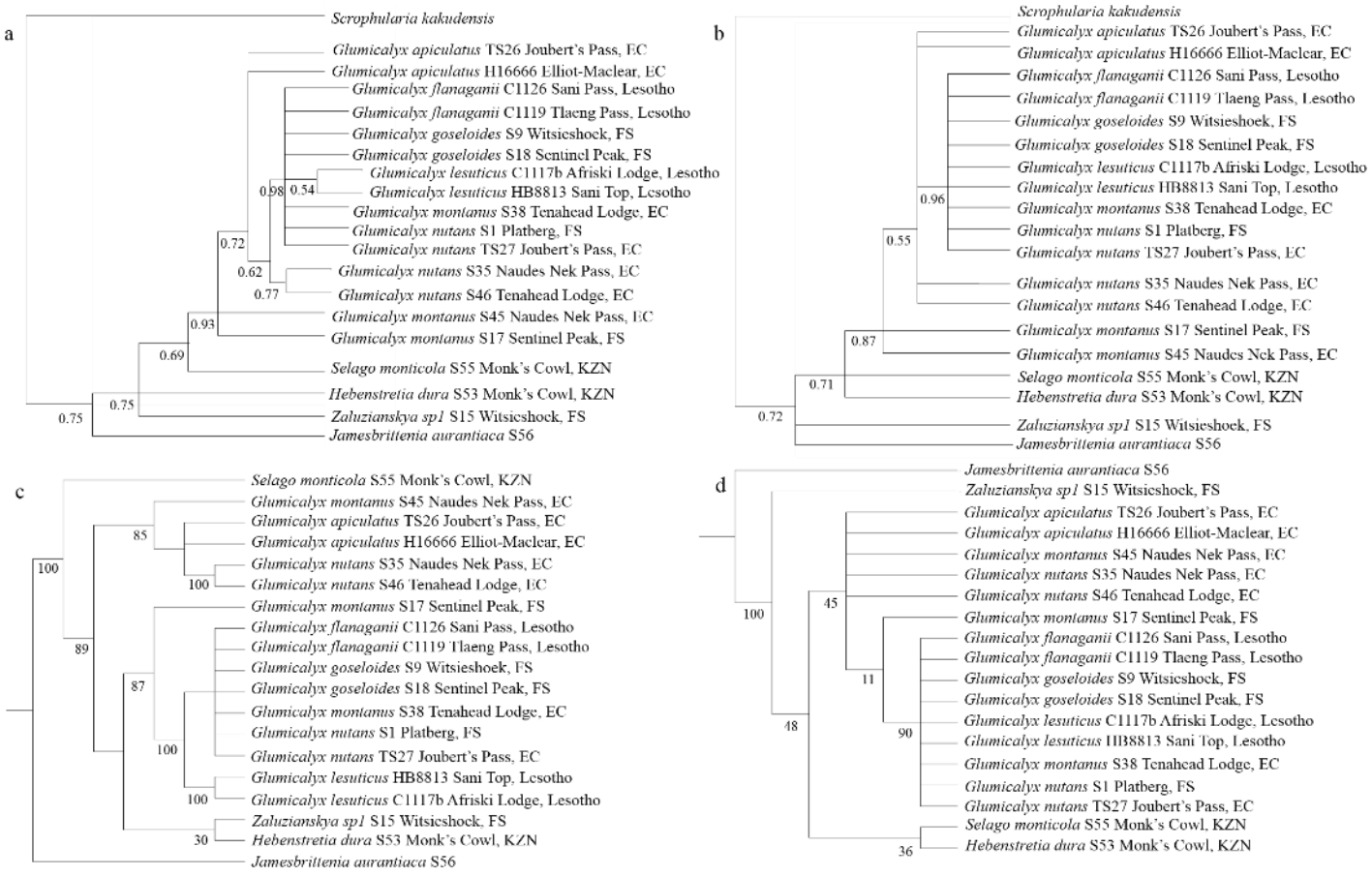
Appendix 2.15. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the maximum likelihood analysis of the *mat*K region (coded indels not included). Bootstrap values are presented below the lines.



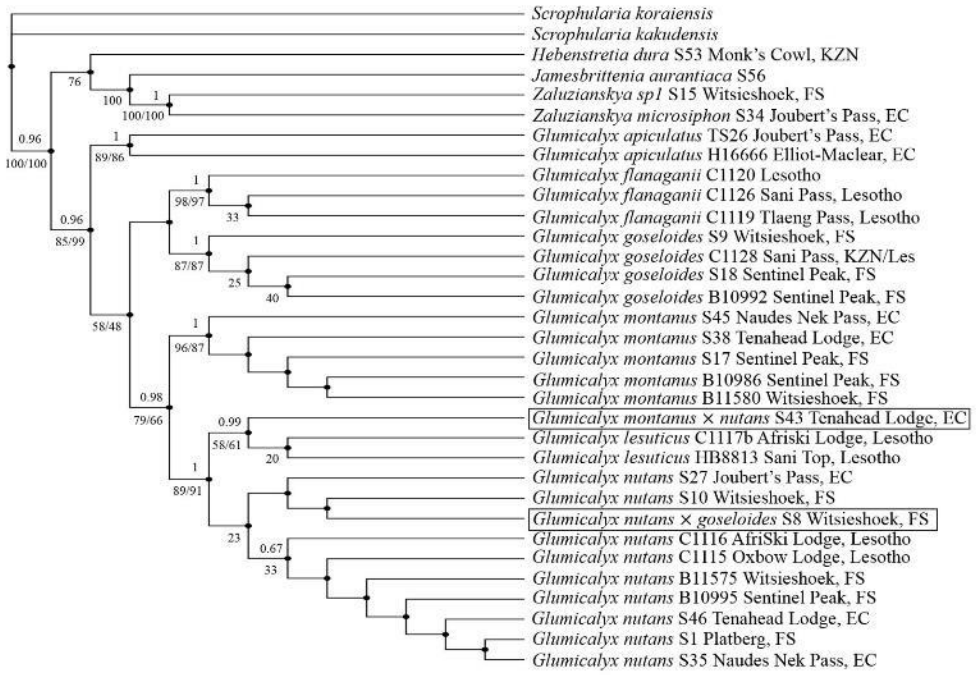
Appendix 2.16. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the *mat*K region with coded indels. Posterior probabilities are presented below the lines.



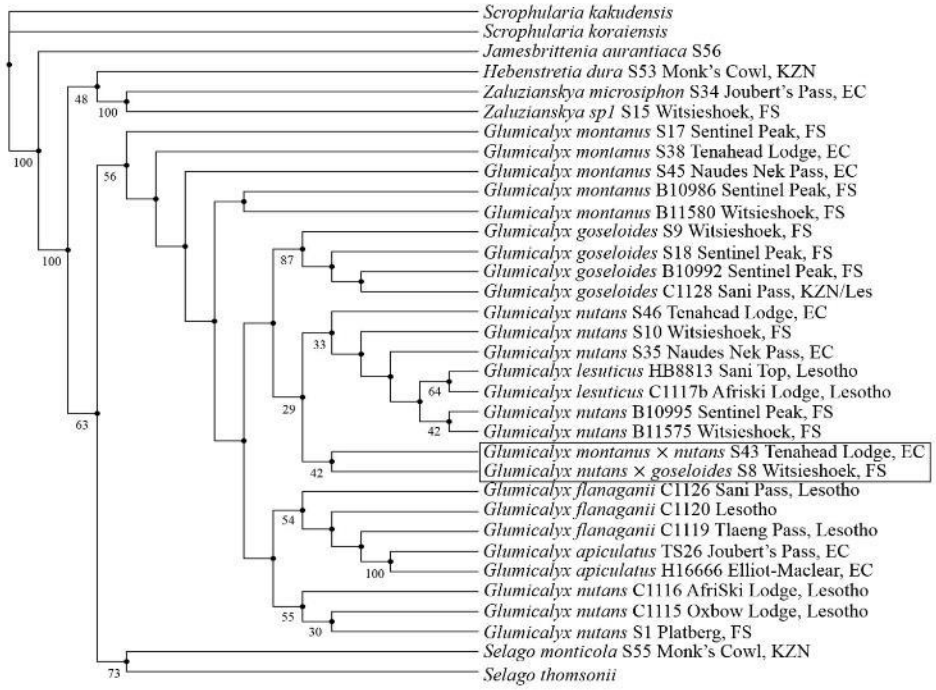
Appendix 2.17. Phylogeny of *Glumicalyx* and the outgroup taxa resulting from the majority rule consensus tree from the Bayesian inference analysis of the *mat*K region with indels not included. Posterior probabilities are presented below the lines.



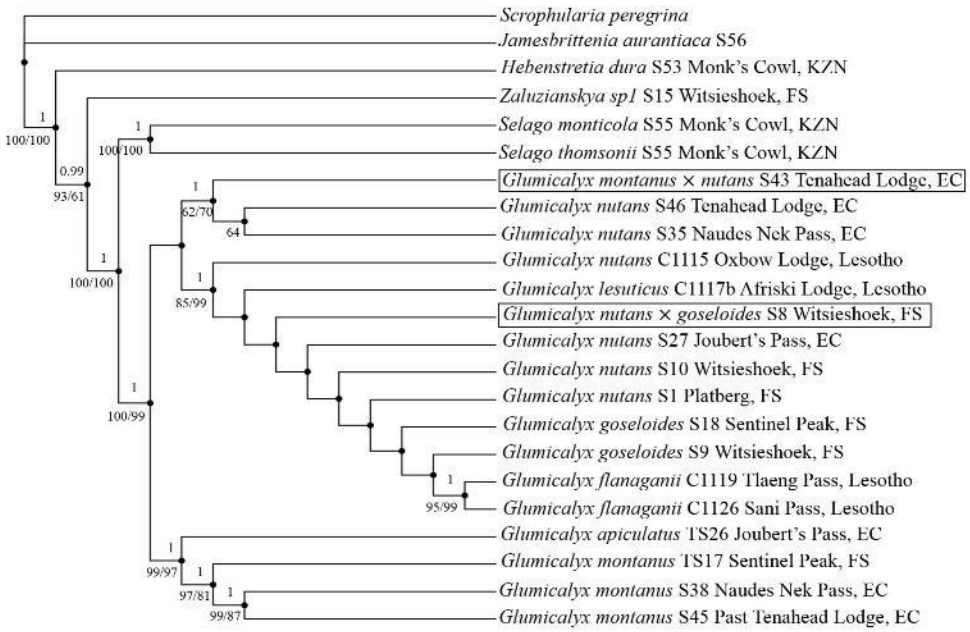
Appendix 2.18. Phylogeny of *Glumicalyx* and the outgroup taxa for the *psb*A-*trn*H region, (a) the majority rule consensus tree from the Bayesian inference analysis with coded indels, (b) the majority rule consensus tree from the Bayesian inference analysis, posterior probabilities are presented below the line, (c) majority rule consensus tree from the maximum parsimony analysis with coded indels, (d) majority rule consensus tree from the maximum parsimony, bootstrap support (%) is presented below the line.



Appendix 2.19. Phylogeny of *Glumicalyx*, its putative hybrids and the outgroup taxa resulting from the maximum likelihood analysis of the ETS region (with indels excluded). Bootstrap values are presented below the lines for maximum likelihood/maximum parsimony, and posterior probabilities are presented above the line.



Appendix 2.20. Phylogeny of *Glumicalyx*, its putative hybrids and the outgroup taxa resulting from the maximum likelihood analysis of the ITS region (with indels excluded). Bootstrap values are presented below the line.



Appendix 2.21. Phylogeny of *Glumicalyx*, its putative hybrids and the outgroup taxa resulting from the maximum likelihood analysis of the *mat*K region (with indels excluded). Bootstrap values are presented below the lines for maximum likelihood/maximum parsimony, and posterior probabilities are presented above the line.

Appendix 2.22. Results of the analysis of variance and Tukey post-hoc analysis of the morphological traits measured on herbarium specimens of *Glumicalyx*.

	Plant height	Leaf length	Leaf width	Inflorescence length	Bract length	Calyx length	Tube length	Limb diameter	Tube diameter
G. flanaganii-G. apiculatus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.130
G. goseloides-G. apiculatus	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
G. lesuticus-G. apiculatus	0.530	0.090	0.650	0.010	0.000	0.890	0.000	0.090	0.510
G. montanus-G. apiculatus	0.090	0.940	0.780	0.640	0.001	0.440	0.180	0.010	0.001
G. nutans-G. apiculatus	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.020	0.630
G. goseloides-G. flanaganii	0.290	0.004	0.970	0.001	0.000	0.990	0.000	0.900	0.150
G. lesuticus-G. flanaganii	0.001	0.000	0.000	0.000	0.007	0.000	0.910	0.500	0.930
G. montanus-G. flanaganii	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.900	0.280
G. nutans-G. flanaganii	1.000	0.015	0.000	0.410	0.970	0.960	0.010	0.730	0.550
G. lesuticus-G. goseloides	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.070	0.220
G. montanus-G. goseloides	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.260	0.000
G. nutans-G. goseloides	0.090	0.000	0.000	0.000	0.000	0.990	0.000	0.110	0.260
G. montanus-G. lesuticus	0.880	0.150	0.010	0.120	0.000	0.190	0.000	0.960	0.050
G. nutans-G. lesuticus	0.001	0.000	0.250	0.000	0.020	0.000	0.000	0.990	0.990
G. nutans-G. montanus	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.990	0.002

Chapter 3

Comparative reproductive biology of three species of the Drakensberg Mountain Centre endemic genus *Glumicalyx*

Abstract

Variation in floral traits among closely related species often reflects differences in pollination system, which may promote reproductive isolation and thereby speciation. The aim was to investigate and compare floral variation, pollination systems and breeding systems in the Drakensberg Mountain Centre of southern Africa by studying three species of the endemic genus *Glumicalyx*. This study provided the first account of the breeding system and pollination in *Glumicalyx*. The species included are representative of the morphological variation within the genus and co-occur at the study site. Exclusion experiments and seed germination trials revealed that although *G. montanus* was not self-incompatible, all three species are dependent on pollinators for successful reproduction. *Glumicalyx goseloides* was the only species found to be pollen limited, and this finding was supported by the lack of effective pollinators observed. *Glumicalyx nutans* was visited by honey bees and noctuid moths, but is considered to be mainly pollinated by noctuid moths based on the analysis of pollen loads and plant-pollinator fit. *Glumicalyx montanus* was mostly likely pollinated by short-tongued flies and noctuid moths, based on visitation and analysis of pollen loads. Among the three species there is substantial variation in floral morphology (corolla tube length and width) and floral traits that act to attract (floral colour and scent) and reward (nectar) pollinators. Floral scent composition varied between species and between day and night, with significant increases in scent emission rate at night. Scent sampled at night was dominated by linalool in *G. nutans*, β-ocimene in *G. goseloides* and benzaldehyde in *G. montanus*. These compounds, together with the increase in scent emission, indicate a possible adaptation to nocturnal pollination. In conclusion the three species of *Glumicalyx* have distinct floral morphology, scent chemistry and pollination, and pollinator shifts may have played a role in the speciation of the genus within the Drakensberg Mountain Centre.

Introduction

Pollinators have been hypothesized to play a role in the diversification of angiosperms (Crepet 1984; Dodd *et al*. 1999; Vamosi and Vamosi 2010), and particularly in the flora of southern African (Johnson 2006; Johnson 2010; van der Niet *et al*. 2014). Pollinators have the potential to drive diversification at the microevolutionary (cf. Harder and Johnson 2009) and the macroevolutionary (Johnson 1996; Johnson 2010; van der Niet *et al*. 2014) scale. Evidence of pollinator driven divergence is evident from species-level angiosperm phylogenies whereby sister taxa are associated with shifts in pollination system and floral traits (Whittall and Hodges 2007; Valente *et al*. 2012; van der Niet and Johnson 2012; Forest *et al*. 2014). The theory of ecological speciation provides a framework to link microevolutionary (adaptation) and macroevolutionary (speciation) processes (van der Niet *et al*. 2014). Ecological speciation occurs when ecologically-based divergent selection results in barriers to gene flow between populations (Rundle and Nosil 2005). Divergent selection on phenotypic traits, may be a result of differences in the ecological niche, the ecological interaction of populations and forms of sexual selection, including adaptation to specific pollinators (Schluter and Nagel 1995; Schluter 2001; Rundle and Nosil 2005). A signature of pollinator-driven adaptation is the divergence of floral features (Johnson 1996), whereas adaptations to the physical environment are reflected predominantly in vegetative characteristics (Verboom *et al*. 2004). Using floral and vegetative functional traits is useful in determining the drivers of ecological speciation, as shown by Johnson (1996) for the flora of the Cape Floristic Region, South Africa.

Southern Africa is a hotspot of plant endemism and diversity (cf. Linder 2003), with two main centres of endemism in the Greater Cape Floristic Region (GCFR) and the Drakensberg Mountains (nested within the Drakensberg is the Drakensberg Mountain Centre, DMC – formerly known as the Drakensberg Alpine Centre; cf. van Wyk and Smith 2001). Ecological speciation has played an important role in the diversification in the GCFR (Linder 1985; van der Niet and Johnson 2009; Schnitzler *et al*. 2011). Ecological shifts were demonstrated for 80% of the 188 sister taxa of Cape species. Sister taxa displayed shifts in floral and vegetative traits, with many shifts only in floral traits, indicating the important role for pollinator-driven speciation (van der Niet and Johnson 2009). Johnson (2010) demonstrated the pollination niches in southern Africa through the identification of 24 specialized pollination guilds that have been documented. Within each guild are multiple species with convergent floral traits (Johnson 2010). Most of the studies addressing the role

of pollinators in the diversification of the southern Africa flora thus far have been carried out within the Cape Floristic Region (e.g. Johnson 1996; Johnson and Steiner 1997; Goldblatt and Manning 1996, 2006). However, pollinators are predicted to also play a role in floral diversification in the eastern regions of southern Africa, in particular the Drakensberg, as there is a wide range of flower shape, size, and colour within genera (Pooley 2003; Johnson *et al*. 2009). Specialized pollination by the long-tongued flies *Prosoeca ganglbaueri* (Goldblatt and Manning 2000; Johnson *et al*. 2002; Anderson and Johnson 2009) and *Philoliche aethiopica* (Johnson 2000; Johnson and Morita 2006; Johnson *et al*. 2009), the oil-collecting bees *Redivivia sp.* (Manning and Brothers 1986; Steiner and Whitehead 1988; Steiner and Whitehead 1990; Steiner and Whitehead 1991; Whitehead *et al*. 2008), the mountain pride butterfly *Aeropetes tulbaghia* (Johnson and Bond 1994; Johnson *et al*. 2009), hawkmoths including *Agris convolvuli* (Alexandersson and Johnson 2002, Johnson *et al*. 2002; Johnson *et al*. 2009, Johnson and Raguso 2016) and small flies including Tachinidae and Muscidae (Johnson *et al*. 2009; van der Niet *et al*. 2010) have been documented in the Drakensberg Mountain Centre. There is still a great deal to investigate about the role of pollinators in diversification of the Drakensberg and this study aims to investigate the association between floral traits and pollinators in the DMC biodiversity hotspot, by investigating the reproductive biology of the endemic genus *Glumicalyx* Hiern (Scrophulariaceae, Limoselleae).

The breeding systems of angiosperms have broad implications for their evolution (Holsinger 2000; Hörandl 2010, Barrett and Harder 2017). The diversification of many plant families have been attributed to the adaptive radiation of breeding and pollination systems, accompanied by changes in ecology and life history (Barrett *et al*. 1996). The majority of plant species produce flowers that are hermaphroditic and therefore have the potential for self-pollination (Hörandl 2010). To avoid the negative consequences that may result from self-fertilization, about 60% of angiosperms have developed mechanisms to prevent self-pollination, including, herkogamy and dichogamy and to avoid self-fertilization through self-incompatibility (Bateman 1952; de Nettancourt 1977; Webb and Lloyd 1986; Takayama and Isogai 2005). Herkogamy is the spatial separation of anther and stigma, of which there are many different forms (Web and Lloyd 1986). The most common form is approach herkogamy in which the stigmas are located above the anthers (Barrett 2003). Dichogamy is the temporal separation of pollen presentation and stigma receptivity; this may be within a flower and/or between flowers on a plant (Barrett 2003). Self-compatibility may be beneficial when pollen receipt is limited, as it allows plants to make use of their own pollen (Larson and

Barrett 2000) and may thereby provide reproductive assurance through autonomous self-pollination (Eckert *et al*. 2006). Pollen limitation can occur in habitats where pollinator availability is low (García-Camacho and Totland 2009), such as would apply to alpine habitats (Totland 1994; Körner 1999). This may result in increased incidence of self-compatibility and self-pollination (Medan *et al*. 2002) and increased generalist pollination (Totland 1993; Larson *et al*. 2001).

For c. 80% of plant species, reproduction is mediated by animal pollinators (Ollerton *et al*. 2011) and these animal-mediated pollination systems may be highly generalized (Waser *et al*. 1996) or highly specialized (Johnson and Steiner 2000). It is also important to consider which of the possibly many floral visitors are effective cross-pollinators (Waser *et al*. 1996; Johnson *et al*. 2009), in that there is successful transfer of pollen from the anthers of one individual to the stigmatic surface of the pistil/carpels of another individual (Kevan 1999), as not all visitors are pollinators (cf. Fenster *et al.* 2004). Specialization is achieved by two mechanisms, the first is when plant morphology limits access to floral rewards, and the second is when floral cues to attract animals are only perceivable by a limited number of animal species (Schiestl and Johnson 2013). A broad range of floral traits may be under pollinator-mediated selection (Harder and Johnson 2009), including variation of corolla tube length and width (Nilsson 1988; Stang *et al*. 2006), floral colour (Bradshaw and Schemske 2003), and floral scent (Dobson 2006). Floral volatiles play an important role in the attraction of pollinators to flowers (Raguso 2008 a,b), as even subtle differences in the floral scent composition can lead to shifts in pollinators, as demonstrated in closely related *Eucomis* species (Shuttleworth and Johnson 2010). Since the same floral volatiles that attract pollinators may also attract herbivores, nectar robbers and inefficient pollinators (cf. Miyake and Yahara 1998, 1999), plants may have temporal differences in scent emission depending on the pollinator activity (Theis *et al*. 2007; Peter *et al*. 2009; van der Niet *et al.* 2015).

The Scrophulariaceae encompass a variety of floral forms, and particularly show a wide variation in the form and colour of the corolla (Pennell 1935, Kampny 1995). Kampny (1995), in her review of the pollination-related floral diversity in the Scrophulariaceae, noted that little information is available on floral scents in the family — possibly a result of their less obvious scent. The family was particularly difficult to classify based on morphology, and was revised based on molecular markers (Kornhall *et al*. 2001, Oxelman *et al*. 2005). Scrophulariaceae *s.s*. is composed mostly of taxa distributed in the southern Hemisphere (Olmstead *et al*. 2001), within which there are eight tribes (Kornhall *et al.* 2001, Kornhall and

Bremer 2004, Oxelman *et al.* 2005). The basic floral form in the Scrophulariaceae is a five-lobed calyx and a campanulate corolla tube with five lobes. Two of the lobes form the upper lip and the remaining three lobes form the lower lip. The ancestral floral form in the Scrophulariaceae is predicted to be an open, bell-shaped, bee-pollinated corolla (Bentham 1876, Pennell 1935, Kampny 1995). Butterfly-pollinated and moth-pollinated flowers are thought to be modified from the campanulate bee flower to form a longer, narrower corolla tube (Kampny 1995). The modified tubular flowers differ in that the butterfly-pollinated flowers have large petal lobes to serve as a landing platform, whereas the moth-pollinated flowers are considered less likely to have landing platforms (Kampny 1995). In both forms, the nectar is produced at the base of the ovary and therefore is only accessible to an insect with a sufficiently long proboscis (Kampny 1995). Modified tubular flowers for butterfly and moth-pollination are found in the Scrophulariaceae tribe Limoselleae. The tribe Limoselleae includes the former tribes Manuleeae and Selagineae, and the genus *Limosella*, as *Limosella* and Selagineae were nested within Manuleeae (Kornhall *et al*. 2001, Oxelman *et al*. 2005, Kornhall and Bremer 2004).

Glumicalyx was in the former tribe Manuleeae. Within the (former) tribe Manuleeae, the floral form is a tubular flower with a five-lobed limb, with variation in the size of the flowers, corolla tube length and width, colour and contrasting colour patterns, and shape and stance of the lobes (Hilliard 1994). Although variation in floral form is suggestive of various pollination syndromes, including bee, butterfly and moth pollination (Vogel 1954, Hilliard and Burtt 1987, Hilliard 1994), the pollinators of the Manuleeae are not well known (Kampny 1995). Pollination of *Zaluzianskya* section *Necterinia* was found to be specialized for hawkmoth pollination, with the exception of one species which is specialized for long-proboscid fly pollination (Johnson *et al*. 2002). "Sniff tests" done to determine the timing of scent emission in *Zaluzianskya* section *Nycterinia* found that the hawkmoth-pollinated species produce a sweet clove-like scent at dusk after the flowers have opened (Johnson *et al*. 2002). *Zaluzianskya natalensis* and *Z. microsiphon*, two closely related species of *Zaluzianskya*, differ in their floral-opening patterns and primary pollinators. The floral scent compounds found in the greatest abundance in *Zaluzianskya natalensis*, *Z. microsiphon* and their natural hybrids were β-myrcene, (E)-ocimene for all three and in *Z. natalensis* there were large amounts linalool and methyl benzoate (Campbell *et al*. 2016). *Zaluzanskya natalensis* flowers at night and emits more floral volatiles than the day-flowering *Z. microsiphon*. *Zaluzianskya natalensis* is pollinated by hawkmoths and *Z. microsiphon* is pollinated by the long-proboscid *Prosoeca* flies, although natural hybridization does occur

between the species as a result in the overlap in flower opening time at dusk (Johnson *et al*. 2002; Campbell *et al*. 2016). In the genus *Jamesbrittenia*, there is a lack of post-zygotic isolation between the species, resulting in their being able to potentially hybridize. Floral isolation related to different pollinators is predicted to play an important role in maintaining species' integrity in *Jamesbrittenia* (Verboom *et al*. 2016).

Glumicalyx is a genus of six closely allied species endemic to the Drakensberg Mountain Centre (DMC), of eastern South Africa and Lesotho. The genus is characterized by nodding, congested inflorescences with tubular corollas with five lobes ranging from pale yellow to brick-red (Hilliard and Burtt 1977; Hilliard 1994). Hilliard (1994) suggested that the similar floral form in *Glumicalyx* may indicate a common suite of pollinators and that *G. goseloides* may be pollinated by diurnal moths based on its floral form. No further research into the pollination biology of the genus has been conducted. Based on substantial variation in the inflorescence size and flower size (corolla tube length and corolla tube width), as well as differences in corolla lobe colour, despite their form being similar, I hypothesise that Hilliard (1994) underestimated the floral variation and pollination in *Glumicalyx*. I predict that the pollination systems differ between the smaller species with pale-yellow corollas and the larger, species with orange-red corollas. There is the possibility that the larger, orange-red species of *Glumicalyx* are pollinated by *Aeropetes tulbaghia*, the mountain pride butterfly. The floral morphologies of *G. goseloides*, *G. flanaganii* and to a lesser extent *G. nutans* correspond to those found in mountain pride butterfly pollination – red and orange flower colour, straight flowers with long and narrow corolla tubes (proboscis length of *Aeropetes tulbaghia* is 28–35 mm). These plants appear to be exclusively pollinated by the mountain pride butterfly and its floral traits are a combination of those typical to bird and butterfly pollination (Johnson and Bond 1994). The red coloration is typical of bird pollination, not of butterfly pollination, and the floral morphology excludes birds (Johnson and Bond 1994). This pollination guild has been found in 21 species in the Cape and Drakensberg mountains (Johnson and Bond 1994, Johnson 2010).

Although *Glumicalyx* is a relatively modest genus, it is the largest of the genera endemic to the DMC. Endemic genera can be used as models to study diversification in the DMC, in similar vain to the way in which studies of Cape clades in the CFR have contributed greatly to improved understanding of the evolution of the region's flora (Linder 2003; Linder 2005). The endemic genera of the DMC, including *Glumicalyx*, are predicted to have recently diversified *in situ* during the Pliocene (Linder and Verboom 2015), and therefore may be less

likely to have experienced extinctions and post-speciation range expansion and provide better insights into the process of divergence (Herrera *et al*. 2006, Johnson 2010).

The aim of this study was to establish whether floral variation in *Glumicalyx* may be associated with different pollination systems. This study focused on three co-occurring species within the genus, *Glumicalyx goseloides* (Diels) Hilliard & Burtt, *G. montanus* Hiern and *G. nutans* (Rolfe) Hilliard and Burtt. Since these species co-occur it is assumed that there is some form of isolating mechanism that inhibit them from hybridizing. The objectives were to establish whether and to what extent *Glumicalyx* depends on pollinators for reproduction, 2) to identify pollinators, and 3) to quantify and compare floral traits including colour, nectar, morphology and scent.

Methods and materials

Study species

The three species of *Glumicalyx* included in this study, *G. goseloides*, *G. montanus* and *G. nutans*, represent both corolla colour morphs of *Glumicalyx*, viz. orange-red and pale yellow coloration, as well as the range in corolla size – from largest (*G. goseloides*) to one of the smallest (*G. montanus*), and are therefore deemed representative of the floral morphological variation. *Glumicalyx nutans* and *G. montanus* both occur in areas of the Drakensberg including KwaZulu-Natal, the eastern Free State, Eastern Cape provinces of South Africa and Lesotho, while *G. goseloides* mainly occurs along the border between KwaZulu-Natal and Lesotho, with a few localities in the eastern Free State and northern Eastern Cape provinces (Figure 3.1). *Glumicalyx goseloides* occurs at elevations of 1600–2800 m above sea level in boulder beds, rocky grass slopes and in bare gravel. *Glumicalyx montanus* grows in stony and rocky habitats and on sparsely grassed slopes at altitudes of 2340–3050 m a.s.l. *Glumicalyx nutans* is the most widely distributed species and occurs over an altitudinal range of 1800–3350 m a.s.l., in bare gravel or silt and around rock sheets (Hilliard and Burtt 1977). The distribution of these three species overlaps near the base of the Sentinel Peak in the northern Drakensberg (Free State), along Naude's Nek Pass in the Eastern Cape Drakensberg (Barkly East District), and along Sani Pass (which extends from KwaZulu-Natal to Lesotho at its summit; Figure 3.1).

Study site

The three species were studied at a site where all three occur — located near the base of Sentinel Peak near Witsieshoek, in the eastern Free State. Sentinel Peak is located in the northern part of the DMC at the junction of the borders of two South African provinces (Free State and KwaZulu-Natal) and Lesotho (Figure 3.1). The three species occur along Witsieshoek Pass and at the base of Sentinel Peak (i.e. the View Point) across an elevational gradient of 450 m in gravel patches and grassy slopes, between 2300 m and 2750 m a.s.l. As a result, all three species were exposed to the same pollinator communities, such that any potential differences in pollination systems detected could be tentatively assigned to specialization. A second, larger population of *G. nutans* on the summit of Platberg (2350 m a.s.l.) near Harrismith, Free State was included in the study as the population density of *G. nutans* at Witsieshoek was much lower than that of *G. goseloides* and *G. montanus* at Witsieshoek, the populations are ± 55 km apart. Further details are given in Table 3.1.

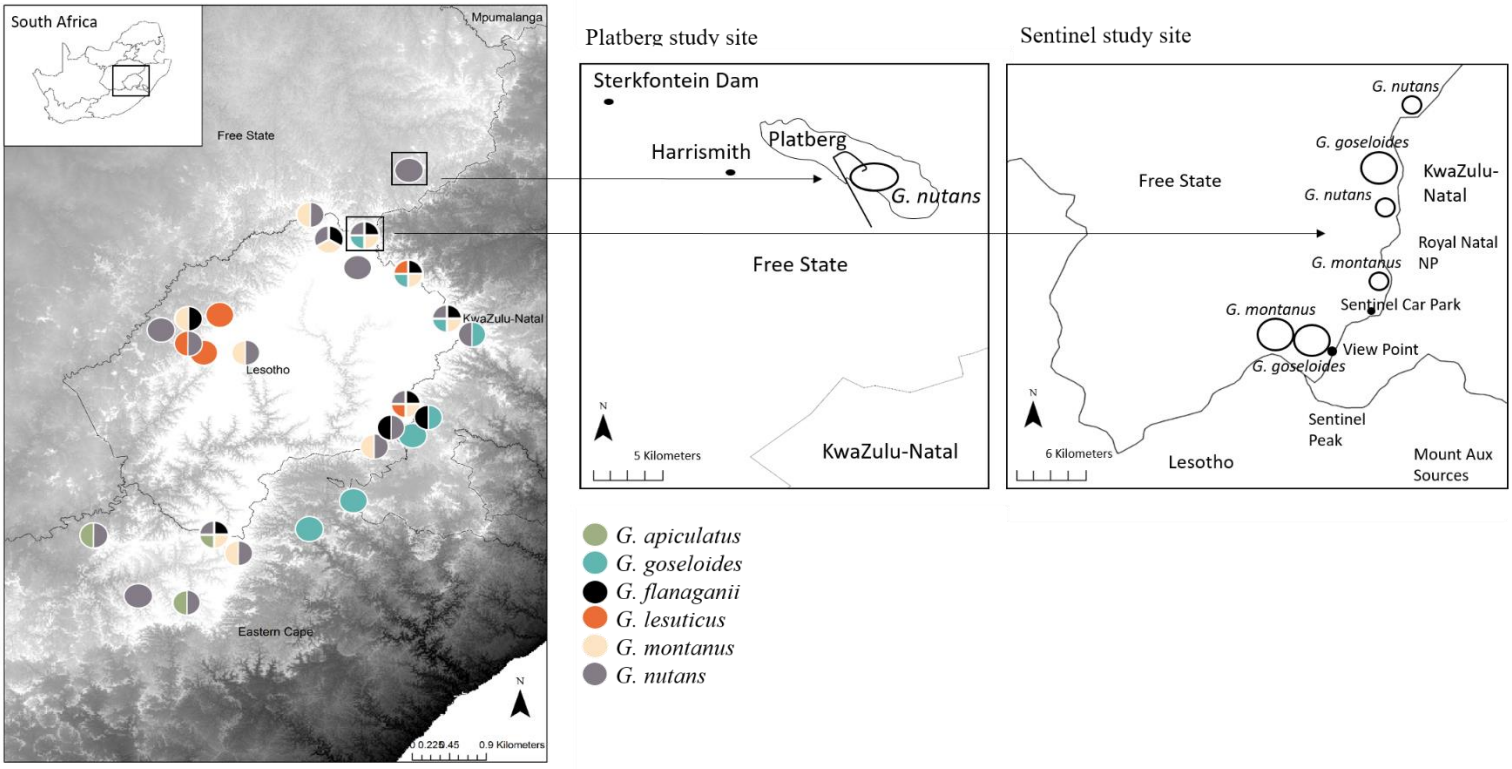


Figure 3.1. Map of the distribution of the species of *Glumicalyx* across the Drakensberg Mountain Centre and the two study sites located in the eastern Free State.

Table 3.1. Study sites, approximate population size and pollinator observation dates and hours for the *Glumicalyx* species included in this study.

Species	Locality	Sites	Longitude (S)	Latitude (E)	Elevation (m)	Population size	Observation dates	Observation time (h)
G. goseloides	Sentinel Peak	Roadside	28° 44.215'	28° 53.699'	2450	± 40	02–03 Dec 17	Day: 31
		Viewpoint	28° 42.562'	28° 53.758'	2750	± 20	11–13 Dec 17	Night: 5.5
G. montanus	Sentinel Peak	Roadside	28° 43.450'	28° 53.369'	2510	± 20	17–18 Jan 18	Day: 16.5
		Viewpoint	28° 44.190'	28° 53.690'	2750	± 100	23–26 Jan 18	Night: 6
G. nutans	Sentinel Peak	Roadside	28° 43.159'	28° 53.755'	2300	± 50	21 Dec 17	Day: 9.5
							4 Jan 18	Night: 3
G. nutans	Platberg, Harrismith	Summit	28° 16.007'	28° 12.561'	2350	± 200	14–15 Dec 17	Day: 22
							23 Dec 17	Night: 8
							19 Jan 18	

Breeding system

Breeding system experiments were conducted on 10 individuals with multiple inflorescences of *G. nutans, G. goseloides,* and *G. montanus* at Sentinel and *G. nutans* at Platberg during the flowering season (November to February). These individuals were located away from the patches where pollinator observations were performed. Newly developing inflorescences were bagged with fine mesh organza bags prior to the opening of the flowers to prevent pollinator access.

Autonomous self-pollination

To test whether the species are capable of autonomous self-pollination, inflorescences were exposed to two treatments, open pollination, and total exclusion of pollinators. In the open pollination treatment, the inflorescences were tagged and left unmanipulated and unbagged for the duration of the flowering period. In the total exclusion treatment, the inflorescences were bagged with fine mesh organza bags prior to the opening of the flowers to prevent pollinator access for the duration of the flowering period.

Self-compatibility

To test whether the species are self-compatible, two flowers within the bagged treatment were exposed to different treatments, hand self-pollination and hand cross-pollination. In the hand self-pollination treatment and hand cross-pollination treatment, pollen was applied to the receptive stigma using an anther held by a pair of forceps. The

reproductive parts were observed with a hand lens to check pollen had stuck to the stigma. In the hand self-pollination treatment pollen from the same inflorescence was placed on the stigma and in the hand cross-pollination treatment pollen from an individual in the same population at least five metres away was placed on the stigma.

Pollen limitation

To test if any of the species was pollen limited, the open pollination treatment was compared to a supplemental cross-pollination treatment. Flowers in the pollen supplementation treatment were hand pollinated by placing pollen from an anther from a separate individual – at least 2 m away, but in the same population – onto the receptive stigmatic surfaces. Cross-pollinated flowers were marked on the floral bracts. The inflorescences were then left unbagged for the remainder of the flowering period.

The inflorescences for each treatment were marked by means of differently coloured ribbon. Once the individuals started to set fruit and the flowers senesced, bags were placed over all inflorescences to avoid damage to the fruit. Mature fruits were collected prior to the dehiscence of the capsules (February 2018), and stored in labelled envelopes. The number of seed containing capsules per inflorescence was counted (small capsules containing non-viable – pale, wrinkled – seed were not considered fruits); capsules were subsequently dissected and seed counts were conducted on up to ten capsules per inflorescence, with the aid of a Zeiss Discovery V8 dissecting microscope. Seed counts were conducted on all capsules that formed in the hand-selfed and hand-crossed treatments, resulting in a sample size of 10 flowers/capsules per treatment.

To compare fruit set among treatments and species, generalised estimating equations in IBM SPSS Statistics version 25 were used, with plant number as the subject variable and treatment as the within-subject variable. The type of model used was a binary logistic (for binomial distribution) with a logit link function and an exchangeable correlation matrix structure. The number of fruit as a function of the the number of flowers was the response variable. The interaction between species and treatment, and species and treatment individually, were the predictor variables. Treatments and species were compared using pairwise comparisons with sequential Sidâk adjustment for multiple comparisons (McCullagh and Nelder 1989; Hosmer and Lemeshow 2000; Field 2009). To compare seed production among treatments for each species (it was not possible to accurately compare between species as each species had different numbers of ovules), generalised estimating equations in IBM SPSS Statistics version 25 were used. Plant number was the subject variable and treatment

was the within-subject variable. The number of seeds was the response variable, and the treatment was the predictor variable. The type of model used was a negative binomial with log link function and an exchangeable correlation matrix. Treatments were compared using pairwise comparisons with sequential Sidâk adjustment for multiple comparisons (McCullagh and Nelder 1989; Hosmer and Lemeshow 2000; Field 2009).

The species were also categorised according to the dependency on pollen vectors for fruit and seed set. If significantly fewer fruit and seed produced from the un-manipulated bagged treatment compared to the open and supplemented cross-pollination treatments then the reproductive output would be significantly reduced in the absence of pollen vectors (Jaimes and Ramírez 1999). To determine if the species were able to autonomously self-pollinate or were dependent on pollen vectors, the index of autonomous self-pollination was calculated (IAS). IAS values range from 0 (completely dependent on pollen vectors) to 1 (completely independent of pollinators). IAS was calculated from the fruit set of the un-manipulated bagged treatment/hand cross-pollination treatment (Jaimes and Ramírez 1999). *Glumicalyx* species that produced significantly fewer fruits and seeds from self-pollination in compared to cross-pollination were considered to have some form of self-incompatibility (SI). Species with results that were not significantly different were considered to be self-compatible (Jaimes and Ramírez 1999); to quantify the degree of SI, the index of self-incompatibility (ISI) was used. ISI values range from 0 (fully self-compatible) to 1 (fully self-incompatible). Species that were self-compatible may be autogamous and self-compatible, non-autogamous and self-compatible or partially self-compatible (Jaimes and Ramírez 1999). ISI was calculated as 1 – the seed set of hand self-pollination/hand cross-pollination (Jaimes and Ramírez 1999) – this calculation of seed set included empty capsules. Species producing significantly less fruit in the open pollination treatment compared to the supplemented cross-pollination treatment were considered to be pollen limited. The pollen limitation index was calculated as $1 – (P_o/P_s)$ where P_o is the open pollination percent fruit set and P_s is the supplemented cross pollination percent fruit set (Larsen and Barrett 2000).

Seed germination

Petri-dish germinations were conducted to determine the germination success of the open, supplement and bagged exclusion treatments, following the guidelines outlined by the International Seed Testing Association (2003). Seed surfaces were rinsed in 2% sodium hypochlorite for 5 min to decontaminate the seed coats and rinsed three times in distilled water (ISTA 2003). The filter paper used was decontaminated with 2% sodium hypochlorite

for 5 min and rinsed three times in distilled water. All laboratory materials were sterilised by autoclaving prior to germination trials (ISTA 2003). Seeds were placed in petri-dishes on two filter papers, saturated with distilled water and a third moist filter paper used to cover them. The seeds were stored in an environmental control chamber at 25 ºC during the day and 15 ºC during the night (a dark/light cycle of 12 h/12 h). Light was supplied by fluorescent bulbs at ~650 nm. Germination was determined by a protruded radical of ~2 mm and the germinated seeds were counted and removed three times a week. The percentage of germinated seeds was calculated after 4 weeks. The percentage seed germination was compared within each species and compared between the treatments for each species, using a chi-square test in RStudio version 3. 5. 1 (R Core Team 2018)

Pollinator observations

Observations of floral visitors were done on days and nights during peak flowering times in November 2017/January 2018. Observations were conducted on *G. nutans*, *G. goseloides* and *G. montanus* at Witsieshoek, Free State and on *G. nutans* at Platberg, Harrismith, Free State (observation times given in Table 3.1). Daytime observations were ideally conducted during warm and sunny weather from mid-morning to mid-afternoon, however observations also occurred in misty weather, as flies were observed even on cold and misty days. Evening observations were conducted on evenings with no thunderstorms, as moths were still observed when some mist was present. Evening observations commenced at 18:30, prior to dusk and continued until moth activity had decreased considerably, usually between 20:00 and 21:00 when mist rolled in.

The activities of the floral visitors were observed (feeding activity and movement between flowers and inflorescences), after which representative individuals of the observed floral visitors were captured using a handheld insect net or a large vial and placed individually into a small killing jar with ethyl acetate fumes present. Ten individuals per morphospecies were collected, or as many as possible if fewer were available (dependent on the type of insect) and then stored in a freezer until further analysis. Insects were observed using a Zeiss dissecting microscope to assess the presence of any pollen. Pollen on different locations on the specimen was noted and extracted by dabbing it with a consistent quantity (~2 mm × 2 mm × 4 mm) of fuchsin gelatin (Beattie 1971); all pollen present on the specimen was removed — excluding pollen baskets on the scopae of bees, as tightly packed pollen in this position is seldom deposited on stigmas (Westerkamp 1991). The pollen collected from each insect was placed onto a separate clean, glass slide, and the gel with

pollen from each location on the specimen was melted under a separate, labelled coverslip. The quantity and identity of the pollen was determined using light microscopy. The identity of the pollen was determined by comparing it to pollen samples of the selected species of *Glumicalyx* and to reference samples collected from flowers growing at the same site as the *Glumicalyx* species (including species of *Kniphofia*, *Senecio*, *Zaluzianskya*, *Craterocapsa*, *Silene* and *Hebenstretia*). The quantity of *Glumicalyx* pollen grains was classified into five categories/classes namely 0, 1–10, 10–99, >100, >1000, and the purity of the pollen was noted. The Coleoptera, Orthoptera and Hymenoptera collected were identified to family, genus and/or species level using collections at the Wits Life Sciences Museum, Johannesburg. Lepidoptera identifications were carried out or confirmed by Dr. Martin Krüger (Lepidoptera curator of the Ditsong National Museum of Natural History, Pretoria, South Africa). Diptera identifications were carried out by Dr. John Midgley (Assistant Director: Natural Science at KwaZulu-Natal Museum). Voucher specimens were appropriately pinned, labelled and accessioned to the Wits Life Sciences Museum Collection. The visitors observed included many different species which may indicate more generalized pollination (Waser *et al*. 1996), however many of the species had similar morphology and possibly exert similar selective pressures on the floral traits (Fenster *et al*. 2004). As a result the collected visitors were categorized into functional visitor groups based on those revised by Fenster *et al*. (2004), including bees and other Hymenoptera (Hymenoptera order, eg. ants), Lepidoptera, Coleoptera, Diptera, and 'other' (i.e. minor visiting groups, eg. thrips, Orthoptera and Blattodea).

Day-Night comparison of Glumicalyx montanus pollination biology

Initial observations suggested visitation during both day and night to *G. montanus* and G. nutans. To distinguish the contribution to pollination of day versus night visitors, the timing of anther dehiscence and reproductive success of day-night exclusion trials were determined in *G. montanus* (the population of *G. nutans* was not accessible throughout the day). To quantify the timing of anther dehiscence and subsequent release of pollen from receptive inflorescences of *G. montanus*, sixteen inflorescences were observed around midday and early evening over three consecutive days (pollen was observed in abundance in *G. goseloides* and *G. nutans* both day and night). To compare the proportion of individuals with visible pollen dehiscence between time periods, generalised estimating equations in IBM SPSS Statistics version 25 were implemented. The subject variable was the individual and within subject variable was the time period, the dependent variable was the proportion with

pollen. The type of model incorporated a binomial probability distribution with logit link function and a autocorrelation AR(1) matrix structure (to account for autocorrelation in pollen dehiscence among consecutive periods).

In a separate population of *G. montanus* to that used to quantify the timing of anther dehiscence, separate inflorescences were bagged during the day or the night to compare reproductive success following from access of diurnal or nocturnal visitors to plants. This separate, smaller population was used because of its greater accessibility compared to the main population of *G. montanus*. In the day treatment, inflorescences were open to pollinators during the day (06:00–18:30) and covered using an organza bag during the night, and in the night treatment inflorescences were open to pollinators during the night (18:30–06:00) and covered using a fine mesh organza bag in the day. To compare the fruit and seed set between the day-exclusion and night-exclusion a generalised linear model in IBM SPSS Statistics version 25 was used. There were too few replicates for seed produced to conduct any statistics. For fruit set, the number of fruit was the events variable and the number of flowers was the trials variable. The type of model incorporated a binomial probability distribution with logit link function.

Floral morphology and floral rewards

In each of the three study species, corolla dimensions that may influence pollinator fit to flowers and nectar production were measured. The floral measurements were done on inflorescences of *G. goseloides*, *G. montanus* and *G. nutans* in the field. Morphological measurements were taken on the same individuals that were used for nectar measurements, with a total of 89 flowers of *G. goseloides*, 42 flowers of *G. nutans* and 128 flowers of *G. montanus* being measured.

To investigate the properties of the nectar released by the three *Glumicalyx* species as a pollinator reward, nectar volume and sugar concentration were measured. The nectar volume measurements were conducted on an average of 20 to 25 inflorescences (from different individuals) with three to four flowers per individual, resulting in an average of 60 recordings per species. Nectar was collected for *G. nutans*, *G. goseloides* and *G. montanus* at the same field sites as where the pollinator/visitor observations were done. The nectary is present as a small dorsal gland at the base of the ovary (Hilliard and Burtt 1977). The corolla tubes of the flowers of the species of *Glumicalyx* included in this study were too narrow to insert a microcapillary tube in order to extract nectar. Therefore, a method adapted from Peter and Venter (2016) was used to extract the nectar to measure its volume and concentration,

whereby individual flowers were removed from the inflorescence and the base of the corolla tube was pierced and the nectar was collected in a 5 µl capillary tube. The length of the nectar column relative to the length of the capillary tube was measured, with digital callipers. The length measured was used to calculate the volume of nectar as a proportion of the 5 µl capillary tube. To measure the sugar concentration of the nectar, nectar from three to four flowers (on one individual) was pooled in a single capillary tube due to the low nectar volumes in individual flowers. The mean nectar sugar concentration was measured using a MISCO Palm Abbe Digital refractometer (0.0–56.0 Refractive Index units, nD).

To compare corolla tube dimensions between species, generalised estimating equations in IBM SPSS Statistics version 25 were implemented. Inflorescence number was the subject variable, the dependent variable was either the corolla tube length or corolla tube diameter, and the species was the predictor variable. The type of model incorporated a gamma probability distribution with log link function and an exchangeable correlation matrix. Species were compared using pairwise comparisons with sequential Sidâk adjustment for multiple comparisons (McCullagh and Nelder 1989; Hosmer and Lemeshow 2000; Field 2009). To compare nectar properties among time period and species, generalised estimating equations in IBM SPSS Statistics version 25 were used, with the inflorescence number as the subject variable and time period as the within-subject variable. The dependent variable was nectar volume or nectar sugar concentration. The type of model incorporated a gamma probability distribution with log link function and an exchangeable correlation matrix. The interaction between species and time period, and species and time period individually were the predictor variable. Treatments were compared using pairwise comparisons with sequential Sidâk adjustment for multiple comparisons (McCullagh and Nelder 1989; Hosmer and Lemeshow 2000; Field 2009).

Floral colour

To determine if there are spectral reflectance differences between the flowers of the species, particularly in those that appear to have similar colours to the human eye (viz. *G. nutans* and *G. goseloides*), the spectral reflectance over the UV-visible range (300–700 nm) was measured for the dorsal and ventral corolla lobes of five flowers in one population of each of *G. goseloides*, *G. montanus* and *G. nutans* using an Ocean Optics S2000 spectrophotometer (Ocean Optics Inc., Dunedin, Fla.) and fibre optic reflection probe (UV/VIS 400 µm) held at 45° to the petal surface (cf. Johnson and Andersson 2002). The light source used was an Ocean Optics DT-mini deuterium tungsten halogen light source with

a ~200–1100 nm spectral range. An Ocean Optics WS-1 diffuse reflectance standard was used to calibrate the spectrophotometer (cf. Johnson and Andersson 2002). Both the dorsal and ventral petal surfaces were measured because of the nodding orientation of *Glumicalyx*, resulting in both surfaces being visible to prospective pollinators. The spectral reflectance data were visualised in RStudio version 3. 5. 1 (R Core Team 2018) using the R package pavo (Maia *et al.* 2013), whereby the data was viewed, smoothed, negative values were removed, and plotted as wavelength (nm) against reflectance (%).

The flower colour perceived by the insect visitors of *Glumicalyx* was investigated by plotting the reflectance spectra of the three *Glumicalyx* species as loci in the bee colour hexagon (Chittka 1992) and the blowfly colour vision model (Troje 1993). The bee colour hexagon is based on the trichromatic visual system of the honeybee (*Apis mellifera*), and is thus well suited as most of the bees observed visiting the *Glumicalyx* species were *Apis mellifera scutellata* (Chittka 1992). The six segments of the bee colour hexagon represent the six colours in bee vision – blue, blue-green, green, UV-green, UV and UV-blue (Chittka 1992). Loci are calculated from the relative stimulation of the three receptor types (UV, blue and green) by the spectral reflectance of the flowers (cf. Shuttleworth and Johnson 2010). Distance between loci in the hexagon correlates with discriminability of the colours by bees (cf. Shuttleworth and Johnson 2010). Colour distance in the hexagon was calculated as the Euclidean distance between loci (cf. Shuttleworth and Johnson 2010), using the package pavo (Maia *et al.* 2013) in RStudio version 3. 5. 1 (R Core Team 2018). Intraspecific distances (hexagon units) represent the range of distances between conspecific samples. Interspecific distances (hexagon units) represent the distances between the mean loci for each species (cf. Shuttleworth and Johnson 2010).

The blowfly colour vision model is based on behavioural experiments with *Lucilia sp.* Calliphorids (Troje 1993) and spectral sensitivities of *Musca* (Muscidae) and *Calliphora* (Calliphoridae) flies (Hardie and Kirschfeld 1983), whereby flies exhibit categorical colour vision (Troje 1993). It was suggested that these flies have similar spectral sensitivities (Troje 1993). Although there is indirect evidence of all studied flies having colour vision, there is no evidence that other species of fly have categorical colour vision (Lunau 2014). The model of Troje (1993) has been applied in studies accessing flower colours and flower visiting flies (Arnold *et al.* 2009), including Syrphidae (Defrize et al. 2010), Calliphoridae, Muscidae and Sarcophagidae (Shuttleworth and Johnson 2010) and Tabanidae flies (Jersáková *et al*. 2012). The flies that visited the *Glumicalyx* species were of the family Muscidae, Syrphidae and

Tachinidae and I considered the model of Troje (1993) to be suitable to these species. The system is based on the relative excitations of the two p-type and two y-type receptors and the perceived colour depends on the receptor of each pair that is stimulated most strongly. The four possible colour categories (pþy þ, pþy2, p2yþ and p2y2) correspond to the difference in excitation between the receptors of each type (Troje 1993). All stimuli within each category are considered indistinguishable by flies (Troje 1993).

Scent sampling

To quantify differences in floral scent between the three species, and between different time periods, floral volatiles were collected. Floral scent was collected between December 2017 and January 2018 from *Glumicalyx goseloides, G. montanus* (Sentinel, Witsieshoek) and *G. nutans* (Platberg, Harrismith) during peak flowering times. For each species, three inflorescences from different plants were sampled, resulting in three day and three night samples (one day sample of *G. nutans* was damaged during transportation and as a result was excluded). To characterise differences in composition and quantity of volatiles emitted during day and night, sampling was performed during the late morning and just after sunset. Scent samples were taken using headspace collection and analysed using gas chromatography-mass spectrometry (GC-MS) methods. The scent was extracted by enclosing one inflorescence per individual (in peak flower) in a polyacetate bag (Kalle Bratschlauch Wiesbbaden, Germany) prior to sampling. To control for volatiles present in ambient air, scent from an empty bag was also sampled. The air present in the bags was pumped through small cartridges containing 1 mg of Tenax® TA 60/80 and 1 mg of Carbotrap® TM 20/40 mesh (both SupelcoTM Analytical; Bellefonte, PA, USA) at a flow rate of 50 ml/min for 30 minutes. The trapped scent cartridges were brought back to the laboratory and stored at -20 °C until analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis of floral scent

GC-MS analyses of the samples were carried out using a Varian CP-3800 GC (Varian, Palo Alto, California) with a 30 × 0.25 mm internal diameter (film thickness 0.25 μm) Alltech EC-WAX column coupled (for two-three of the day samples and two of the night samples) and DB5 column (one night sample from each species) to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode. Cartridges were placed in a Varian 1079 injector equipped with a Chromatoprobe thermal desorption device (Gordin and Amirav 2000). The flow of helium carrier gas was 1 ml min^{-1}. The injector was held at 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C/min in splitless mode

for thermal desorption. After a 3-min hold at 40 °C, the temperature of the GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 min (van der Niet *et al.* 2014). Compounds were identified using the Varian Workstation software with the NIST05 mass spectral library (NIST/EPA/NIH Mass Spectral Library [data version: NIST 05; MS search software version 2.0 d]) and verified with published Kovat's indices (references in the NIST 05 library). Compounds present in similar abundance in the controls were considered to be contaminants and excluded from the analyses. For quantification of scent emission rates per hour and per minute, known amounts of methyl benzoate (1087 ng) were injected into thermodesorption cartridges and thermally desorbed under identical conditions to those that the samples were subjected to (cf. van der Niet *et al.* 2015). Although methyl benzoate were not found in the samples of *Glumicalyx*, methyl benzoate was used as a standard for the quantification of scent emission rate.

Statistical analysis of floral scent data

To assess the variability in the floral scent of the 14 *Glumicalyx* samples of the three different species, I used the program Past3 (PAleontological Statistics, Hammer *et al.* 2001). For the scent data, proportional abundance of compounds (relative amounts with respect to total peak areas, excluding volatiles present in the ambient air) was used as a result of the total amount of emitted volatiles varying greatly among samples (cf. van der Niet *et al.* 2010). To visualize variation among samples, multivariate analyses using non-metric multidimensional scaling (NMDS) were conducted (Clarke and Warwick 2001). The data were square root transformed before calculating Bray-Curtis similarities (Clarke and Warwick 2001), which performs well if the data matrix contains many zero values. To test for differences in scent profiles between species, a one-way Analysis of Similarity ANOSIM (Clarke 1993) with 10,000 random permutations was used, whereby the day and night samples were combined for each species – the sample number was too low to test for variation between time period and species. Similarity Percentage (SIMPER) analysis was used to identify the compounds responsible for similarities/dissimilarities among species and time of day (Clarke 1993).

Results

Breeding systems

In both *Glumicalyx goseloides* and *G. nutans*, significantly more seeds were produced in the hand cross-pollinated treatment than the self-pollinated treatment (*G. goseloides*: $\chi^2 = 30$, $P < 0.001$; *G. nutans*: $\chi^2 = 30.7$, $P < 0.001$, Figure 3.2). The index of self-incompatibility for *G. goseloides* was 0.99 and for *G. nutans* was 0.93, indicating that *G. goseloides* and *G. nutans* are fully self-incompatible (Table 3.2). *Glumicalyx montanus* was considered partially self-compatible, as there was no significant difference between the hand cross-pollinated treatment and the self-pollinated treatment ($\chi^2 = 3.1$, $P > 0.05$, Figure 3.2) and the index of self-incompatibility was 0.59 (Table 3.2). All three species of *Glumicalyx* included are not completely dependent on pollen vectors for fruit and seed production as some fruit and seed were produced in the un-manipulated bagged treatment, however significantly fewer fruit were produced in the un-manipulated bagged treatment than the open treatment (Figure 3.2). The index of autonomous self-pollination based on fruit set for *G. goseloides* was 0.05, for *G. montanus* was 0.47 and for *G. nutans* was 0.16, indicating that *G. montanus* is partially able to autonomously self-pollinate and *G. goseloides* and *G. nutans* are not able to autonomously self-pollinate (Table 3.2). In *G. montanus* and *G. nutans* there was no significant difference between the open and supplement treatments (Figure 3.2) and the pollen limitation index based on fruit set was 0.16 in *G. montanus* and 0.14 in *G. nutans* suggesting that these species were not pollen limited. In contrast, there was a statistical difference between the open and supplement treatments in *G. goseloides*, with the open treatment resulting in lower fruit set than the supplement treatment ($\chi^2 = 25.5$, $P < 0.001$, Figure 3.2) and the pollen limitation index was 0.65, suggesting that *G. goseloides* was pollen limited (Table 3.2). Seed germination was significantly different between the open, supplement and bagged treatment, and was greatest in the supplementary treatment and lowest in the bagged treatment in *G. goseloides*. In *G. montanus* and *G. nutans* no seeds germinated in the bagged treatments, with overall very low seed germination in *G. montanus* (Figure 3.3). Seed germination did not differ significantly between the supplementary treatment and open treatment in *G. montanus* ad *G. nutans* (Figure 3.3).

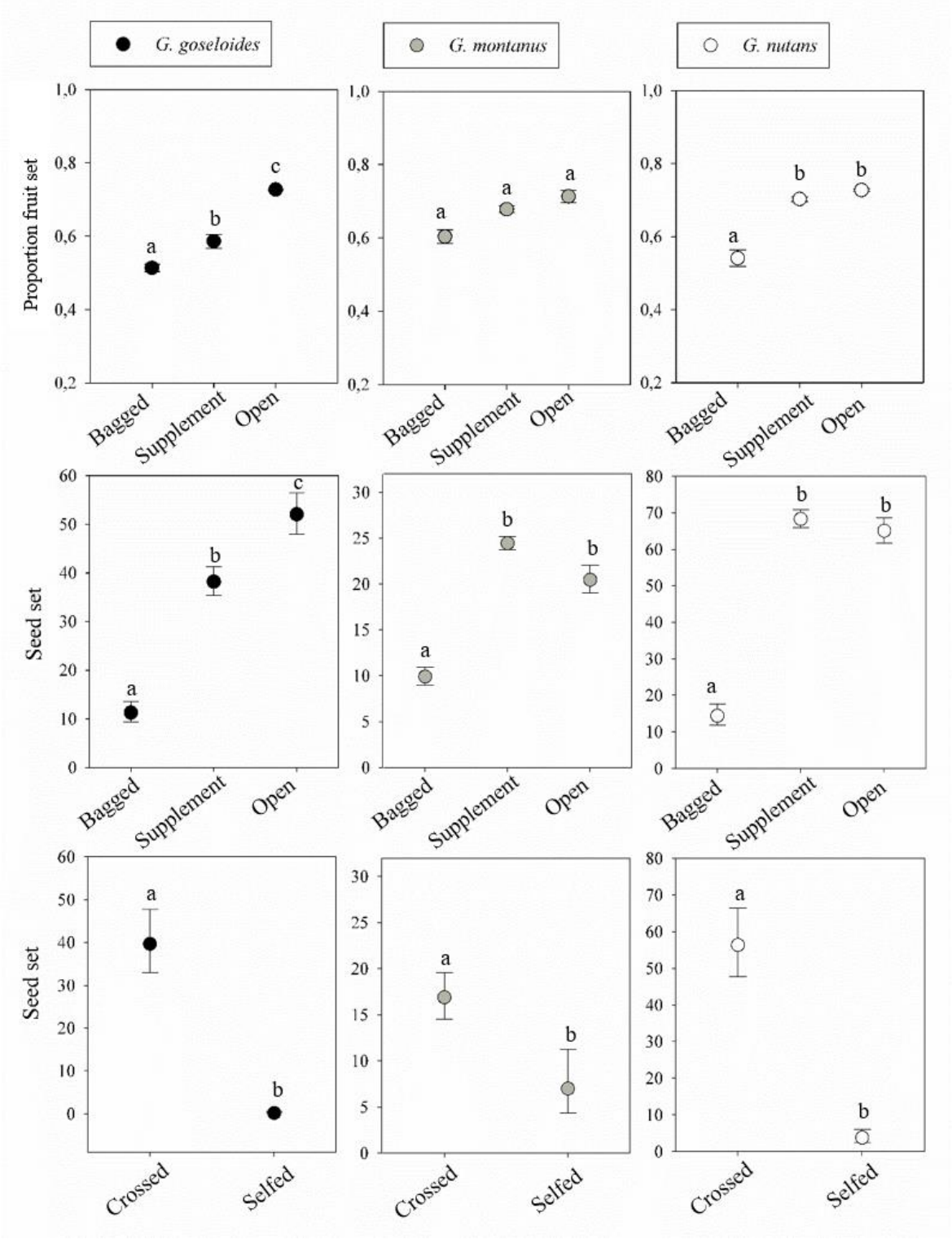


Figure 3.2. Mean proportion fruit set (±SE) of the bagged, open and supplement treatments, total seed set (±SE) of the flowers that produced fruit of the bagged, open and supplement treatments and the total seed set (±SE) of the hand-crossed and hand-selfed treatments for the three *Glumicalyx* species. Means for a particular attribute that have different superscript letters are significantly different ($P < 0.05$).

Table 3.2 Breeding system indices, including index of self-incompatibility, index of autonomous self-pollination and pollen limitation index, calculated from seed or fruit set from the three *Glumicalyx* species. IAS = 1, completely autofertile, ISI = 1, completely self-incompatible; PLI = 0, no pollen limitation.

Breeding system indices		*G. goseloides*	*G. montanus*	*G. nutans*
Index of self-incompatibility (ISI)	Seed	0.99	0.59	0.93
Index of autonomous self-pollination (IAS)	Fruit	0.05	0.47	0.16
Pollen limitation index (PLI)	Fruit	0.65	0.16	0.14

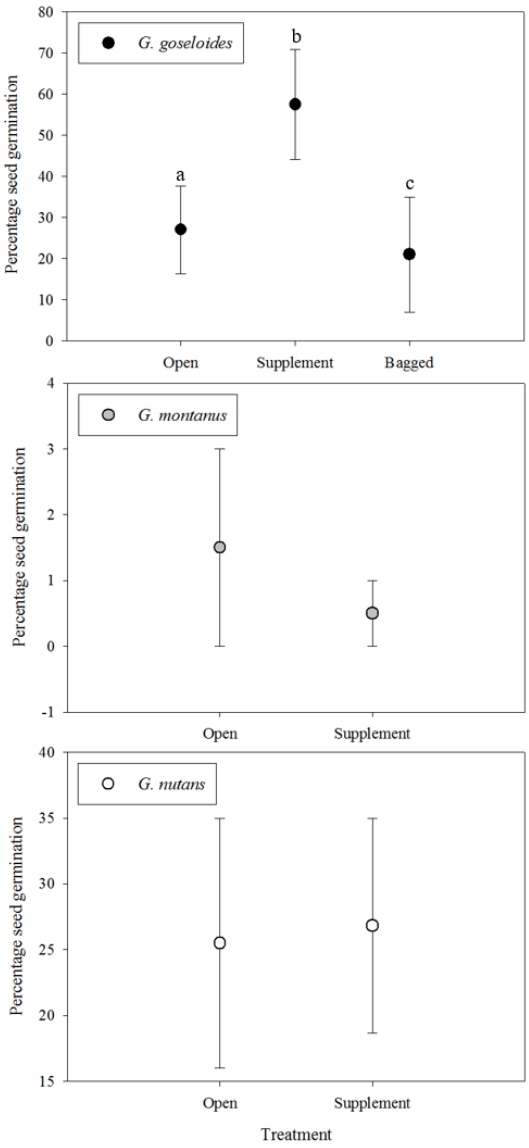


Figure 3.3. Average percentage of seeds germinated in three species of *Glumicalyx* for the open, supplement and bagged treatment. No seeds germinated in the bagged treatment of *G. montanus* and *G. nutans*. Means for a particular attribute that have different superscript letters are significantly different ($P < 0.05$).

Pollinator observations

During a total of 101.5 h of observations, visitation by insects to *Glumicalyx* flowers was observed for all three species, with photographs taken where possible (Figure 3.4). Highest visitation was to *G. nutans*, and lowest visitation was to *G. goseloides* (Figure 3.5). Insects were observed both during daytime and evening, but moths were mostly observed at night whereas bees, flies, beetles and grasshoppers were observed in the daytime (Figure 3.5). No insects carrying pollen were observed and caught on *G. nutans* at Witsieshoek, and therefore all observations and pollen loads presented are from the Platberg populations of *G. nutans* (Table 3.3). Honeybees (*Apis mellifera scutellata*) were observed in large numbers collecting pollen from flowers of *G. nutans* (Table 3.3). Settling moths visited *G. nutans* in the evenings and comprised mostly of various species of Noctuidae (Table 3.3). In contrast, large numbers of Diptera from various families visited *G. montanus* during the day, but only a low percentage of the total insects caught had pollen present (Table 3.3). The families of flies with pollen present were Syrphidae, Muscidae and Tachinidae. Settling moths also visited *G. montanus* in the evenings and consisted mostly of *Mythimna* sensu lato *corax* (Noctuidae, Table 3.3). Photographs of the moths foraging at the flowers revealed thick deposits of pollen along the length of the proboscis, as well as on the abdomen and appendages (Figure 3.3). Large pollen loads were found on the majority of the moths caught visiting both *G. montanus* and *G. nutans* (Table 3.3). Species of Orthoptera, Coleoptera, Hymenoptera and a single species of Blattodea were observed visiting *G. goseloides*. Of the insects caught visiting *G. goseloides*, only three of the insects carried pollen, species of an ant, a grasshopper and a cockroach (Table 3.3). Insects that were observed feeding but were not caught and analysed for pollen are given in Appendix 3.1.

Figure 3.4. (over page) Insect visitors foraging on *Glumicalyx* species. (a) Settling moth (Noctuidae, Lepidoptera) feeding from *G. nutans*, pollen present on the proboscis and legs. (b) Honey bee, *Apis mellifera scutellata*, (Apidae, Hymenoptera) visiting *G. nutans*, pollen baskets present on the hind legs. (c) Short-tongued bee (Hymenoptera) visiting *G. nutans*, pollen baskets present on the hind legs. (d) Settling moth (Noctuidae, Lepidoptera) feeding from *G. montanus*, pollen present on the proboscis. (d) Short-tongued fly (Muscidae, Diptera) visiting *G. montanus*, pollen present on legs. (e) Short-tongued fly (Muscidae, Diptera) feeding from *G. montanus*. (g) Blattodea visiting *G. goseloides*. (h) Ant (Hymenoptera) visiting *G. goseloides*. (i) Orthoptera feeding on *G. goseloides* corolla lobes. All photographs, apart from (d) taken by T. Springer, photograph (d) courtesy of T. van der Niet.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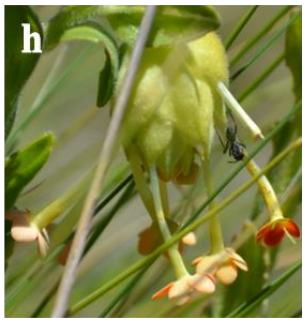
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Table 3.3. Captured floral visitors of the three *Glumicalyx* species, identified to lowest taxonomic rank possible (number of individuals caught given in parentheses) and pollen load.

Plant species	Time period	Insect order	Insect family	Insect species (*N*)	Pollen load category			
					0-100	> 100	> 1000	Pollen basket
G. nutans	Day	Diptera		Morphospecies 1 (1)		1		
G. nutans	Day	Hymenoptera	Apidae	*Apis mellifera scutellata* Lepeletier 1836 (5)		1	3	5
G. nutans	Day	Hymenoptera		Morphospecies 2 (1)				1
G. nutans	Day	Hymenoptera		Morphospecies 3 (2)	1			1
G. nutans	Night	Lepidoptera	Noctuidae, Cucullinae	*Cucullia hutchinsoni* Hampson 1902 (2)			2	
G. nutans	Night	Lepidoptera	Noctuidae, Plusiinae	*Trichoplusia exquisita* (Felder and Rogenhofer, 1874) (1)	1			
G. nutans	Night	Lepidoptera	Noctuidae, Heliothinae	*Heliothis xanthiata* Walker 1865 (1)	1			
G. nutans	Night	Lepidoptera	Noctuidae, Acronictinae	*Athetis pigra* (Guenée 1852) (1)	1			
G. nutans	Night	Lepidoptera	Noctuidae, Cuculliinae	*Cucullia terensis* Felder and Rogenhofer 1874 (13)	3	8	2	
G. nutans	Night	Lepidoptera		Morphospecies 4 (1)		1		
G. nutans	Night	Lepidoptera	Noctuidae, Plusiinae	*Thysanoplusia angulum* (Guenée 1852) (8)		3	4	
G. nutans	Night	Lepidoptera	Noctuidae, Noctuinae	*Agrotis segetum* ([Denis & Schiffermüller] 1775) (2)		2		
G. nutans	Night	Lepidoptera		Morphospecies 5 (1)	1			
G. nutans	Night	Lepidoptera		Morphospecies 6 (1)	1			
G. montanus	Day	Diptera	Syrphidae	*Betasyrphus intersectus* Wiedemann 1824 (2)	2			
G. montanus	Day	Diptera	Muscidae	Morphospecies 7 (2)	2			
G. montanus	Day	Diptera	Tachinidae	Morphospecies 8 (1)		1		
G. montanus	Day	Diptera	Muscidae	*Helina* sp. 1 (1)	1			
G. montanus	Day	Diptera	Muscidae	*Helina* sp. 2 (1)	1			
G. montanus	Day	Diptera	Tachinidae	Morphospecies 9 (1)		1		
G. montanus	Night	Lepidoptera	Hesperiidea, Hesperiinae	*Metisella metis* (Linnaeus 1764) (1)	1			
G. montanus	Night	Lepidoptera	Noctuidae	*Mythimna* sensu lato *corax* Krüger 2005 (9)	5	3		
G. montanus	Night	Lepidoptera	Scopariinae	*Scoparia* sp. (1)		1		
G. goseloides	Day	Coleoptera		Morphospecies 10 (1)	1			
G. goseloides	Day	Hymenoptera		Morphospecies 11 (1)	1			
G. goseloides	Night	Blattodea		Morphospecies 12 (1)			1	

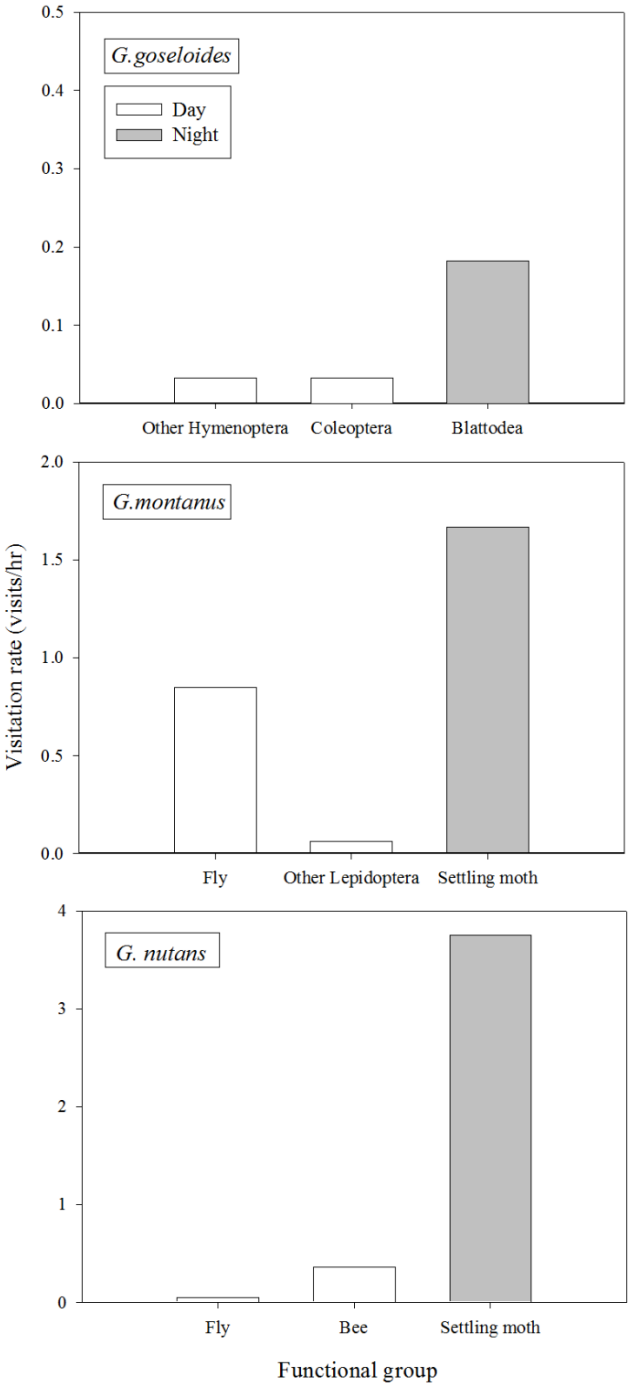

Figure 3.5. Visitation rates per hour of seven functional pollinator groups to three *Glumicalyx* species. Insects included were insects that were caught and had *Glumicalyx* pollen present. Short-tongued bees that only had pollen present in the form of pollen baskets (on their scopae) were excluded. White shading indicates insects observed and caught during daytime observations and grey shading indicates insects observed and caught during evening observations.

Day-Night comparison of Glumicalyx montanus pollination biology

A greater proportion of individuals were observed with visible anther dehiscence and subsequent release of pollen at night compared to the day (χ^2 = 18.8, P < 0.001, Figure 3.6). However, there was no difference in the number of fruits (χ^2 = 0.52, P > 0.05) and seeds produced in individuals open to pollinators during the day compared to the night. In both the day-exclusion and night-exclusion treatments, two out of five and two out of four fruit respectively produced no seed, but statistical analyses were not possible due to limited sample size.

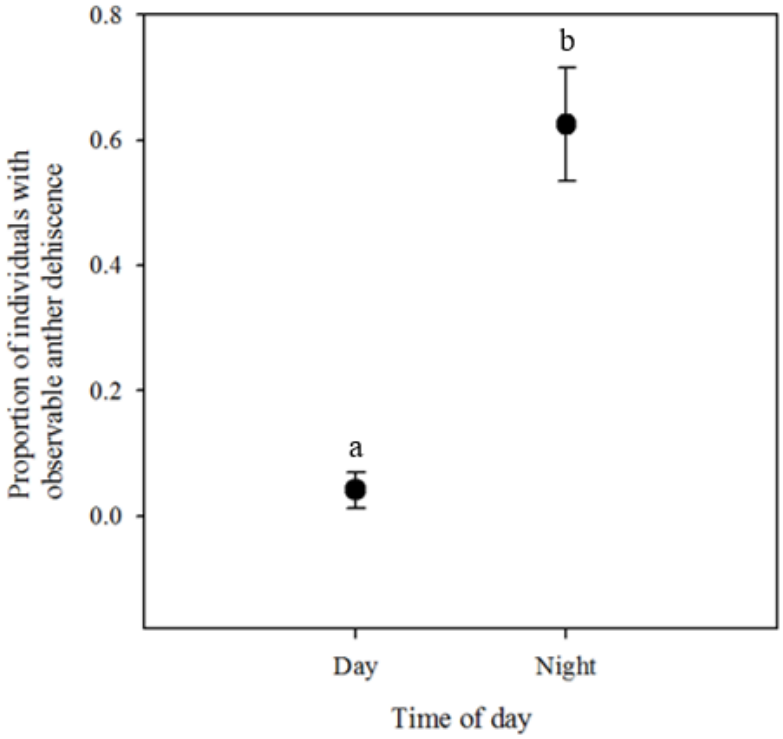

Figure 3.6. Mean ± SE of the observed anther dehiscence and subsequent release of pollen from receptive inflorescences of *G. montanus* during observations around midday and at dusk over three consecutive days. Means for a particular attribute with different superscript letters are significantly different (P < 0.05).

Floral morphology and nectar properties

Corolla tube length varies between c. 5 mm in *G. montanus* to c. 26 mm in *G. goseloides* and was significantly different between the three species (Table 3.4). The width of the corolla tube opening varies between c. 1.1 mm in *G. goseloides* and *G. nutans* and 1.6 mm in *G. montanus* and was significantly wider in *G. montanus* compared to the other two species

(Table 3.4). All three species produce nectar when measured at peak day and peak night pollinator activity (Table 3.4). Nectar volume varied from c. 0.3 µL in *G. montanus* to 0.9 µL in *G. goseloides* (Table 3.4). Nectar volume differed between the three species, except between the *G. nutans* and *G. montanus* night samples. Nectar volume differed between the day and night only in *G. nutans*. Nectar sugar concentration varied from c. 17% in *G. montanus* to c. 32% in *G. nutans*, and differed between the three species. Nectar sugar concentration differed between the day and night samples in *G. goseloides* and of *G. montanus*, in both the nectar sugar concentration was higher at night. The nectar sugar concentration was consistent between the day and night in *G. nutans* (Table 3.4).

Table 3.4. Corolla tube dimensions and nectar properties for three *Glumicalyx* species. Values are means ± SD with the sample size given in parentheses. Means for a particular attribute with different superscript letters are significantly different ($P < 0.05$). (Generalized Estimating Equation with pairwise comparison, compared between species – all measurements – and time period – for nectar volume and nectar sugar concentration).

Trait		*G. goseloides*	*G. montanus*	*G. nutans*
Corolla tube length (mm)		25.7^{a} ± 2.7 (94)	5.4^{b} ± 0.8 (128)	14.7^{c} ± 2.5 (73)
Corolla tube diameter (mm)		1.1^{a} ± 0.1 (94)	1.6^{a} ± 0.2 (128)	1.1^{b} ± 1.4 (73)
Nectar volume (µL)	Day	0.9^{a} ± 0.4 (74)	0.3^{b} ± 0.2 (98)	0.4^{c} ± 0.2 (49)
	Night	1.1^{a} ± 0.7 (20)	0.3^{bd} ± 0.1 (30)	0.2^{d} ± 0.2 (24)
Nectar sugar concentration (%)	Day	24.7^{a} ± 2.8 (20)	17.2^{b} ± 8.3 (30)	31.9^{c} ± 6.7 (10)
	Night	28^{d} ± 2.8 (7)	21.4^{e} ± 4.2 (8)	41.0^{c} ± 14.7 (6)

Spectral reflectance of flowers

For all three species examined, there is minimal reflectance in UV wavelengths (300–400 nm) on the petal surface (Figure 3.7). The dorsal petal surfaces of *G. montanus* are perceived as pale yellow and the ventral petal surface as cream-white to humans; both reflect all wavelengths over 400 nm, with the dorsal petal surface showing a sharp increase in reflectance at 550 nm (green to red; Figure 3.7). The dorsal petal surfaces of *G. goseloides* and *G. nutans* show an increase in reflectance after 500 nm (blue) with a slight decrease around 660–700 nm (orange to red; Figure 3.7). In all three species there was generally low reflectance of the corolla lobes, particularly in *G. nutans*.

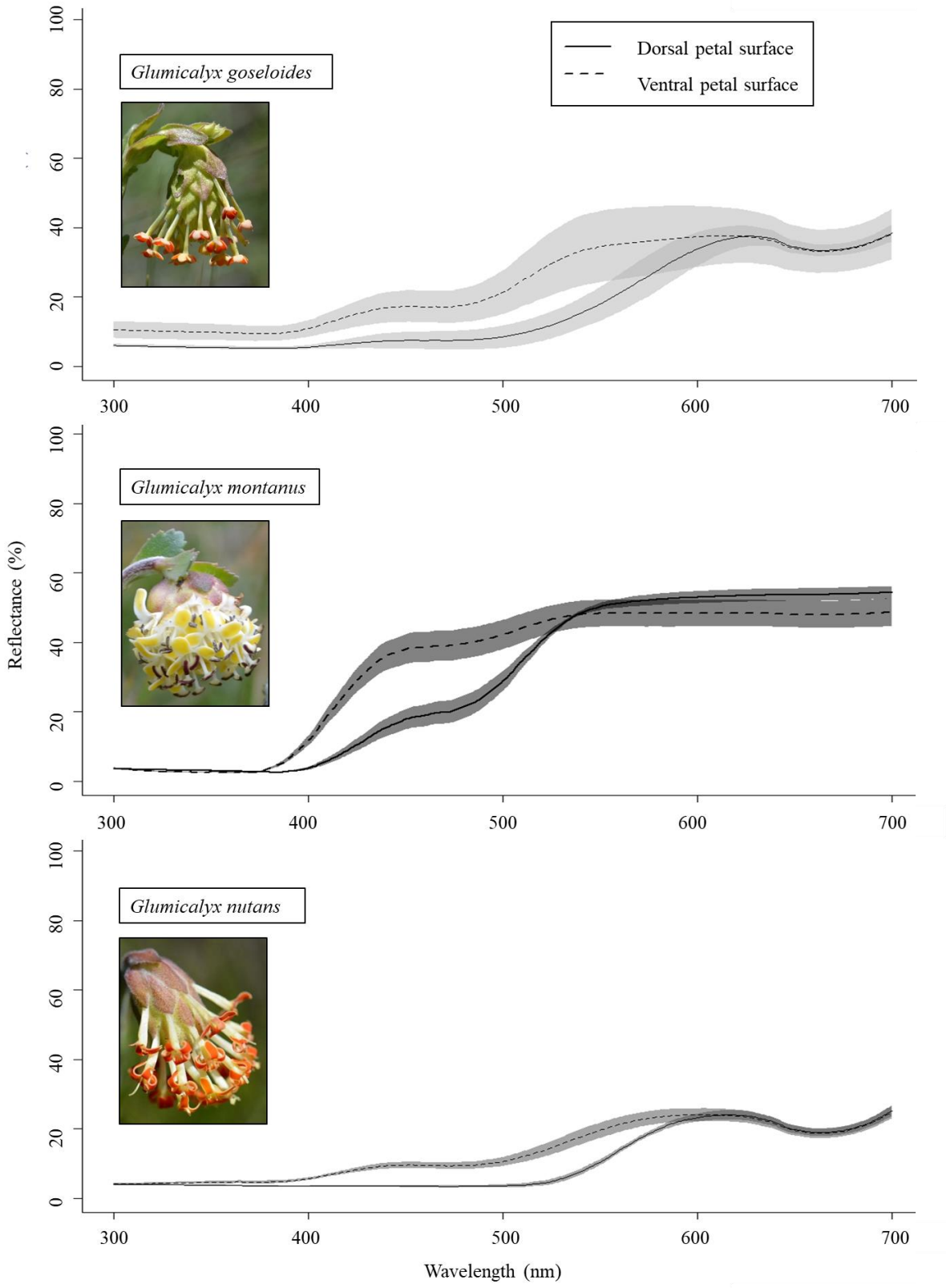

Figure 3.7. Average spectral reflectance patterns of the upper petal surface (solid line) and lower petal surface (dotted line) of three *Glumicalyx* species. The shaded area represents the standard error of the reflectance.

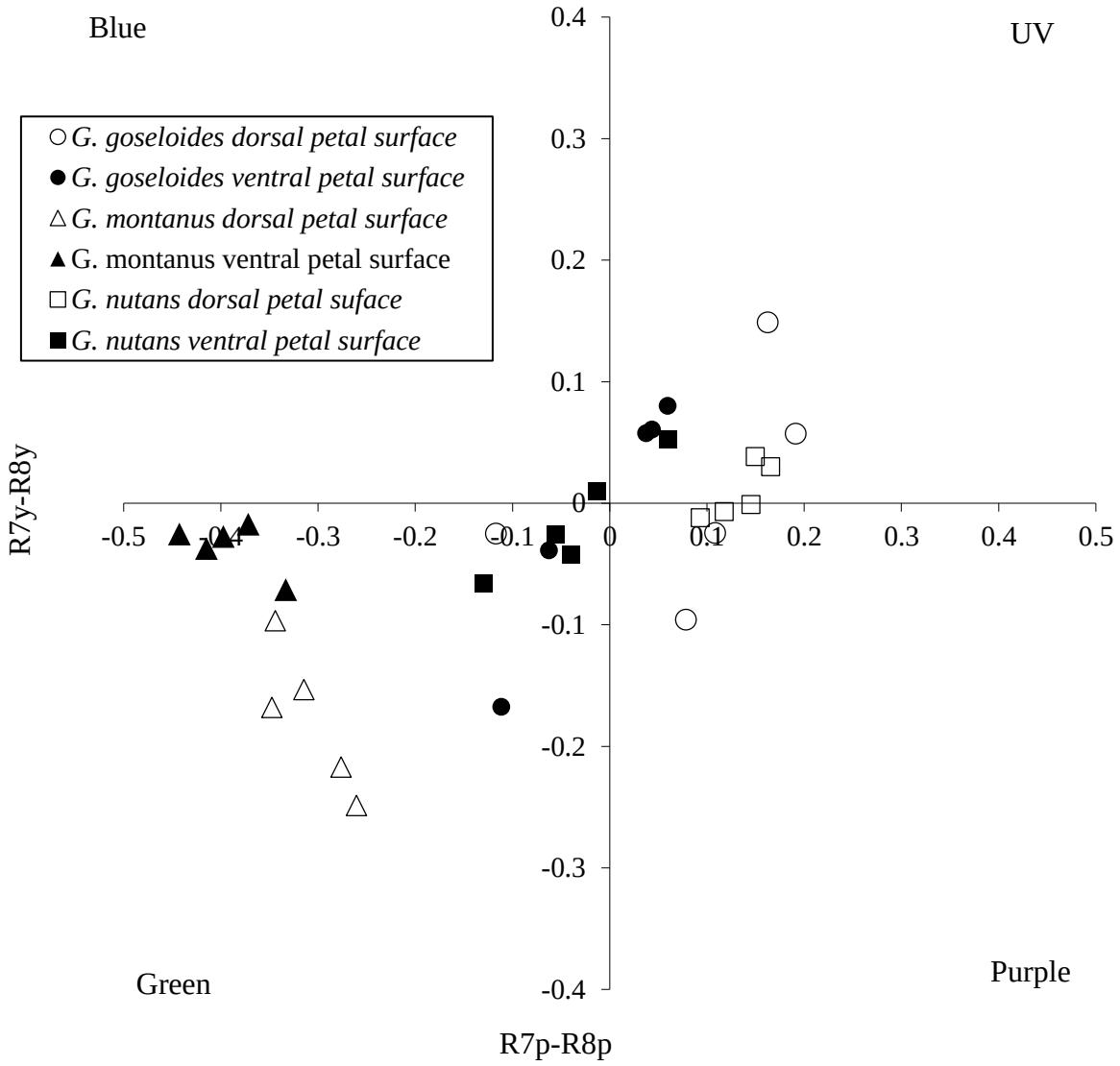

Figure 3.8. Floral colours of the three *Glumicalyx* species for the dorsal and ventral petal surfaces in the blowfly colour vision model (Troje 1993).

The spectral reflectance of the dorsal and ventral petal surface in *G. montanus* fall closely together in both the blowfly colour vision model (Figure 3.8) and bee colour hexagon (Figure 3.9). The spectral reflectance of the dorsal petal surface in *G. nutans* and *G. goseloides* fall closely together, as does the spectral reflectance of the ventral petal surface in both the blowfly colour vision model (Figure 3.8) and bee colour hexagon (Figure 3.9). Floral colour for *G. montanus* (dorsal and ventral petal surfaces) falls within the green quadrant of the blowfly colour vision model (Figure 3.8). The dorsal petal surfaces of *G. goseloides* and *G. nutans* fall between the purple and UV quadrants and the ventral petal surfaces of *G. goseloides* and *G. nutans* fall between the green and UV quadrants of the blowfly colour vision model (Figure 3.8). Floral colour for *G. montanus* (dorsal and ventral petal surfaces)

falls within the blue-green region of the bee colour hexagon (Figure 3.9). The orange-red floral colours of *G. goseloides* and *G. nutans* mostly fall within the centre of the bee colour hexagon, representing the colour of the green foliage background (Figure 3.9).

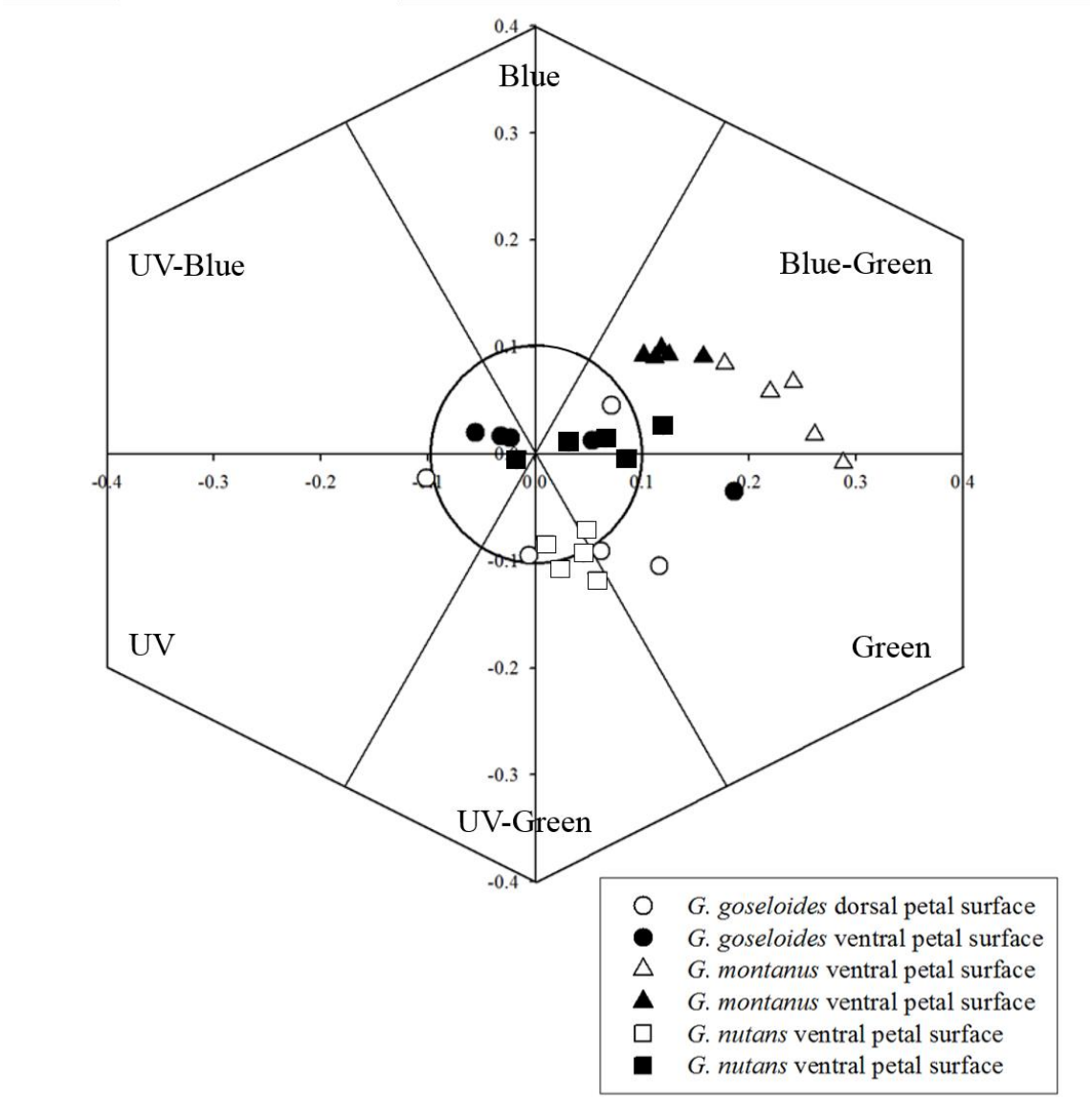

Figure 3.9. Floral colours of the three *Glumicalyx* species for the dorsal and ventral corolla surface in the bee colour hexagon (Chittka 1992). The six segments represent the six categories of bee colour vision.

Floral scent composition

The three species produced a combined total of 99 scent compounds, of these 61 were unknown compounds. The unknown compounds contributed a total of 33.3% to all the samples. Of the total number of compounds, 94 were species specific (Table 3.5). The total number of compounds was 51 in *G. goseloides,* 26 in *G. montanus* and 34 in *G. nutans.* The

total number of scent compounds and the mean scent emission per inflorescence for each species in the day and night samples differed – in all three species there was a greater scent emission at night compared to the day (Table 3.5). In *G. goseloides* and *G. montanus* there was a greater number of scent compounds at night compared to the day, in *G. nutans* there was only a difference of three compounds between day and night (Table 3.5). All replicates of the sampling period for each species clustered closely together in the multivariate ordination analysis (Figure 3.10).The day and night scent samples of *G. montanus* clustered in the same area of the hexagon, together with the day samples of *G. nutans* (Figure 3.10), whilst the night samples of *G. goseloides* and *G. nutans* clustered together (Fig. 3.10). The day samples of *G. goseloides* did not cluster closely to the other samples. There was a significant difference in the scent composition between the species ($P < 0.001$) based on a one-way ANOSIM using Bray-Curtis similarities. An unidentified compound with dominant mass fragments (in descending order) 147, 105, 162, 91, 120, 119, 77, 79, 92, 115 (Kovats retention index = 1655), benzaldehyde and linalool account for the greatest dissimilarity between the species (Table 3.6). The dominant compounds between the day and night samples differed between the species (Figure 3.10; Appendix 3.2).

Table 3.5. Average relative amounts (%) of main floral scent compounds of three *Glumicalyx* species. Compounds are listed in order of increasing retention time within each compound class, identification using CWAX. KRI = Kovats retention index; CAS# = unique numerical identifiers of compounds assigned by the Chemical Abstracts Service. CIC = compound identification criteria and notes: a = comparison of MS with published data; b = comparison of MS and retention time with published data. Locality: SN = Sentinel Peak, PB = Platberg.

	CAS #	KRI	CIC	*G. goseloides*		*G. montanus*		*G. nutans*	
Locality				SN	SN	SN	SN	PB	PB
Sample type (day versus night)				Day	Night	Day	Night	Day	Night
Number of samples				3	2	3	2	3	2
Mean of scent emission per inflorescence ($ng \cdot min^{-1}$)				1.7	181.5	0.8	26.1	6.9	98.5
Mean of scent emission per inflorescence ($ng \cdot h^{-1}$)				100.3	10892.1	49.2	1568.6	413.7	5912.0
Number of compounds				19	38	12	25	21	18
Alcohols									
Isopentyl alcohol	123-51-3	1114	b	-	-	-	-	10.48	-
Isopentyl alcohol, acetate	123-92-2	1128	b	-	-	-	-	31.08	1.90
1-Hexanol, 2-ethyl-	104-76-7	1449	a	1.26	-	8.54	0.07	-	-
4-Hexen-3-ol	6126-50-7	1954	a	-	0.02	-	-	-	-

Esters									
Butyl acetate	123-86-4	1094	b	-	-	-	0.28	-	-
Butanoic acid, 2-methylpropyl ester	539-90-2	1151	b	-	-	-	-	10.22	-
Acids									
Propanoic acid	79/09/04	1497	b	-	0.03	-	-	-	0.01
Propanoic acid, 2-methyl-	79-31-2	1528	b	-	0.89	-	0.46	-	0.16
Isovaleric acid	503-74-2	1632	b	-	-	0.40	4.29	-	1.17
Octanoic acid	124-07-2	2030	b	-	-	-	-	-	0.17
Benzenoids									
Toluene	108-88-3	1076	b	-	-	39.26	4.90	-	-
Ethylbenzene	100-41-4	1129	b	-	-	-	1.48	-	-
Styrene	100-42-5	1231	b	-	-	22.40	1.79	16.21	-
Anisole	100-66-3	1309	b	-	-	5.14	1.35	-	-
1,4-Dichlorobenzene	106-46-7	1413	a	-	-	1.36	0.15	0.55	-
Benzaldehyde	100-52-7	1495	b	-	-	10.19	65.37	-	-
Benzeneacetaldehyde	122-78-1	1617	b	-	-	-	11.91	-	-
Naphthalene	91-20-3	1722	b	-	-	-	0.08	0.91	0.06
Benzyl alcohol	100-51-6	1848	b	-	-	-	2.54	-	-
Phenylethyl alcohol	60-12-8	1886	b	-	-	0.25	4.14	-	-
Monoterpenes									
β-Pinene	127-91-3	1163	b	-	0.22	-	-	-	-
Eucalyptol	470-82-6	1197	b	-	-	-	-	26.67	-
Limonene	138-86-3	1198	b	-	-	-	0.19	-	-
β-Ocimene	13877-91-3	1243	b	-	64.11	-	-	-	-
(Z)-Linalool oxide	5989-33-3	1437	b	-	-	-	-	-	0.62
Linalool	78-70-6	1505	b	1.15	-	-	-	0.53	70.47
β-Cyclocitral	432-25-7	1601	b	-	0.02	-	-	-	-
3,7-Octadiene-2,6-diol, 2,6-dimethyl-	13741-21-4	1906	b	-	-	-	-	-	0.02
Sesquiterpenes									
β-Bourbonene	5208-59-3	1507	b	1.19	-	-	-	-	-
β-Cubebene	13744-15-5	1524	b	0.81	-	-	-	-	-
β-Caryophyllene	87-44-5	1590	b	-	-	-	-	0.54	-
cis-Muurola-4(14),5-diene	157477-72-0	1592	a	-	-	-	-	0.45	-
Dihydro-β-agarofuran	5956-09-2	1775	b	11.30	0.03	-	-	0.09	-
Irregular terpenes									
2,6,6-Trimethyl-2-cyclohexene-1,4-dione	98419-16-0	1673	b	-	-	-	-	-	2.06
Unknowns									
m/z: 55,70,41,42,43,39,56,57,69,31		1179		-	-	-	0.17	-	-
m/z: 43,70,71,45,41,55,69,39,27,29		1249		-	-	-	-	0.39	-
m/z: 70,85,57,71,43,69,41,55,103,42		1272		-	-	-	-	-	0.38
m/z: 105,134,91,92,104,77,67,43,103,119		1283		-	-	3.08	0.46	-	-
m/z: 67,82,43,66,41,81,39,68,54,55		1285		2.10	-	-	-	-	-
m/z: 45,75,43,119,44,134,46,91,41,42		1300		-	-	7.92	-	-	-
m/z: 126,43,68,58,125,59,39,40,83,69		1309		-	0.03	-	-	-	-
m/z: 119,91,134,67,95,41,53,77,65,59		1337		-	0.04	-	-	-	-
m/z: 67,82,41,54,55,39,69,42,68,43		1342		0.13	-	-	-	-	-
m/z: 121,136,85,105,91,79,119,93,77,41		1346		-	0.02	-	-	-	-
m/z: 150,135,121,91,79,107,95,105,77,53		1368		-	0.01	-	-	-	-

m/z: 132,117,115,131,91,116,133,118,105,128	1375	-	-	-	0.04	-	-
m/z: 147,105,162,91,120,119,77,79,92,115	1395	57.29	0.12	-	-	-	-
m/z: 43, 45, 60, 59, 94, 93, 111, 68, 55, 67	1408	-	-	-	-	-	0.05
m/z: 59,103,45,43,72,73,58,41,104,57	1432	-	-	-	-	0.08	-
m/z: 41,39,69,97,42,53,98,43,79	1435	-	0.02	-	-	-	-
m/z: 59,103,45,73,41,58,31,104,43,72	1440	-	-	-	-	0.06	-
m/z: 79,81,77,53,80,91,93,72,43,193	1454	-	0.04	-	-	-	-
m/z: 91,107,92,79,135,43,90,77,65,39	1469	-	0.03	-	-	-	-
m/z: 147,105,162,91,120,119,77,79,92,115	1478	4.26	0.12	-	-	-	-
m/z: 71,93,43,67,82,69,83,55,81,68	1482	0.09	-	-	-	-	-
m/z: 79,91,93,162,81,78,77,41,67,105	1541	0.13	-	-	-	-	-
m/z: 93,119,41,69,43,55,91,71,67,79,107	1553	0.42	-	-	-	-	-
m/z: 71,67,113,55,43,70,83,84,57,66	1553	-	-	-	-	-	0.51
m/z: 109,81,79,83,43,124,41,53,108,55	1560	-	0.01	-	-	-	-
m/z: 161,121,162, 93,105,91,119,107,79,77	1566	-	-	-	-	0.93	-
m/z: 45,72,59,73,31,43,60,75,58,44	1580	-	-	-	-	0.10	-
m/z: 133,91,93,69,79,105,161,77,67,41	1589	2.88	0.02	-	-	-	-
m/z: 71,57,43,85,41,55,105,42,70,69	1591	-	-	1.46	-	-	-
m/z: 204,161,105,91,119,41	1591	-	-	-	-	0.06	-
m/z: 93,108,96,95,94,81,79,91,111,107	1644	-	-	-	-	0.48	-
m/z: 79,161,77,120,121,105,93,91,81,136	1652	0.48	-	-	-	-	-
m/z: 56,85,125,43,69,41,55,83,168,153	1655	-	32.04	-	-	-	22.36
m/z: 68,96,152,40,41,39,109,55,121,67	1667	-	1.01	-	-	0.05	-
m/z: 91,79,105,150,107,77,135,78,121,93	1701	-	0.07	-	-	-	-
m/z: 91,108,150,65,84,107,89,43,77,79	1701	-	-	-	0.09	-	-
m/z: 71,43,99,59,70,53,55,41,85,69	1757	-	0.03	-	-	-	-
m/z: 104,91,105,65,92,103,78,90,73,117	1763	-	-	-	0.05	-	-
m/z: 126,108,43,81,107,93,82,67,83,69	1764	-	-	-	-	0.12	-
m/z: 126,108,43,81,109,67,93,82,83,107	1764	5.65	-	-	-	-	-
m/z: 43,109,152,81,79,91,67,93,77,59	1770	-	0.02	-	-	-	-
m/z: 105,77,51,65,50,75,43,106,32,120	1788	-	-	-	0.02	-	-
m/z: 43,69,59,84,85,56,68,41,58,39	1798	-	0.07	-	-	-	-
m/z: 162,133,159,107,108,77,134,79,105,78	1848	-	0.03	-	-	-	-
m/z: 58,59,57,116,43,55,42,41,69,70	1875	-	0.03	-	-	-	-
m/z: 43,109,124,125,81,99,116,85,70,83	1892	-	-	-	-	-	0.01
m/z: 57,85,55,56,31,43,39,41,67,84	1931	-	-	-	0.10	-	-
m/z: 97,41,68,72,67,43,69,95,57,77	1932	-	0.01	-	-	-	-
m/z: 72,97,68,41,43,95,67,69,79,39	1969	-	0.05	-	-	-	-
m/z: 43,125,168,83,107,55,79,41,81,95	1975	-	0.01	-	-	-	-
m/z: 97,72,41,68,69,43,95,55,77,67	1991	-	0.02	-	-	-	-
m/z: 43,141,99,170,98,83,109,85,154,55	1993	-	-	-	-	-	0.01
m/z: 59,71,113,55,43,79,70,73,41,84	2000	-	0.01	-	-	-	-
m/z: 150,107,91,79,135,95,77,105,108,67	2037	-	0.01	-	-	-	-
m/z: 67,82,55,124,41,54,77,107,96,69	2087	-	-	-	0.03	-	-
m/z: 79,108,77,43,80,111,82,67107,81	2132	-	0.06	-	-	-	-
m/z: 43,95,59,153,125,97,55,83,69,79	2148	-	0.02	-	-	-	-
m/z: 43,95,152,109,91,153,119,81,55,125	2156	-	0.20	-	-	-	-
m/z: 43,108,126,93,67,111,71,69,77,79	2323	1.23	-	-	-	-	-
m/z: 73,57,256,60,43,55,41,129,69,61	2911	1.51	-	-	-	-	-
m/z: 69,81,68,136,95,41,67,121,137,93	3272	8.13	-	-	-	-	-

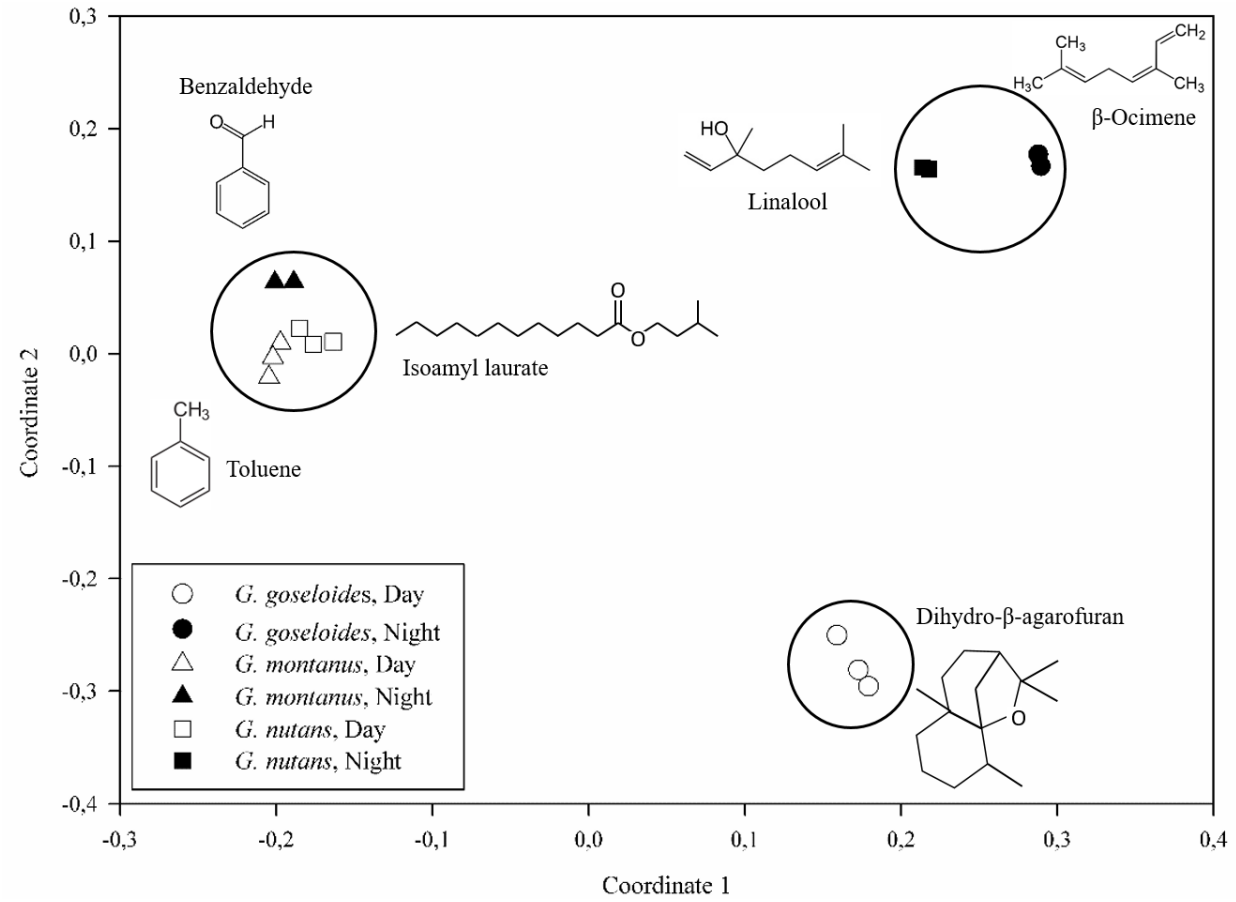

Figure 3.10. Non-metric multidimensional scaling of scent samples of three *Glumicalyx* species based on Bray-Curtis similarities of square-root transformed proportions of compounds in the blend.

Table 3.6. Compounds (as proportions) contributing to the first 50% of dissimilarity between the scent profiles of the three species of *Glumicalyx* (from the SIMPER analysis).

Compound	Average dissimalarity	Cumulative percentage	*G. goseloides*		*G. montanus*		*G. nutans*	
			Day	Night	Day	Night	Day	Night
m/z: 147,105,162,91, 120,119,77,79,92,115	11.1	11.55	57.3	0.125	0	0	0	0
Benzaldehyde	10.45	22.43	0	0	10.2	65.4	0	0
Linalool	10.03	32.87	1.15	0	0	0	0.526	70.5
β-Ocimene	8.962	42.19	0	64.1	0	0	0	0
Toluene	8.074	50.6	0	0	39.3	4.9	0	0

Discussion

Using a multidisciplinary approach to compare the reproductive biology of three co-flowering species of the largest endemic DMC genus, this study shows that the three species all relied on animal visits for pollination. Furthermore, there is substantial variation in floral traits and breeding and pollination system. This study also represent the first pollinator records for two of the three species.

Although some seed was produced when pollinators were excluded the species do depend on pollinators for successful reproduction. *Glumicalyx goseloides* and *G. nutans* appear to be self-incompatible. *Glumicalyx montanus* is partially self-compatible and can autonomously self-pollinate to some extent. Of the seeds produced by autonomous self-pollination, none germinated in *G. montanus* and *G. nutans*, and very few germinated in *G. goseloides*. Indicating that although seed were produced these seed may not be viable. In a study of 21 grassland flower species in eastern South Africa, two thirds of the species were self-incompatible and did not appear to be limited by pollinator availability (Johnson *et al*. 2009). Autonomous self-pollination becomes more common when visitation by pollinators is unreliable (Kevan 1972; Wyatt 1983).

The position of the anthers differed between the species, in *G. nutans* and *G. goseloides*, two anthers are inserted in the corolla tube and two anthers are exerted, and this difference in anther length increase the physical contact between anthers and pollinator. In *G. montanus* all four anthers are exerted from the corolla tube and of equal length. The stigma was observed to become receptive after anther dehiscence, whilst pollen was still observable, and self-pollen was observed on the stigmas of bagged flowers. The same was observed in *Zaluzianskya*, a close relative of *Glumicalyx*, whereby the stigma elongates after anther dehiscence and in doing so comes into contact with self-pollen (Johnson *et al*. 2003). The two species of *Zaluzianskya* studied were self-incompatible, thus the self-pollination did not result in self-fertilization (Johnson *et al*. 2003).

Pollen limitation appears to be a widespread occurrence amongst plants in general (Burd 1994; Larson and Barrett 2000; Ashman *et al*. 2004), including in high altitude and high latitude habitats (Totland 1994; García-Camacho and Totland 2009). Species with a narrow distribution range, may be at greater risk of pollen limitation, primarily in combination with self-incompatibility (Alonso *et al*. 2010). Pollen limitation is positively related to regional plant diversity and is more common in biodiversity hotspots (Vamosi *et al*.

2006). In a study of pollen limitation in South African biodiversity hotspots, South African plants exhibited pollen limitation comparable to other regions (Rodger and Ellis 2016). However, in my study only the population of *G. goseloides* was pollen-limited. There appears to be less pollen limitation in self-compatible and autogamous species, as these species are less reliant on pollen vectors and are able to use their own pollen to ensure reproduction (Larson and Barrett 2000). *Glumicalyx goseloides* appears to be self-incompatible, and the populations studied were located at 2450 m a.s.l and 2750 m a.s.l. The *G. goseloides* study population at 2750 m a.s.l is located at the upper limit of the species' altitudinal range and this may have influenced the pollen limitation.

The insects observed visiting the three species of *Glumicalyx* indicate a diverse pollinator fauna, as there were insects from multiple orders, including important pollinators from Diptera, Hymenoptera and Lepidoptera. Effective pollinators were observed for *G. montanus* and *G. nutans*, but not for *G. goseloides*. The lack of observed effective pollinators in *G. goseloides*, over a total of 36.5 observation hours, supports the finding that the populations were pollen limited. A single opportunistic visit by *Philoliche aethiopica* (Diptera, Tabanidae) was the only observed visit by a long-proboscid insect to *G. goseloides*. A number of species belonging to Coleoptera, Orthoptera and Blattodea were observed visiting *G. goseloides*, but observations of these insects showed a lack of contact between the insect and the reproductive parts and they were subsequently found to have little to no pollen present. The Coleoptera and Orthoptera were observed feeding on floral parts (corolla and anthers) rather than nectar result these floral visitors were not considered effective pollinators. Coleoptera and Orthoptera are known florivores, florivory is the damage to developing flora buds or mature flowers prior to seed development (Burgess 1991; McCall and Irwin 2006). The lack of observed pollinators may be a result of phenology, as the flowering of *G. goseloides* peaked in early December (early summer) and during this time considerably fewer flying insects were observed in the area surrounding the study site, in comparison to when *G. montanus* and *G. nutans* flowered later in the season (T. Springer, per. obs).

Glumicalyx nutans was visited primarily by African honey bees (*Apis mellifera scutellata*) in the day and settling moths (family Noctuidae) in the evening, with pollen present on both. Observations showed that honey bees were able to efficiently transfer pollen to their scopae, and some of the bees caught had no pollen present other than in their pollen baskets. It is unlikely that the bees fed on the nectar, as the nectary is located at the base of

the ovary (Hilliard and Burtt 1977) and corolla tube length was ~16 mm in *G. nutans*. Nectar in long corolla tubed species may well up if visited infrequently and thus allow a shorter tongued insect to feed on the nectar, as shown in *Tritoniopsis revolute* and *Amegilla* bees (de Merxem *et al*. 2009). It is not known how efficient the bees were at transferring pollen to the stigma when collecting pollen, and since bees can collect considerable amounts of pollen (Müller *et al.* 2006), this may negatively impact plant reproduction (Thomson and Thomson 1992, Hargreaves *et al*. 2009). However as there is no separation between the exserted anthers and stigma in *G. nutans*, there is an increased likelihood of pollen transfer by the bees (cf. Hargreaves *et al*. 2009). The nocturnal visitors to *G. nutans* were settling moths predominantly of the family Noctuidae, and were predominantly the species *Cucullia terensis* and *Thysanoplusia angulum*. These moths were observed feeding from the flowers with their long proboscis, and were able to reach the nectar. *G. nutans* pollen was found along the entire length of the moth's proboscis, as well as on the head, abdomen and legs of the moths. Settling moths visited *G. nutans* in the greatest number and carried the most pollen, indicating that they are important visitors and the likely pollinators.

Glumicalyx montanus was visited by various small Diptera species in the day, including members of Syrphidae, Muscidae and Tachinidae, and settling moths in the evening. Both the moths and the flies were observed feeding on nectar and both carried pollen, although a greater proportion of the moths carried pollen than the flies. There was a greater observable amount of anther dehiscence and subsequent pollen release during the evening, compared to midday. The greater amount of pollen released may explain why a greater proportion of nocturnal settling moths had pollen present compared to the diurnal Diptera, despite higher visitation by the flies.

Floral shape both attracts pollinators to the floral display (cf. Herrera 1989) and facilitates pollination efficiency through plant and pollinator mechanical fit (cf. Nilsson 1988; Campbell *et al*. 1997). Pollinator fit is influenced by the width (Campbell *et al*. 1997) and the length of the corolla tube (Nilsson 1988; Johnson and Steiner 1997). Hilliard (1994) suggested that based on its floral morphology, *Glumicalyx* was pollinated by diurnally active moths and that the six species within the genus were pollinated by a common suite of pollinators. All three species studied here have nodding, congested inflorescences and corolla tube with five lobes, with distinct corolla tube dimensions: the corolla tube is longest in *G. goseloides*, intermediate in *G. nutans* and shortest in *G. montanus*, whilst the corolla tube is widest in *G. montanus* and equally narrow in *G. goseloides* and *G. nutans*. In *G. nutans*, the

length of the corolla tube more closely matches the proboscis length of the Noctuid settling moths than the short-tongued bees. On the other hand in *G. montanus*, the length of the corolla tube is one of the shortest within the genus and the settling moth pollinators had proboscis lengths two to three times that of the corolla tube length; as a result *G. montanus* pollen was predominantly found on the end of the proboscis. The Diptera species had proboscis lengths much closer in length to the corolla tube of *G. montanus*, indicating a closer plant-pollinator fit. Pollen transfer is effected by physical contact between the reproductive organs of the plant (anthers and stigma) and the pollinators (Nilsson 1988). Darwin (1862) reasoned that the floral tube length should exceed the pollinator proboscis length in order to maximise pollen removal. However for maximum nectar feeding by the long proboscis pollinator, the proboscis length should exceed the floral tube, thus creating a coevolutionary arms race between plant and pollinator (Pauw *et al*. 2009).

Nectar plays an important role in plant reproduction by providing a reward to visitors (Simpson and Neff 1983). Nectar volume and sugar concentration varied between the *Glumicalyx* species. The nectar sugar concentration between *G. montanus* and *G. nutans* differed, with *G. nutans* producing more concentrated nectar than *G. montanus*. Lepidoptera feed by active suction and the optimal nectar concentration for moths ranges from 30% to 40% (Kingsolver and Daniel 1979; Pivnick and McNeil 1985; Kim *et al*. 2011). This closely matches the nectar concentration of *G. nutans* that ranges from 32% (day) to 41% (night), but not of *G. montanus* which is from 17% (day) to 21% (night). The optimal nectar concentration appears to vary widely for flies, and may not be a determining factor in the search for flowers to feed on (Machado and Loiola 2000). Bees feed by dipping their tongue into viscous nectar and the optimal concentration for the genus *Apis* is 55% (Kingsolver and Daniel 1995; Kim *et al*. 2011). My results therefore suggest that none of the species of *Glumicalyx* fall within the optimal nectar sugar concentration for the genus *Apis.*

Pollinators differ in their perception and preference of floral colours (Faegri and van der Pijl 1979; Gumbert *et al*. 1999; Fenster *et al*. 2004; Lazaro *et al.* 2008), resulting in flower colour to be under pollinator-mediated selection (Kevan and Baker 1983; Rodriguez-Girones and Santamaria 2004; Tastard *et al*. 2008; Waser 1983; Whibley *et al*. 2006). The dorsal corolla lobes are a dark orange to brick red in *G. goseloides* and *G. nutans*, with the corolla lobes being strongly reflexed in *G. nutans* (Hilliard and Burtt 1977) – this trait results in the corolla lobe coloration in *G. nutans* being more visible than in *G. goseloides*. In contrast, the dorsal corolla lobes are a pale yellow in *G. montanus*. The corolla lobe

coloration of *G. goseloides* and *G. nutans* within the bee colour hexagon indicates it is indistinguishable from the background. This may be as a result of the limited sensitivity of Hymenoptera photoreceptors in the red end of the spectrum (Peitsch *et al*. 1992; Chittka and Waser 1997). *Apis* species have trichromatic vision that includes peaks in sensitivity in the UV, blue-green and yellow spectrum (Kevan and Baker 1983). UV is an important wave band in insect vision, particularly for bees and flies (Kevan and Baker 1983), however the three species of *Glumicalyx* studied show little to no reflectance in UV. Flowers visited by flies, particularly muscid flies, tend to be white or yellow to the human eye (Lázaro *et al*. 2008), corresponding to the colour of *G. montanus*. The corolla lobe coloration of *G. nutans* differs from that of the typical pale-coloured moth-pollinated flowers. The contrast of pale flowers against a dark background is particularly important for nocturnal hawkmoths that primarily use sight to locate flowers (Farina *et al*. 1994; Kebler *et al*. 2003). This may not apply to settling moths, as olfactory cues may be the primary cue to locate flowers for feeding (Nilsson *et al*. 1993; Raguso 2001; Dötterl *et al*. 2006; Peter and Venter 2016).

Floral scent is thought to play an important role in plant-pollinator interactions in angiosperms (Pellmyr and Thien 1986; Svensson *et al*. 2005; Raguso 2008b; Smith 2010). In the three species of *Glumicalyx* each species had a characteristic fragrance profile that differed between sampling periods, both in terms of relative proportion among dominant scent compounds and by the number of species-specific compounds (van der Niet *et al*. 2015). *Glumicalyx nutans* day samples were dominated by the alcohol, isopentyl alcohol acetate (~31%), and by the monoterpene, eucalyptol (~27%). The night samples were dominated by linalool accounting for ~70% of the floral composition and only small amounts of linalool were detected in the day samples of *G. nutans*. Linalool is a compound commonly associated with moth-pollination, although it is one of the most common floral scent compounds (Knudsen and Tollsten 1993; Knudsen *et al*. 2006) and settling moths have been shown to be attracted to linalool (Dötterl *et al*. 2006, Meagher and Landolt 2008). Bees were the most frequent diurnal visitor to *G. nutans*, and bees have been found to detect a wide range of floral volatiles (Galizia *et al*. 2000; Wright *et al*. 2002; Paldi *et al*. 2003; Dobson 2006). Floral scents tend to be dominated by monoterpenoids, with low amounts of benzoids and fatty acid derivatives in bee-pollinated plants (Dobson 2006). There was a 14 fold increase in scent emission between day and night scent samples in *G. nutans*. Moth pollinated flowers usually emit a strong scent at night and have been shown to produce scent during the main period of moth activity, namely dusk and early morning (cf. Knudsen and Tollsten

1993; van der Niet *et al*. 2015). The dominance in linalool in only the night samples suggests an adaptation to moth pollination in *G. nutans*.

The floral scent of *G. montanus* both in the day and night samples was dominated by benzenoid compounds, the day samples being dominated by the benzoids toluene (~39%) and styrene (~22%), and the night samples by benzaldehyde (~65%) and benzeneacetaldehyde (~12%). Benzaldehyde is associated with moth-pollinated taxa (Knudsen and Tollsten 1993), including the noctuid moth-pollinated *Silene* (Caryophyllaceae), but is also a common floral volatile in many flowers (Knudsen *et al*. 2006). The two dominant day sample compounds for *G. montanus*, toluene and styrene, are not common floral volatiles, although they have been identified as fruit volatiles, in fruits such as mango (MacLeod and Snyder 1985; Pino *et al*. 2005), nectarine (Takeoka *et al*. 1988) and star apple (Pino *et al*. 2002). Styrene, together with anisole (a minor compound in the day and night samples of *G. montanus*), was found in bird-pollinated species of grassland *Protea* (Steenhuisen *et al*. 2012). The occurrence of styrene was noted to be unusual, as it is seldomly emitted by plants (Steenhuisen *et al*. 2012). Styrene was emitted in minor quantities by a butterfly-pollinated species of *Buddleja* (Andersson *et al*. 2002). Anisole, an aromatic ether, is usually a minor or trace compound (Knudsen *et al*. 2006), except in the cyclocephaline scarab pollinated night blooming water lilies, *Nymphaea*, where anisole is the dominant compound in some species (Maia *et al*. 2014). The scent of the day samples in *G. montanus* were of an unusual composition, and contained the lowest number of compounds in comparison to the other species. There was also a ~30 fold increase in scent emission from day to night.

The floral traits in *G. goseloides* suggest pollination by a specialist long-proboscis insect and I initially hypothesized pollination by the mountain pride butterfly, *Aeropetes tulbaghia*. However the phenology of *G. goseloides* differs, as flowers in this guild tend to flower in late summer, when the mountain pride butterfly is most abundant, and *G. goseloides* flowers in early summer. Flowers in this guild are generally unscented, whilst *G. goseloides* produces scent both in the day and night. Many of the compounds emitted by *G. goseloides* were unidentified, including the dominant scent compound in the *G. goseloides* day samples, accounting for ~57%. The second most abundant compound was the sesquiterpenoid dihydro-β-agarofuran, this compound is often found in essential oils (Bhuiyan *et al*. 2009; Naef 2011). Isolated dihydro-β-agarofuran was found to have antifeeding and insecticidal effects against some species of insects (Céspedes *et al*. 2001; Gao *et al*. 2007; Tang *et al*. 2013). However its role in *G. goseloides* is unknown. There was a 100

fold increase in floral scent emission from day to night in *G. goseloides*, suggesting that the likely pollinator of *G. goseloides* is a crepuscular long-proboscis insect.

The combination of floral traits, pollinator observations and pollinator efficiency (i.e. pollen load) all support the conclusion that *G. nutans* is pollinated by noctuid moths, and is likely only visited (robbed?) by honeybees. *Cucullia terensis* was the most frequent moth visitor to *Glumicalyx nutans*. Pollination by species of *Cucullia* has been documented in the following species *Satyrium* (including *Cucullia terensis*; van der Niet *et al*. 2015), *Monadenia ophrydea* (Johnson 1995), *Struthiola ciliata* (Makholela and Manning 2006) and in *Gladiolus* (Goldblatt *et al*. 2001). The floral morphology between these species is variable, but common among all of them and *G. nutans* is a long corolla tube/nectar spur and increased floral sent in the evening.

With an increase in altitude there is an overall decrease in pollinator abundance and diversity (Kearns 1992; Totland 1992). The proportion of Diptera, particularly flies, increases with increasing altitude and latitude (Eberling and Olesen 1999). Muscid flies in alpine habitats in the arctic-subarctic zone have been found to be frequent and important generalist pollinators (Elberling and Olesen 1999). Bees however dominate in montane and subalpine habitats (Lázaro *et al*. 2008). The abundance of bees observed visiting *G. nutans* at the Platberg site (2350 m a.s.l) compared to the abundance of flies visiting *G. montanus* at the Sentinel Peak site (2750 m a.s.l.) may be related to the altitudinal differences, although no flies or bees were observed visiting *G. nutans* at the lower Sentinel Peak site (2300 m a.s.l.). The high altitude specialist moth *Mythimna corax* was the most common moth visitor to *G. montanus* (Krüger 2005). The combined floral traits of *G. montanus* are neither typical of settling moth pollination, nor of short-tongued fly pollination. The pale yellow dorsal and creamy white ventral corolla lobe coloration could indicate pollination by settling moths, as pale coloured flowers are preferred (Faegri and van der Pijl 1979; Dobson 2006), and food-seeking flies, as yellow coloured flowers with sweet scents are preferred (Kugler 1956; Dobson 2006). The dominance of unusual benzenoid compounds in the day samples, and dominance of benzaldehyde in the night samples, together with a 30 fold increase in scent emission and double the number of compounds from day to night, would indicate moth pollination. However, the small, compact inflorescences with relatively short and wide corolla tubes do not indicate pollinate by a long proboscis insect. A possibility may be that *G. montanus* has a bimodal pollination system, including settling moths and flies, or the population studied may be adapting to their local pollinator assemblage. The flies were

observed in a much greater abundance than what was documented visiting the flowers – throughout each day of observations; even when misty, windy and cold, flies visited the flowers. The number of moths observed was accurately recorded, as they visited for short periods of time in lower numbers. This likely resulted in a bias towards moth pollination when calculating visitation rate and measuring pollen loads. Further research is needed on multiple populations of *G. montanus* to investigate if their pollinators vary. As well as more rigorous day-night pollinator exclusion experiments are needed to determine if nocturnal or diurnal pollinators are more important for their reproductive success.

In conclusion, the three species of *Glumicalyx* studied show considerable differences in floral morphology and floral traits, particularly scent, that in combination act to attract specific pollinators. Future sampling of floral traits, pollinators and breeding systems of low altitude *G. goseloides*, and of the other three species of *Glumicalyx* would allow us to map possible reproductive transitions onto the phylogeny of *Glumicalyx* (Chapter 2). This would allow us to explicitly determine if shifts in reproductive biology and pollinators have occurred within the genus and between sister species of *Glumicalyx* (Givnish and Sytsma 1997; Weller and Sakai 1999; Johnson 2010). Increased pollinator observations and measurements of floral traits, particularly scent, at multiple sites across each species' altitudinal range should reveal if the populations studied are adapted to different local pollinator assemblages (Johnson *et al*. 2005).

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Appendices

Appendix 3.1. Insects observed feeding from the three *Glumicalyx* species' flowers, numbers caught, and those subsequently found to have *Glumicalyx* pollen. Insects are arranged by functional group and time of observation.

Species	Locality	Time period	Functional group	Number observed feeding	Number observed feeding and caught	Number caught with *Glumicalyx* pollen
G. goseloides	Sentinel	Day	Long-tongued fly	1	0	0
G. goseloides	Sentinel	Day	Coleoptera	18	14	1
G. goseloides	Sentinel	Day	Other hymenoptera	10	8	1
G. goseloides	Sentinel	Day	Orthoptera	35	9	0
G. goseloides	Sentinel	Night	Coleoptera	3	3	0
G. goseloides	Sentinel	Night	Orthoptera	2	2	0
G. goseloides	Sentinel	Night	Blattodea	1	1	1
G. montanus	Sentinel	Day	Butterfly	1	1	1
G. montanus	Sentinel	Day	Short-tongued fly	>200	73	14
G. montanus	Sentinel	Day	Short-tongued bee	3	0	0
G. montanus	Sentinel	Night	Settling moth	59	18	10
G. montanus	Sentinel	Day	Orthoptera	2	1	0
G. montanus	Sentinel	Day	Coleoptera	>100	30	0
G. nutans	Platberg	Day	Short-tongued bee	>50	20	8
G. nutans	Platberg	Day	Short-tongued fly	1	1	1
G. nutans	Platberg	Night	Settling moth	>50	33	30

Appendix 3.2. Compounds (as proportions) contributing to the first 50% of dissimilarity between the scent profiles between the day and night samples for each species of *Glumicalyx* (from the SIMPER analysis).

Species	Compound	Average dissimilarity	Cumulative %	Day	Night
G. goseloides	β-Ocimene	16.82	17.47	0	0.801
	m/z: 147,105,162,91,120,119,77,79,92,115	15.18	33.24	0.752	0.0346
	cis-muurola-4(14),5-diene	11.87	45.57	0	0.566
	m/z: 43,109,152,81,79,91,67,93,77,59	5.468	51.25	0.27	0.0176
G. montanus	Benzaldehyde	10.49	15.25	0.273	0.807
	Toluene	8.597	27.75	0.511	0.215
	Benzeneacetaldehyde	6.508	37.21	0	0.332
	Styrene	6.213	46.24	0.439	0.134
	m/z: 45,75,43,119,44,134,46,91,41,42	5.481	54.21	0.273	0
G. nutans	Linalool	16.45	18.08	0.0636	0.839
	Eucalyptol	10.89	30.06	0.516	0
	cis-muurola-4(14),5-diene	9.978	41.02	0	0.472
	Isopentyl alcohol, acetate	8.978	50.89	0.554	0.134

Chapter 4

General discussion and recommendations for future study

General overview

This dissertation aimed to investigate the role of pollinators as ecological drivers of diversification in the Drakensberg Mountain Centre (DMC). The endemic genus *Glumicalyx* was used as a model to study speciation in the DMC. Prior to this study very little was known about *Glumicalyx* with regards to its species relationships and reproductive biology. This study provides the first phylogenetic hypothesis for the genus, allowing for hypotheses of the speciation and evolutionary trends. A complete phylogeny of *Glumicalyx* was produced using both nuclear and plastid DNA regions, with multiple accessions for each species. Although the relationships within the *Glumicalyx* phylogeny require greater resolution, it was established that *Glumicalyx* is a monophyletic group and the species are very closely related with incongruence between plastid and nuclear data sets suggesting past hybridization, introgression and/or incomplete lineage sorting. Four of the six species of *Glumicalyx* were previously placed in other genera (*Selago*, *Walafrida* and *Zaluzianskya*) prior to the revision of the genus by Hilliard and Burtt (1977).

The low number of synapomorphies between and within the species of *Glumicalyx* indicate a relatively recent radiation, likely within the last 5 My. This would correspond to tectonic uplift during the Miocene-Pliocene boundary, during which uplift of the Lesotho plateau occurred to form the modern alpine zone (Partridge and Maud 2000; Linder 2014). The creation of novel habitats in the subalpine and alpine zones of the DMC would have initiated speciation (Linder 2014), and by physically isolating plant populations through erosional fragmentation, allopatric speciation may have been promoted in various biota. The speciation of *Arrowsmithia* (=*Macowania*; Bentley *et al*. 2014, 2017) has been attributed to vicariance caused by this tectonic uplift. *Glumicalyx* occurs in the subalpine and alpine zones of the DMC. Speciation of *Glumicalyx* may have been initiated by tectonic uplift with the resulting novel habitats allowing for adaptive radiation. However, based on the current distribution of the extant taxa, geographical isolation through erosional fragmentation is unlikely to have played a role in speciation of the genus as a whole. The species have overlapping distributions, with many species co-occurring at some localities. Incongruence

between the nuclear and plastid reconstructions have made hypothesises of the modes of evolution between sister taxa challenging. In all reconstructions, *G. lesuticus* is nested in *G. nutans*, and *G. lesuticus* is therefore likely to have speciated in sympatry with *G. nutans* at high altitudes in Lesotho, where it is known to co-occur (G. Cron, pers. com. 2019). In the nuclear reconstruction, the clade of *G. nutans* and *G. lesuticus* is sister to *G. montanus*, which is morphologically and ecologically distinct from *G. nutans*. *Glumicalyx montanus* and *G. nutans* have overlapping distributions and do co-occur, but utilize different microhabitats and pollinators – thereby being reproductively isolated. In the nuclear phylogeny, *G. apiculatus* is sister to *G. flanaganii*; these species are morphologically and ecologically distinct, differing in growth form, floral traits and habitat. Their distributions overlap minimally, and it likely that they speciated in allopatry. In contrast to the relationships in the nuclear phylogeny, in the plastid phylogeny *G. apiculatus* and *G. montanus* are sister taxa and they are morphologically and ecologically very similar. Based on floral morphology, these species are likely to share pollinators, although this needs to be confirmed by observations for *G. apiculatus*. This suggests that ecological speciation did not occur between these two species (if indeed they are sister species), and they may have been isolated as a result of vicariance. The distribution of *G. apiculatus* is restricted to the Eastern Cape Drakensberg, where it overlaps with *G. montanus*, however this may not depict the ancestral distribution of *G. montanus. Glumicalyx montanus* mainly occurs in KwaZulu-Natal and Lesotho, but also extends into the northern Eastern Cape and eastern Free State.

Variation in both vegetative and floral traits suggest that the genus has adapted ecologically to both the biotic and abiotic components of its habitats. Some of the species have overlapping distributions, but occur in specific microhabitats allowing for their co-occurrence but maintaining their isolation. There is significant variation in floral morphology within the genus, most notably in corolla dimensions and coloration. This is indicative of a role played by pollinators in their speciation and in maintaining reproductive isolation amongst the species. The reproductive biology, including breeding system, pollination system and floral traits, were further investigated for three of the six species. The three species chosen are representative of the floral morphological variation within the genus, particularly floral size and corolla lobe coloration. Breeding system experiments determined that all three species are dependent on pollinators for reproduction. *Glumicalyx goseloides* is self-incompatible and unable to autonomously self-pollinate. *Glumicalyx montanus* appears to be partially self-compatible and partially able to autonomously self-pollinate. However none of the seeds produced in the exclusion treatment germinated, indicating that they are not be able

to successfully reproduce in the absence of pollinators. Floral traits vary significantly between the species, including floral morphology, nectar volume and sugar concentration and floral scent composition. *Glumicalyx nutans* is pollinated by noctuid moths, and its pollen may robbed by African honey bees. The nocturnal floral scent emission, dominant scent compounds (linalool), and nectar sugar concentration are associated with noctuid moth pollination, confirming this conclusion. *Glumicalyx montanus* is pollinated by short-tongued flies from various families and also by noctuid moths. There is however a size disparity between the lengths of the corolla tube in *G. montanus* and of the proboscis in the noctuid moths, suggesting that the noctuid moths may not be the most effective pollinators. Effective pollinators were not observed for *G. goseloides*. The long, narrow corolla tube indicates pollination by a long-proboscis insect and increased scent emission at night in *G. goseloides* suggests nocturnal pollination.

Future research

Future research on *Glumicalyx* would focus on producing a better-resolved phylogenetic hypothesis. There is currently poor resolution within the plastid phylogeny, and supported incongruence between the nuclear and plastid phylogeny. Although the nuclear phylogeny is better resolved, the reconstructions of sister relationships are not well supported. Improvements to the phylogeny would involve increased DNA regions (both plastid and nuclear), particularly low-copy nuclear genes (LCNG). Although it requires both time and effort to develop useful LCNG for non-model organisms (Feliner and Rosselló 2007). Of the many LCNG available, the pentatricopeptide repeat (PPR) gene family may be very useful. The PPR gene have a rapid rate of evolution, with Lamiales-specific primers available (Yuan *et al*. 2009; Yuan *et al*. 2010). Three PPR genes together with the ETS region and three plastid regions were used to reconstruct the phylogenetic relationships in Buddlejeae, Scrophulariaceae (Chau *et al*. 2017). Next generation sequencing, possibly of the whole genome, is a feasible option in *Glumicalyx* because of its small size. The possibility of past hybridization as the cause of the incongruence is worth considering, as many of the species co-occur and putative hybrids have been reported. Although the ITS region is the most widely used non-plastid region for species-level phylogenetic studies (Baldwin *et al*. 1995, Álvarez and Wendel 2003, Feliner and Rosselló 2007), Álvarez and Wendel (2003) suggested that ITS sequence data were less suitable for plant phylogenetic studies than single-copy or low-copy nuclear genes (LCNG). This is because the ITS region of the 18S-5.85-26S nuclear ribosomal cistron shows higher homoplasy than other DNA sequence data sets (Álvarez and

Wendel 2003), and as a result of evolutionary phenomena, ITS gene trees may be incongruent with species trees. This study forms one of the few southern African clades that are fully sampled with multiple accessions per species. If other studies implemented a similar sampling design, incongruence may be more prevalent.

Glumicalyx, as well as the other endemic Drakensberg lineages, are hypothesised to have speciated following major uplift at the Miocene-Pliocene boundary (Galley *et al*. 2007). Explicitly testing this hypothesis by generating a dated phylogeny for *Glumicalyx* and its sister genus *Strobolopsis* would be very useful in furthering our understanding of speciation in the DMC. Although the DMC is species rich, there are no large endemic clades, rather many small clades (Linder 2014), with *Glumicalyx* being the largest of these clades. Ecological speciation through adaptations to habitat and altitudinal zones has been implicated in the speciation of the near-endemic clades, *Killickia* (Kgaboesele 2019), *Galtonia* (Minaar 2018) and *Craterocapsa* (Uys and Cron 2013). Tectonic uplift and post-uplift habitat production by erosive processes has been implicated in the speciation of the DMC clade of *Arrowsmithia* (=*Macowania*; Bentley *et al*. 2014, 2017). The role of erosion in diversification may be further explored, particularly in genera that are geographically isolated like *Killickia* (Kgaboesele 2019) and *Craterocapsa* (Uys and Cron 2013).

To further explore the role of pollinators as ecological drivers of diversification in *Glumicalyx*, a more resolved phylogeny in combination with a more extensive study of the reproductive biology of the genus are needed. From this study adaptations to nocturnal pollination and pollination by long-proboscid insects have have been investigated in *Glumicalyx*. I hypothesise that species with similar floral morphology, i.e. *G. montanus* and *G. apiculatus*, may share a common suite of pollinators, whilst morphologically distinct species, viz. *G. apiculatus* and *G. flanaganii*, are likely to have different pollinators. The position of the anthers in *G. goseloides* and *G. nutans* (two inserted in the corolla tube and two exerted from the corolla tube) differs from the other species (all four exerted from the corolla tube) and may be an adaptation to pollinators, whereby the deposition of pollen on the same pollinator differs between species, and presumably therefore the deposition on the stigma of the recipient plant. The variation in habitats, as well as altitudinal ranges, results in the species coming into contact with different insect (Kearns 1992; Totland 1992). For example, *G. montanus* occurs in the subalpine and alpine zones, whereas *G. apiculatus* occurs only in the subalpine zone. Diptera dominate in the alpine zone, whilst Hymenoptera dominate in the subalpine zone (Eberling and Olesen 1999; Lázaro *et al*. 2008), therefore

although these species are floristically similar, they may be exposed to different pollinator communities. There is often overlap in the floral traits in short-tongued fly and short-tongued bee pollinated taxa, such that short-tongued bee and Diptera form a pollen-collecting or nectar-feeding functional group (Fenster *et al*. 2004).

This study is the first to have elucidated the potential role of pollinators in speciation of a DMC endemic genus through detailed observations and measurements. This role of pollinators may be further explored in the larger endemic lineages, for example within *Erica*. The Cape species of *Erica* have significant variation in pollination syndromes and floral forms (Rebelo *et al*. 1985). Specialized pollination guilds within the DMC have also been identified, including pollination by the long-tongued flies *Prosoeca ganglbaueri* (Goldblatt and Manning 2000; Johnson *et al*. 2002; Anderson and Johnson 2009) and *Philoliche aethiopica* (Johnson 2000; Johnson and Morita 2006; Johnson *et al*. 2009), the oil-collecting bees *Rediviva spp.* (Manning and Brothers 1986; Steiner and Whitehead 1988; Steiner and Whitehead 1990; Steiner and Whitehead 1991; Whitehead *et al*. 2008), the mountain pride butterfly *Aeropetes tulbaghia* (Johnson and Bond 1994; Johnson *et al*. 2009), hawkmoths including *Agris convolvuli* (Alexandersson and Johnson 2002, Johnson *et al*. 2002; Johnson *et al*. 2009; Johnson and Raguso 2016) and small flies including Tachinidae and Muscidae (Johnson *et al*. 2009; van der Niet *et al*. 2010).

Further studies on the aspects mentioned above would better our understanding of the likely drivers and modes of speciation and the reproductive biology of *Glumicalyx*, but mostly importantly provide further insights into the diversification of the flora of the DMC. Future studies may also extend beyond *Glumicalyx* to the other endemic lineages of the DMC, and further investigate the role of specialist pollination in the diversification of the region's remarkable biodiversity.

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