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# A SYSTEMATIC STUDY OF THE SOUTH AFRICAN GENUS *PRIONIUM* (THURNIACEAE)

**BY**

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## ABSTRACT

The South African monocotyledonous plant genus *Prionium* E. Mey (Thurniaceae; Cyperid clade) is an old, species-poor lineage which split from its sister genus *Thurnia* about 33–43 million years ago. It is a clonal shrubby macrophyte, widespread within the Fynbos biome in the Cape Floristic Region (CFR) with scattered populations into the Maputaland-Pondoland Region (MPR). This study of the systematics of the genus *Prionium* investigates whether this old lineage comprising of a single extant species *P. serratum*, is morphologically, genetically and ecologically impoverished, and identifies apomorphic floral developmental traits in relation to its phylogenetic position as sister to the Cyperid families, Juncaceae and Cyperaceae. Sampling for morphological, molecular and ecological studies was done to obtain representatives from its entire distribution range, falling within the phytogeographic regions of the CFR (North West, NW; South West, SW; Agulhas Plain, AP; Langeberg, LB) and extending into Eastern Cape (South East, SE) and KwaZulu Natal (KZN). Samples for the floral ontogenetic study were collected to obtain representatives of the Cyperid clade families: Cyperaceae (*Eriophorum*, *Scirpus*), Juncaceae (*Juncus*, *Luzula*) and Thurniaceae (*Prionium*).

Morphometric analysis revealed the absence of morphological differentiation between *P. serratum* populations from the entire distribution range. To explore genetic diversity in *P. serratum*, levels of genetic variation and patterns of population structure were investigated using inter-simple sequence repeat (ISSR) markers and two chloroplast markers (*rpl32-trnL* and *rps16* intron). Chloroplast markers revealed that *P. serratum* populations have low variability in DNA sequences of *rpl32-trnL* and *rps16* intron which are amongst the most variable plastid markers and are routinely used in generic level phylogenetics. Phylogenetic analyses produced weakly supported nodes and polytomies for samples between and within *P. serratum* populations. Haplotype network analyses of the DNA sequence data revealed two networks, with the most widespread (ancestral) haplotypes occurring in KZN and NW, but with no apparent geographical structure. The presence of one (or few) common haplotypes in all populations suggests that the species experienced a bottleneck after which new allelic variants have originated in low frequencies. Moreover, a phylogenetic inference, based on *rps16* intron DNA sequence data for *P. serratum* and representative taxa of Poales, shows comparable branch length (from stem to crown nodes) for all three Cyperid families, although *Prionium* has unusually short branch lengths. The lack of DNA sequence variability may be linked to life form, but is still unusually low for a lineage with its stem age in


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Oligocene. These findings from phylogeographic and phylogenetic analyses, point to the possibility that extant populations of *P. serratum* have a more recent origin, suggesting the taxon experienced a recent genetic bottleneck.

ISSR results revealed relatively low levels of polymorphism at the population level with the percentage of polymorphic bands (PPB) ranging from 6.83 % to 34.4 %. A high level of genetic differentiation between populations was detected based on Nei's genetic diversity analysis ($G_{ST}$ = 0.743 %) and limited gene flow (Nm = 0.173). AMOVA revealed that there were highly significant ($P < 0.001$) genetic difference between populations and $F_{ST}$ analog ($\Phi_{ST}$) was 0.529, which indicated that 53 % of total genetic variability occurred between populations and 47 % within populations. AMOVA supported the results of Nei's gene diversity statistics and Shannon's Information index that there is a high degree of population differentiation. Limited gene flow between populations, perhaps limited to particular drainage systems and separated by unsuitable habitats, is a plausible reason for the high inter-population genetic differentiation observed for this species. Genetic relationships between the *P. serratum* populations examined by UPGMA cluster analysis and by PCA using Nei's pairwise genetic distance ($\Phi_{ST}$) showed that there is no positive correlation between geographic and genetic distance. Populations in nearby CFR Mountains (NW, SW, and LB) are not similar to each other, yet distant coastal populations in the CFR (AP) and the MPR (KZN) are genetically most similar.

Soil samples from the CFR and MPR obtained from streams with and without *P. serratum* were analysed to investigate whether the occurrence of the species was limited by available nutrients. Nested ANOVA of the soil nutrient levels showed that *P. serratum* occurred on acidic soils with wide variation in nutritional values for the measured parameters. *P. serratum* sites have nutritional levels comparable to the non-*Prionium* sites for all the measured parameters, and the habitat it occupied had nutrient regimes similar to other habitats supporting the Fynbos biome. This implies that the absence of *P. serratum* in the non-*Prionium* sites is not associated with nutritional levels of the soil, and that perhaps other ecological parameters (e.g. hydrological regime) dictate occurrence of *P. serratum.* A species may be absent at a particular habitat due to chance, or failure to disperse to an otherwise suitable site.

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Species representative of the Cyperid clade were investigated to clarify long-standing queries on floral homologies and evolution within the Cyperid clade and to identify new ontogenetic characters towards supporting (or refuting) phylogenetic hypothesis. The results revealed that Cyperid clade species shared a floral developmental pattern and uncovered evolutionary patterns in the development of the perianth, androecium, and gynoecium. *Prionium* possesses plesiomorphic features among the Cyperids, including a typically monocotyledonous trilocular ovary which matures into a dehiscent capsule. This strongly supports the current molecular phylogenetic hypothesis that Thurniaceae is sister to a clade comprising of Juncaceae and Cyperaceae. Juncaceae and Cyperaceae gynoecia have an annular ovary primordium which develops into a single locule, but in the former numerous ovules are enclosed, whereas in the latter only one ovule is surrounded. In Thurniaceae, the trilocular ovary differentiates into three congenitally fused carpels and the ovary envelops numerous bitegmic ovules.

The overall aim of this study was to investigate whether the species-poor lineage *P. serratum* is morphologically, genetically and ecologically impoverished. The conclusion drawn from this study is that *P. serratum* is slightly genetically differentiated, when one considers lack of variability in DNA sequence and ISSR data. In addition, *P. serratum* occurs on soils with different nutritional levels, and in both winter and summer rainfall areas, suggesting that the species is not ecologically impoverished.

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## DECLARATION STATEMENT

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# CHAPTER 1
# GENERAL INTRODUCTION AND LITERATURE REVIEW

## 1.1. Problem statement

The South African monocotyledonous plant genus *Prionium* E. Mey (*Thurnia*ceae; Cyperid clade, *sensu* Bremer, 2002) is an old, species-poor lineage which split from its sister genus *Thurnia* about 33-43 million years ago (Bremer, 2002; Michelangeli *et al*., 2003; Chase *et al*., 2006; Givnish *et al*., 2006b, 2010). Its single species, *Prionium serratum* (L.f.) Drège ex E. Mey, is widespread within the Fynbos biome in the Cape Floristic Region (CFR) and has scattered populations in the Maputaland-Pondoland Region (MPR) (Munro & Linder, 1997; Munro *et al*., 2001; Goldblatt & Manning, 2002). Both the CFR and MPR are characterised by exceptionally high species richness and endemism, and numerous factors have been postulated to account for the high diversity.

The other two lineages belonging to the Cyperid clade (Cyperaceae and Juncaceae) have a cosmopolitan distribution, and are species rich. For example, Cyperaceae is amongst the 15 largest families in the CFR (Goldblatt & Manning, 2000). Cyperaceae in the CFR occur in diverse habitats (Archer, 2000) and are represented in all soil types (e.g. sandstone, limestone, granite, shale) and wetness regimes (e.g. mountain streams, salt pans, seasonally wet soil). The family has varied life forms including therophytes, hemicryptophytic geophytes, and halophytes (Govaerts *et al*., 2007). It exhibits wide morphological variation in vegetative and reproductive features (Archer, 2000) and shows genetic variability for common molecular phylogenetic markers (Muasya *et al*., 2009).

On the other hand, *Prionium* is morphologically uniform across its range (Munro *et al*., 2001), hence the recognition of a single species. Thus, *Prionium serratum* is an ideal plant lineage for studying traits and processes limiting diversification in the midst of the 'orgy of speciation' (Linder, 2003) in the CFR. This raises the question(s) regarding why an old lineage would be depauperate while its sister lineage(s) have high species number. Despite the growing numbers of morphological and molecular phylogenetic studies, aspects of genetic diversity, population structure, gene flow, rates of molecular evolution in plant system and floral ontogenetic hypotheses in species poor lineages in the CFR have received little attention.



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## 1.2. The Cape Floristic Region as a centre of diversity and endemism

The CFR is the most species rich region in Southern Africa, with about 9000 species in an area of about 90,000 km$^2$ (Cowling & Pressey, 2001; Goldblatt & Manning, 2002). The CFR has been globally recognized as one of 25 global biodiversity hotspots (Myers *et al.*, 2000; Mittermeier *et al*., 2004). Goldblatt & Manning (2000) calculated levels of vascular plant endemism in the CFR as being 70 % at specific level and 16.2 % at generic level. The CFR has a level of richness which is more comparable to that of islands, including the Philippines, Madagascar, Western Australia, Chile, Taiwan and New Zealand (Linder, 2003), rather than to other Mediterranean-type areas. The degree of endemism in the CFR has never been observed in any other equivalent temperate region (Cowling *et al*., 1996; Linder *et al*., 2003).*et al*The CFR has provoked intense interest in the origins of its flora (Linder *et al*., 1992; Linder, 2003; Galley & Linder, 2006). Given the CFR's remarkable diversity and well-circumscribed area, it provides an excellent model for studying the causes of plant diversification (Schnitzler *et al*., 2011).

The historical events underlying the genesis of CFR diversity, endemism, as well as the time frame over which it occurred, have been the subject of considerable discussion in the literature (e.g. Levyns, 1964; Linder *et al*., 1992; Linder, 2003, 2005). This "orgy of speciation" (Linder, 2003) has been hypothesized to have been triggered by the climatic changes near the Miocene/Pliocene boundary, with the establishment of the Benguela current leading to a substantially cooler and more arid climate (Goldblatt & Manning, 2000). Recent analyses using dated molecular phylogenies indicate that this might not be a general feature of the Cape flora, but that the radiation of several plant lineages had started well before these climatic changes took place (Linder & Hardy, 2004; Linder, 2005; Verboom *et al*., 2009). There are several relictual lineages in the CFR, hypothesized to occur in areas protected from summer drought and fires (Linder, 2003), that had originated before Miocene (Warren & Hawkins, 2006; Forest *et al*., 2007)). Old relictual lineages (pre-Miocene) occur in typical of forest patches (e.g. *Smelophyllum*) and and stream margins (*Prionium*, *Metrosideros*, *Brabejum*, and *Platylophus*) (Warren & Hawkins, 2006, Forest *et al*., 2007). Several other lineages in the CFR pre-date the burst of radiation associated with onset of Mediterranean climate. For instance, the Restionaceae in the Cape region had begun diversification into the Oligocene continuing into the early Eocene (Linder & Hardy, 2004; Hardy *et al*., 2008). Linder (2006) and Barraclough (2006) concluded that the high diversity in the region might reflect high levels of species persistence and sustained diversification rather than recent, rapid

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diversification, perhaps explained by the relative climatic stability combined with continuing geomorphological dynamics (Cowling *et al*., 2009).

### 1.2.1. Drivers of speciation in the CFR

Speciation is a fundamental process responsible for the generation of biodiversity on Earth. It was well understood by Charles Darwin as the evolutionary process by which new species form (Darwin, 1859). The speciation process occurs as inherent barriers to gene flow evolve between formerly interbreeding populations (Schluter, 2009; Rundle & Nosil, 2005). The barriers to gene flow accumulate over time and evolve most readily if populations experience geographic isolation or divergent selection (Rundle & Nosil, 2005). Whereas some barriers to gene flow are the consequences of differences in chromosome structure or intrinsic genetic incompatibilities, barriers to gene flow often result from divergent selection and local adaptation (Jiggins *et al*., 2001; Schluter, 2009). Some of the main factors postulated to be playing a major role in driving speciation within lineages occurring in the CFR include pollinator specialization, fire, edaphic conditions (e.g. soil type and nutrient levels); phenology (e.g. flowering time); geographic isolation (e.g. habitat fragmentation); polyploidy or hybridization (Linder, 2003; Richardson *et al*., 2001). These factors have been explored by several researchers in the CFR as well as in many other floristic regions of the world.

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**Pollination specialization**

The exact role that pollination plays a role in the speciation process remains controversial (Johnson, 1996). Studies focussing on the role of pollinators include Johnson (1996), Johnson *et al*. (1998), Vamosi *et al*. (2005) and van der Niet & Johnson (2009). van der Niet & Johnson (2009) showed that most diversification in Cape plant lineages was associated with floral features, suggesting that there is strong selection for pollinator specializations. For example, a study by Johnson & Steiner (2003) noted that the CFR is characterized by a remarkably high number of specialized pollination systems. Another feature of Cape clades is that many (but not all) display relatively little variation in vegetative morphology but striking differences in floral morphology and pollinators (Johnson, 1996). Divergence of pollinator types between two parts of a population, whether caused by drift or by divergent selection pressures imposed by abiotic environment or pollinator preference, would lead to automatic reproductive isolation between the two populations (Johnson, 1996). The Cape is typified by low abundance of pollinators, for reasons that are obscure but could include low soil fertility,

although we are unaware of empirical data to support this (Johnson, 1996). As a result, plants may be under strong selection to attract pollinators.

**Fire**

Fire plays a key role in global ecosystem patterns and processes, and has had a distinct effect on the evolution of biota (Bowman *et al*., 2009). According to Bond *et al*. (2005a, 2005b), the distribution of various ecosystems around the world would be considerably different without regular fire. Linder (2003) suggested that shifts in fire survival strategy can drive speciation associated with differences in growth form and phenological differences which could isolate populations from each other. Another aspect is based on the premise that fire-induced plant mortality increases generation turnover of reseeders, thereby providing potential for more rapid evolution than in resprouters (Cowling & Pressey, 2001). For example, Linder & Kurzweil (1999) found that many Orchid species are rarely found in unburnt areas, which suggests that fire plays an important role in their reproduction processes (Linder, 2003). Alternatively, even if fire did not play a direct role in driving speciation in these clades, it might create the ecological space for groups of plants such as *Ficinia* radiata (field observation), *Disa conferta* and *Disa obtusa* (Linder & Kurzweil, 1999; Bytebier *et al*., 2011).

**Geographic isolation**

Geographic isolation is expected to have significant effects on the genetic structure of populations (Smith, 1999). A population may be physically divided when its original habitat becomes divided by a natural barrier (e.g. a river, shoreline, mountain range or glacier) or even an artificial barrier (e.g. a man-made canal or highway). These barriers can restrict or prevent gene flow, resulting in the genetic differentiation of isolated subpopulations. This might have promoted parapatric speciation in the CFR (Bauert *et al*., 1998; Nesbo *et al*., 1998). Changes in altitude provided an opportunity for considerable geographical isolation, which provides potentially steep climatic and ecological gradients, which might also promote parapatric speciation (Goldblatt & Manning, 2002). Loveless & Hamrick (1984) indicated that gene flow largely shapes the genetic structure of plant populations. A further consideration of the research on genetic diversity and gene flow is presented in Chapter two of this dissertation.

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**Edaphic heterogeneity**

In the CFR, edaphic factors have not received as much attention as some of the other drivers of speciation. The CFR is characterized by a mosaic of sharply distinct soil types; a complex topography of mountain ranges, valleys and plains; and differences in rainfall availability and seasonality. Ecological factors such as soils, climate and topography have been suggested as major drivers of speciation (Linder & Vlok, 1991; Linder, 2003, 2005; van der Niet & Johnson, 2009). The CFR receives an annual rainfall of between 300 and 2000 mm $yr^{-1}$, although some montane sites in the west receive as much as 3000 mm $yr^{-1}$ (Deacon *et al*., 1992). According to Linder (2003), combinations of these physical parameters can result in a large number of distinct niches, often in close proximity to each other. A striking phenomenon in the CFR flora is the common occurrence of closely related sister taxa on different soil types (e.g. limestone and sandstone). For example, *Protea compata* R. Br is endemic to sandstone whereas *Protea obtusifolia* is endemic to limestone (Shane *et al*., 2008); and *Aspalathus callosa* occurs on sandstone while *A. cephalotes* grows in clay soil (Goldblatt & Manning, 2002). This could be the outcome of strong divergent selection promoting genetic divergence and speciation. Again, it is clear that many speciation events are associated with shifts between soil types, but whether this explains the CFR's diversity is less clear (Barraclough, 2006). In contrast to the ecological factors discussed above, edaphic factors have not received as much attention as some of the other drivers of diversification and speciation. This study focuses on edaphic heterogeneity in terms of soil types and nutrient concentrations to explore their role in diversification of genus *Prionium*. A more detailed introduction on soils and nutrient concentrations is presented in Chapter three of this dissertation.

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### 1.2.2. Causes of variation among species

Explaining why some taxa and geographical regions contain more species than others is an important goal of evolutionary biology, and phylogenetic approaches are increasingly used to explore the timing and rates of diversification, and to determine the net accumulation of species through time (Rabosky, 2006; Ricklefs, 2007). Biological traits differ between and within lineages and such variants differ in their ability to promote speciation (Jablonski, 2008). However, there has been some disagreement regarding the historical events underlying the genesis of the diversity of lineages, as well as the time frame over which it occurred. Cardillo *et al*. (2003) postulated two ways that variation in species richness among lineages can arise. Firstly, variation among genera may be simply by chance: unpredictable sequences

of historical events may have left different lineages with the different numbers of species that we see today (Raup *et al*., 1973). The second way is by age: some lineages are older than others, so have had more time to accumulate species (Ricklefs & Schluter, 1993; Barraclough *et al*., 1998). According to Heard & Hauser (1995), the key traits used to explain differences between lineages encompass a broad range of mechanisms, because different lineages appear to have specific traits that promote speciation (Jablonski, 2008; Rabosky, 2006). Some lineages are linked with geographic range size (Jablonski & Hunt, 2006), whereas others are associated with pollinator specificity (Sargent, 2004). For example, Johnson *et al*. (1998) noted that radiation in the genus *Disa* (Orchidaceae) has encompassed nearly all major groups of pollinating insects, with the 27 species in their study found to have 19 different specialized pollination systems. Furthermore, some lineages exhibit lower extinction rates than others (Roy *et al*., 2009). Restricted gene flow between populations is thought to promote speciation in the CFR (Linder, 1985). Restricted gene flow promotes population divergence via natural selection and genetic drift, which if persistent lead to speciation. Some lineages vary in the number of species they contain within a genus and possible causes for this variation include differences in the environment experienced by those lineages (Kerr & Packer, 1997). This study uses the species-poor *Prionium serratum* as a model system to investigate some of the factors which lead to depauperate genera, with particular emphasis on the role of gene flow and edaphic heterogeneity, in the CFR.

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## 1.3. The genus *Prionium* E. Mey

### 1.3.1. Classification history and phylogenetic position of *Prionium*

Based on morphological data, *Prionium* has traditionally been included in Juncaceae (e.g. Cutler, 1964, 1969; Dahlgren *et al*., 1985; Simpson, 1995; Plunkett *et al*., 1995). The single species in the genus was initially described as *Juncus serratus* L.f., and its inclusion within Juncaceae was primarily due to shared (typical juncaceous) flower morphology (Simpson, 1995). Simpson (1995) reconstructed phylogenetic relationship, based on morphological data, which placed *Prionium* within Juncaceae with *Thurnia* Hook.f as its sister taxon. Earlier work by Bentham and Hooker (1883) had treated *Thurnia* as a member of the Juncaceae, but *Thurnia* was later placed in segregate family *Thurnia*ceae by Engler (1907). DNA data resolve a slightly different pattern, where *Prionium* was sister to *Thurnia* (*Thurnia*ceae) and this clade was sister to a clade including Juncaceae and Cyperaceae (Bremer, 2002; see Figure 1.1).

Various morphological and anatomical features of *Prionium* led to the suggestion that it be removed from Juncaceae. Cutler (1965) was the first to suggest that the strange leaf anatomy of *Prionium* warranted its removal from the Juncaceae. In addition, other unique features of *Prionium* include fused carpels and lack of a style (Cutler, 1969). *Prionium* has unique chemistry, Williams & Harborne (1975) indicated the presence of flavone c-glycosides in *Prionium* tissues, which are absent from the rest of Juncaceae and Cyperaceae. However, Plunkett *et al*. (1995) downplayed the utility of the woody habit to separate *Prionium* since it was also found in some members of Cyperaceae, which include the monotypic *Macrodracoides* from tropical Africa, arguing that such growth habit and distribution in the Old world may be primitive for the entire Juncaceae and Cyperaceae complex. The phylogenetic position of *Prionium* remained a subject of debate for many researchers, with several studies (e.g. Chase *et al*., 2000; Muasya *et al*., 2000) supporting Simpson's (1995) morphological data. It is now accepted that *Prionium* is sister to *Thurnia* with the two taxa classified in Thurniaceae family (Bremer, 2002; Linder & Rudall, 2005). Juncaceae, Thurniaceae and Cyperaceae form the Cyperid clade (Bremer, 2002; Figure 1.1). A more thorough discussion on relationships within the Cyperid clade is presented in Chapter four.

Currently, Thurniaceae has two genera (*Thurnia* and *Prionium*) with four species: *T. macrocephala* Schnee, *T. sphaerocephala* (Rudge) Hook.f. *T. jenmanii* Hook.f and *Prionium serratum* (L.f.) Drège ex E. Mey (Bremer, 2002). The species of *Thurnia* are large, amphibious herbs which occur on sandy lowlands in the Guayana Shield and Amazon Basin in northern South America (Givnish *et al*., 1999). The sister relationship between *Prionium* and *Thurnia,* together with their restriction to limited areas of sand or sandstone on either side of the Atlantic Ocean, led Givnish *et al*. (1999) to hypothesize an origin of this lineage in western Gondwana before the rifting of the Atlantic separated Africa and South America. Such a western Gondwanan common ancestor and the occurrence of *Prionium* and *Thurnia* in different continents would imply a vicariant origin in the Mesozoic, but such an age is not supported by dates inferred from dated phylogenies (e.g. Bremer 2002, Figure 1.1) which show a Cretaceous origin of the taxa, perhaps involving a long distance dispersal event.

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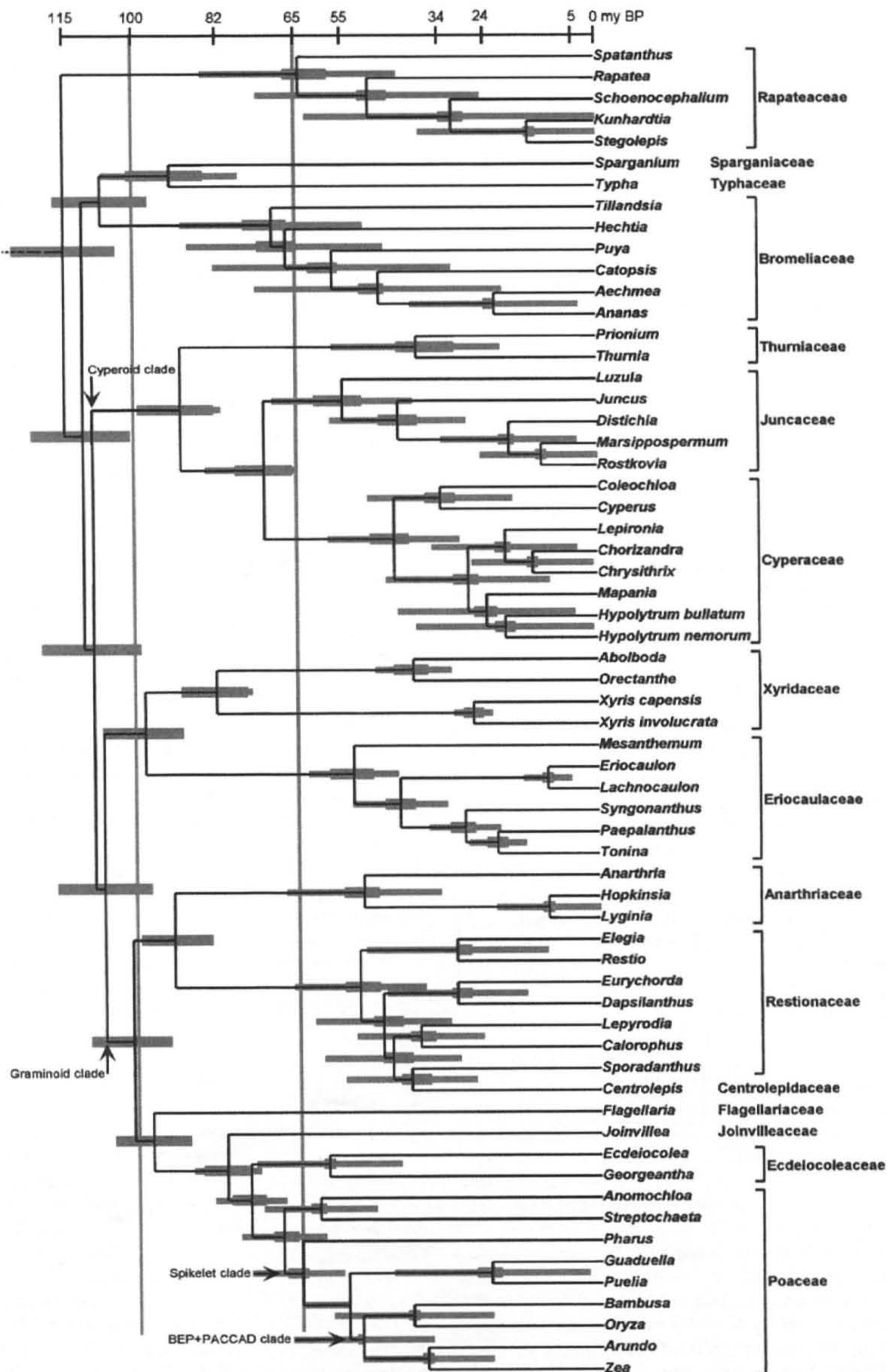


**Figure 1.1** A phylogenetic tree showing divergence times and relationship between *Prionium* with *Thurnia* (Thurniaceae), Juncaceae and Cyperaceae (from Bremer, 2002).

### 1.3.2. Vegetative and reproductive morphology, distribution and ecology of *Prionium*

The vegetative morphology of *P. serratum* was detailed by Balslev (1996), Munro and Linder (1997), and Munro *et al*. (2001). Munro *et al.* (2001; see Figure 1.2) described *Prionium* as a perennial hygrophyte with an aerial rhizome and a woody, decumbent habit. The aerial rhizomes are enclosed by old leaf sheaths. *Prionium serratum* has numerous leaves inserted in dense terminal rosettes. Each leaf has a rigid blade, which is V-shaped in the middle and triangular near the apex of the leaf, and keel with upward-pointing prickly serrulations. The inflorescence is comprised of a much-branched panicle, with numerous branchlets bearing many flowers. Each branchlet is subtended by funnel-shaped sheathed bracts. *Prionium serratum* has glumaceous perianth parts, which consist of six scale-like outer and inner tepals. Each flower has six basifixed stamens, glabrous filaments and latrorse anthers. The androecium surrounds the trilocular ovary, with the locules separated by septa. The ovary has numerous, bitegmic, anatropous ovules which mature into numerous minute seeds per locule.

*Prionium serratum* produces numerous seeds, which germinate in open spaces along river banks (Munro & Linder, 1997). It exhibits clonal growth form, forming new plants by budding along the woody rhizome. Clonality is the dominant form of plant growth in environments with suboptimal environmental conditions, such as at the edge of the geographical range (Eckert *et al*., 1999) or in small and isolated populations (Hooftman & Diemer, 2002; Wilcock & Neiland, 2002). Most perennial plants combine sexual reproduction through seed with clonal reproduction through vegetative propagation (Dorken & Eckert, 2001, Eckert, 2001). Variation in reproductive mode is expected to be a major determinant of population genetic structure (Eckert *et al*., 1993, 1999).This may directly affect the genetic structure of natural populations, because clonal reproduction yields offspring that are genetically identical to both the maternal plant and each other, hence clonal plant populations are expected to exhibit low genotypic diversity (Eckert *et al*., 2003). The production and recruitment of sexual and clonal progeny often varies widely within a species in response to various ecological and genetic factors that limit a species' reproductive mode (Eckert, 1999). Basic understanding of species biology, age, life history and the analysis of present-day spatial patterns of genetic diversity can yield insights into past evolutionary processes. It is imperative to be acquainted with the population genetic structure and level of gene flow within and between populations of a particular species (Liu *et al*., 2011).

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Bremer (2002) and Warren & Hawkins (2006) indicated that *P. serratum* originated 33-43 million years ago (see Figure 1.1). The age of the species suggests that it may have occupied its current niche for an extended time in evolutionary history, thereby constraining the development of evolutionary traits in other species that could potentially occupy this habitat (Sieben, 2012). *Prionium serratum* has clonal integration which aids it in surviving resource competitions with other species. The abundance of plants along streams and rivers suggests that it is adapted to habitats with year-round supplies of water (Sieben, 2012). *Prionium serratum* has a narrow distributional range. Generally, species with narrow distributional ranges possess lower levels of genetic diversity than their widespread congeners, because they are associated with historically smaller and less continuously distributed populations (Hamrick & Godt, 1996). Clonal macrophytes, which are long lived, have few open niches for recruitment, unless major disturbance occurs, and have lower levels of genetic diversity than widespread species (Hamrick & Godt, 1996). The genetic diversity of P. serratum will be detailed in Chapter two of this dissertation.

*Prionium serratum* has a geographically restricted distribution in South Africa. It occurs along mountains streams and rivers, on oligotrophic soils, from the Giftberg (Western Cape) through the Eastern Cape to the southern coast of KwaZulu Natal. *Prionium serratum* occurs in both the Cape Floristic Region and the Maputaland-Pondoland Region (MPR). *Prionium serratum* populations in MPR are mostly found on sandstone areas in coastal Nature Reserves (Mkambati, Mpenjati, and Umtamvuna) and the Fraser Falls area. The MPR is associated with the Indian Ocean Coastal Belt of South Africa, previously referred to as the Tongaland-Pondoland Regional Mosaic, which is a floristic region in which two (Maputaland and Pondoland) smaller centres of endemism are found (Van Wyk & Smith, 2001). The MPR is the home of 7000 vascular plant species (Scott-Shaw, 1999, Van Wyk & Smith, 2001), and supports a diverse range of vegetation, including grassland types, as well as forest, savanna, thicket and aquatic communities.

Most of the *P. serratum* populations predominantly occur in the CFR, an area generally associated with low nutrient soils and winter rainfall. The CFR has a highly dissected, rugged topography and a diversity of climates with rainfall mostly falling in the winter months and varying from 2000 mm locally to less than 100 mm (Cowling *et al.*, 1996; Goldblatt & Manning, 2002), and the MPR which also has low nutrient soils with summer rainfall ranging from an average of 400 mm to 1200 mm per annum (van Wyk & Smith, 2001). In terms of

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soils, the CFR consists of various edaphic types, which are sharply delimited, and thus form a mosaic of edaphic habitats (Linder, 1985). CFR soils are typically acidic and nutrient poor, particularly with regard to nitrogen and phosphorus (Kruger *et al*., 1983). Soil nutrients play an important ecological role in shaping vegetation and species distributions in the CFR. Richards *et al*. (1997b) showed that soil nutrient content (total nitrogen, total phosphorus, organic carbon and various cations, including calcium, magnesium, sodium and potassium) was significantly different between 18 sites of different soil and vegetation type in the Soetanysberg communities. Hence, they concluded that spatial variation in soil nutrient availability might be important in explaining landscape-level species distribution and community composition of nutrient-poor Mediterranean-climate ecosystems. However, the extent to which the distribution of the *P. serratum* is linked to edaphic factors is unknown and will be detailed in Chapter three.

### 1.3.3. Ecosystem value and uses of *Prionium serratum*

*Prionium serratum* is an important indigenous medicinal plant, used within the Xhosa communities as traditional medicine (Field observation and personal interview within the Amapondo community in KwaZulu Natal). The tribal medicinal men and women of Mtambala and Mkambati villages use leaf extracts for chest pain and as medication for insect bite. *Prionium serratum* leaves are used for making baskets, hats, mats and thatching of houses. It produces numerous flowering shoots from September to February, most of which are broken off before flowering and eaten by monkeys. Along the rivers, *P. serratum* is considered to be an ecosystem engineer (Jones *et al*., 1994, 2010) that changes its own environment to support itself and its associates growing in the same habitat. Apart from providing the organic material that builds the peatland, *P. serratum* also changes the hydrology of the ecosystem (Sieben, 2012), as its deep and extensive rooting system together with its clonal growth and ability to withstand strong flood events provide the key traits that help the plant to transform its own environment. Pugnaire and Valladeres (2007) observed that *P. serratum*'s extensive rooting system probably plays a role in ''leaking'' oxygen into the peat substrate which would otherwise be completely anoxic, making the habitat suitable for other species and other functional groups that do not usually occur in peatlands. The mass of its woody rhizomes act to bind the soil, thereby building up river beds, ameliorating effects of flooding events, and filtering water (Munro & Linder, 1997; Richardson *et al*., 2001). This species occurs in large populations which are widespread in South Africa and do not face

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increased pressure due to human activities, and its conservation status was recently listed as Least Concern (LC; Rebelo *et al*., 1992).

**Figure 1.2** *Prionium serratum* life-forms, distribution and ecology. Pictures taken from Oliphants and Bainskloof River (pictures taken by Dr Muthama Muasya and Rabelani Munyai 2010).

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### 1.4. Aims and objectives of the study

The overall aim of this study was to investigate whether the old lineage comprising a single extant species is morphologically, genetically and ecologically impoverished and study its floral developmental patterns in relation to its systematic position between the Cyperid clade. *Prionium serratum* has a geographically patchy distribution, restricted within drainage system (e.g. a particular river or valley), and such catchment areas are separated by geographical barriers between phytogeographic regions. We hypothesized that *P. serratum* populations will have high genetic diversity between populations, and limited gene flow. Thurniaceae (*Prionium serratum*) is sister to Juncaceae and Cyperaceae, we hypothesized that there is a similarity on the floral developmental pattern compared to the sister taxa (Cyperid clade). The study clarifies long-standing queries on floral homologies and evolution within the Cyperid clade. The specific objectives for this study were:

1. To investigate the morphological diversity between *P. serratum* across its entire range;
2. To investigate the evolutionary history of genus *Prionium*, and genetic diversity and structure between *P. serratum* populations;
3. To investigate edaphic heterogeneity in the sites of *P. serratum* in South Africa; and
4. To investigate the floral developmental pattern within the Cyperid clade.

**1.5. Thesis Outline**

**Chapter 1** provides a general introduction to the dissertation.

**Chapter 2** investigates whether the species-poor *P. serratum* was impoverished by its morphological diversity, evolutionary history and genetic diversity and structure. Morphological diversity was investigated using both herbarium and freshly collected specimens. Evolutionary history was investigated using DNA sequences of *P. serratum* and other monocots which include other members of Poales from the GenBenk. Population genetic diversity was investigated using inter-simple sequence repeat (ISSR) and chloroplast markers (*rpl32-trnL*, *rps16* intron).

**Chapter 3** investigates species distributions in relation to edaphic heterogeneity. Soil samples collected across *P. serratum* natural habitats were used to investigate the nutritional difference between *Prionium* and non-*Prionium* site. Soils were analysed for the following parameters: pH, concentration of Phosphorus (P Bray II), total nitrogen (N), potassium (K), iron (Fe), calcium (Ca), magnesium (Mg), sodium (Na), and the T-value which is the sum of the exchangeable cations.

**Chapter 4** documents floral developmental pattern within the Cyperid clade. It was done to clarify long-standing queries on floral homologies and evolution within the Cyperid clade and to identify new ontogenetic characters towards testing phylogenetic hypotheses. Inflorescence materials were collected from the field in South Africa and Belgium. Examination of floral development was conducted using scanning electron microscopy (SEM). The chapter presents evolutionary novelties focusing on perianth parts, androecium, and gynoecium.

**Chapter 5** briefly revisits the study objective and key objective/questions, and then integrates results, discussions and conclusions of various chapters to bring out collective findings. It also provides recommendations and future research prospects.

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## CHAPTER 2

## MORPHOLOGY, GENETIC DIVERSITY AND STRUCTURE, AND EVOLUTIONARY HISTORY OF THE GENUS *PRIONIUM*

### 2.1. Introduction

The distribution of genetic variants in plant populations is strongly affected by current patterns of microevolutionary forces, such as gene flow and selection, mating system, method of seed dispersal, by the mode of reproduction of the species, and by the phylogenetic history of populations (Hamrick *et al*., 1992; Schaal *et al*., 2003). All these forces can lead to complex genetic structure within populations and their study is imperative and serves as an effective method to infer processes associated with of a particular species (Mayr, 1963). Understanding the interplay of shared history and current evolutionary events is particularly confounding in plants due to the sometimes reticulating nature of gene exchange between diverging lineages (Schaal *et al*., 2003). Furthermore, phylogeographic analyses provide insights about the historical processes responsible for restricted distributions of populations or species within a particular locality (Cruzan & Templeton, 2000). According to Avise (2000) and Schaal *et al*. (1998), phylogeography characterizes population subdivision by recognizing geographical patterns of genealogical structure across the range of populations of species, synthesizing the influence of both history and current genetic exchange. Cladistic gene genealogies can form the basis of historical approaches to the study of intraspecific processes (Schaal *et al*., 1998; Templeton, 2004). Certain gene sequences provide historically-ordered neutral molecular variation that can be converted to gene genealogies which trace the evolutionary relationships between haplotypes (alleles). Gene genealogies can be used to understand the evolution of specific DNA sequences and relate sequence variation to plant phenotype. Avise (2000) emphasised that a basic understanding of species biology, life history and the analysis of present-day spatial patterns of genetic diversity can yield insights into past evolutionary processes. In addition, an examination of rates of molecular evolution is essential for answering fundamental questions about molecular evolution such as how much mutational change has taken place since the genus split from its common ancestor (Welch & Bromham, 2005). Evolutionary rates are known to vary significantly between different genes and the gene expression level is presently considered as a major determinant of evolution (Pal *et al*., 2006).

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There have been numerous advances in the field of evolutionary biology over the past two decades. Clearly one of the most important developments has been the use of DNA sequences and markers for understanding both the patterns and processes of evolution. At the population level, the use of DNA sequences to infer past evolutionary processes has been enhanced by John Avise's concept of phylogeography, which examines the distribution of genealogical lineages of specific DNA sequences in a geographical context. Phylogeographic approaches offer a way of determining the types of contemporary and historical processes that have influenced the current geographic distribution of variation (Avise, 2000). DNA sequence data used for phylogenetic inference in plants are from plastids (mainly the chloroplast), the mitochondrion and the nuclear genomes (Ankel-Simons & Cummins, 1996; Mogensen, 1996; Petit *et al*., 2005).

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For plant genomes, nucleotide sequences of chloroplasts have proven to be a primary source of data for molecular genetic relationship studies. Many early publications usually focused on several coding-regions of chloroplast DNA (cpDNA) sequences such as *rbc*L*, mat*K*, atp*B and *ndh*F genes to elucidate genetic relationships between higher-level taxa (e.g. Chase *et al*., 1993; Olmstead & Sweere, 1994; Steele & Vilgalys, 1994). For example, the suprageneric relationships of angiosperms were first inferred using the *rbcL* gene (Chase *et al*., 1993), which encodes the large subunit of the photosynthetic enzyme Rubisco, a major carbon acceptor in all photosynthetic eukaryotes and cynobacteria (Nabors, 2004). In addition, Bremer (2002) explored the early evolution of Poales, including phylogenetic interrelationships between the 18 families, age and geographic origin, by cladistic analysis of chloroplast DNA *rbcL* and *atpB* sequences including *P. serratum* samples. In Cyperaceae, family-level studies to explore phylogenetic relationships have been based mainly on *rbcL* sequence data (e.g. Muasya *et al*., 1998; Simpson *et al*., 2007); whereas at tribal or subfamilial levels other plastid and nuclear regions have been used. For example, *rps16* intron, *trnL* intron and *trnL-F* intergenic spacer have been used in studies of subfamily Mapanioideae (Simpson *et al*., 2003). The utility of coding markers such as the *rbcL* gene is limited by their slow rate of change which renders them less informative in the inference of phylogenetic relationships between closely related taxa such as genera and species. For studies below family level, much attention has been paid towards using non-coding regions of the chloroplast genome (Shaw *et al*., 2007). The most variable regions identified by Shaw *et al*. (2007) are: *rpl*32-*trn*L $^{(\mathrm{UAG})}$, *trn*Q (UUG)-$^{5}$´*rps*16, 3´*trn*V $^{(\mathrm{UAC})-}$*ndh*C, *ndh*F-*rp*l32, *psb*D-*trn*T $^{(\mathrm{GGU})}$, *psb*J-*pet*A, 3´*rps*16–5´1*trn*K $^{(\mathrm{UUU})}$, *atp*I-*atp*H, and *pet*L-*psb*E.

Chase *et al*. (2006) and Esselman *et al*. (1999) have stressed the importance of using more than one class of molecular markers to estimate and infer genetic diversity between and within population because they provide valuable information about the genetic structure of natural plant populations ( Castr-Felix *et al*., 2008). Recently, numerous studies have directly compared genetic diversity estimates based on RAPD (random amplified polymorphic DNA; Williams *et al*., 1990), ISSR (inter-simple sequence repeats; Zietkiewicz *et al*., 1994) and the somewhat more technically demanding AFLP (amplified fragment length polymorphism; Vos *et al*., 1995). Chloroplast DNA markers have been widely used in the investigations of genetic structure (Meister *et al*., 2005; Parducci *et al*., 2001) and phylogeography (Rendell & Ennos, 2002) of tree and grass species. Both chloroplast markers and ISSR have been widely used in the study of genetic diversity and structure (Li & Jin, 2008), genetic diversity (Sheeja *et al*., 2009), and genetic relationship (Li *et al*., 2009). ISSRs have proven to be a rapid, simple and inexpensive way to assess genetic diversity (Jin *et al*., 2003; Tanyolac, 2003) and to study evolutionary processes such as gene flow (Wolfe & Liston, 1998). Esselman *et al*. (1999) pointed out that ISSRs can generate a higher percentage of polymorphic loci than other PCR methods. For example, in higher plants and animals, ISSR markers are known to be abundant, reproducible, highly polymorphic, and highly informative (Zietkiewicz *et al*., 1994, Bornet & Branchard, 2001; Bornet *et al*., 2002). The use of these genetic markers allows identifying genetic individuals (genet), which may be composed of many independent units (ramets). However, one caveat for population genetics studies involving ISSR or other dominant markers is that the method of analysis must be selected carefully due to inherent limitations, such as assumptions of Hardy–Weinberg equilibrium and random mating in diploids (Lynch & Milligan, 1994). For this study I use molecular data, both sequence and ISSR, to infer evolutionary patterns in *Prionium*.

The morphological diversity, genetic diversity and evolutionary history of *P. serratum* is poorly known. This study will be the first to describe population genetic patterns for this species, and will provide valuable baseline information for future population studies and management actions. *Prionium serratum* has relatively uniform relatively uniform morphology*,* across its entire range (Munro *et al*., 2001), hence its treatment as a single species. *Prionium serratum* has a geographically patchy distribution, with gene flow expected to be restricted within drainage system (e.g. a particular river or valley), and such catchment areas are separated by geographical barriers which are expected to limit gene flow between



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populations. Similar patterns are observed in the *Podostemun irgangii* C. Philbrick & Novelo (Podostemaceae) a species with narrow geographic range occurring in several locations of the Paraná River basin, in the south-central Paraná and central-western Santa Catarina (Philbrick *et al*., 2010). I therefore hypothesized that *P. serratum* populations will have high genetic diversity between and low within population, and limited gene flow between catchments. Research questions addressed include: 1) how is the level of genetic diversity partitioned within and between *P. serratum* populations? and 2) how is the evolutionary rate within genus *Prionium* compared to other monocots especially member of Poales?

### 2.1.2. Objectives of this chapter

The objectives for this chapter were:

(a) To investigate the morphological diversity between *P. serratum* populations;

(b) To investigate the evolutionary history of genus *Prionium* compared to other members of Poales; and

(c) To investigate genetic diversity and structure between and within *P. serratum* populations.

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## 2.2 Materials and methods

### 2.2.1. Sampling for molecular and morphological studies

A total of 105 samples of *P. serratum* were collected from independent populations in 21 sites in South Africa. These covered the entire range of the species, in the six phytogeographic regions, namely North West (NW), South West (SW), Langeberg (LB), Agulhas Plain (AP), South East (SE) which extends to Eastern Cape and KwaZulu Natal (KZN) (Goldblatt & Manning, 2000; Figure 2.1 and Table 2.1). A population in a locality was defined as a group of 20 to 30 plants at a locality and separated from another group by habitats such as mountain slopes. All the localities within each phytogeographic region were lumped, *a priori*, into group. At each sampling locality, fresh leaf materials were collected from five plants for DNA extraction and herbarium specimen. Individual DNA tissue samples were placed in separate coffee filter bags which were then placed in a closed container with silica gel to directly dry and preserve them until DNA extraction. Voucher specimens were dried in a plant press using blotting papers, and well-preserved for morphometric measurements and herbarium contribution. For each species, a standard herbarium label was prepared to capture data on *P. serratum*.

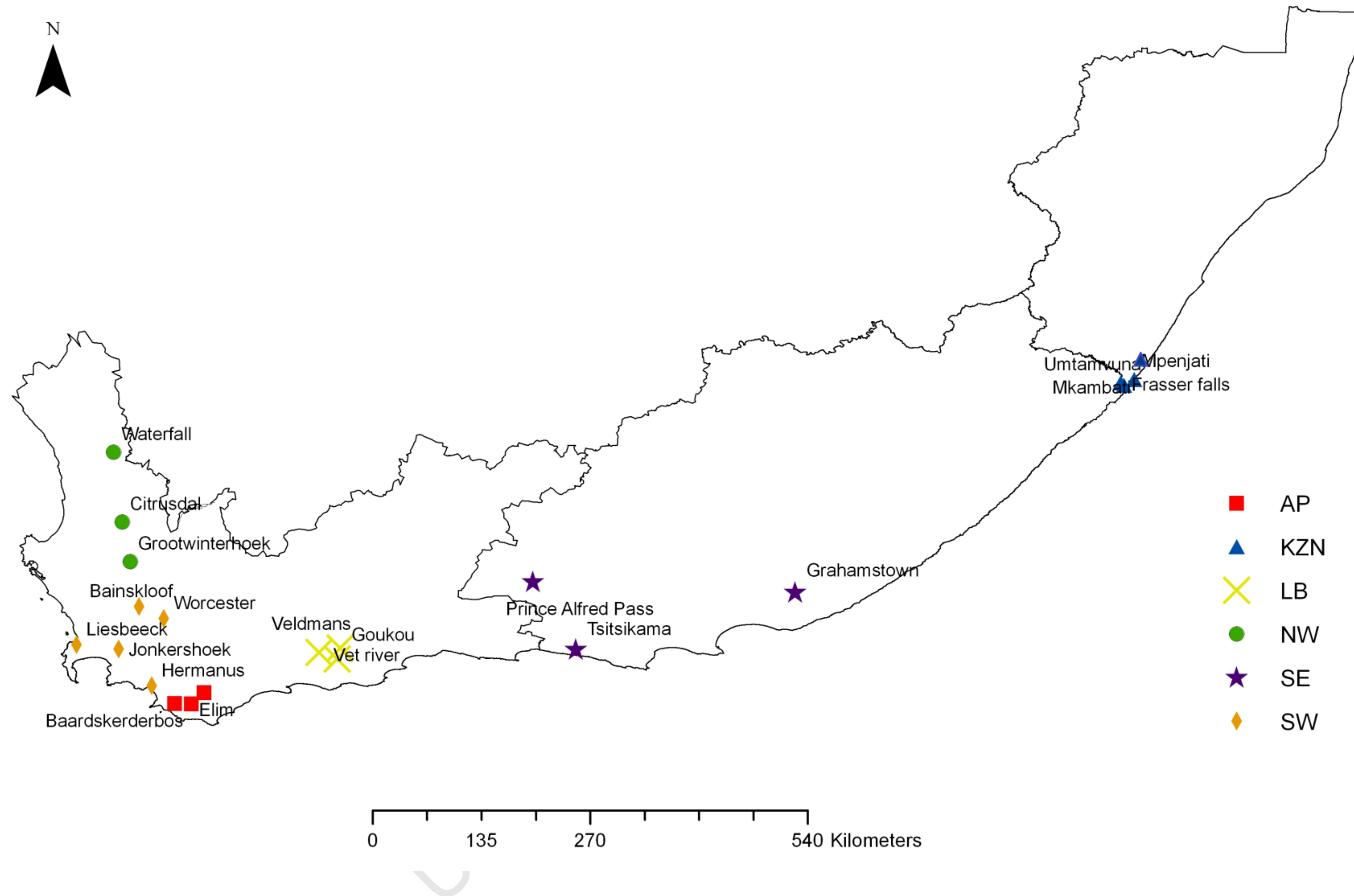


**Figure 2.1** A map of localities for *Prionium serratum* sampled for this study. Abbreviations for sampled localities: Agulhas Plain AP, (red), KwaZulu Natal (KZN, blue), Langeberg (LB, yellow), North West (NW, green).South East and Eastern Cape (SE, purple), and South West (SW, orange).

**Table 2.1** Distribution of *Prionium serratum* collections included in molecular studies.

| Phytogeographic region | Locality | Latitude | Longitude | Collector/ sample no. | *rpl32-trnL* | *rps16* | ISSR |
|---|---|---|---|---|---|---|---|
| North West (NW) | Waterfall | -33° 00’ | 19° 07’ | Munyai-13B | √ | | √ |
| North West (NW) | Waterfall | -33° 00’ | 19° 07’ | Munyai -13C | √ | √ | √ |
| North West (NW) | Waterfall | -33° 00’ | 19° 07’ | Munyai -14A | √ | | |
| North West (NW) | Waterfall | -33° 00’ | 19° 07’ | Munyai -14D | √ | | √ |
| North West (NW) | Waterfall | -33° 00’ | 19° 07’ | Munyai -15E | √ | | √ |
| North West (NW) | Cederberg | -32° 56’ | 18° 98’ | Muasya -5716A | √ | | |
| North West (NW) | Cederberg | -32° 56’ | 18° 98’ | Muasya- 5716B | √ | √ | √ |
| North West (NW) | Cederberg | -32° 56’ | 18° 98’ | Muasya -5716D | √ | | |
| North West (NW) | Noordhoek | -32° 56’ | 18° 98’ | Munyai -10A | √ | | √ |
| North West (NW) | Groot Winterhoek | -31° 78’ | 18° 88’ | Munyai -1C | √ | | √ |
| North West (NW) | Groot Winterhoek | -31° 78’ | 18° 88’ | Munyai -3B | √ | √ | √ |
| North West (NW) | Groot Winterhoek | -31° 78’ | 18° 88’ | Munyai -5E | √ | √ | √ |
| North West (NW) | Groot Winterhoek | -31° 78’ | 18° 88’ | Munyai -6B | √ | | |
| North West (NW) | Groot Winterhoek | -31° 78’ | 18° 88’ | Munyai -6D | √ | | |
| South West (SW) | Bainskloof | -34° 39’ | 19° 31’ | Munyai -7A | √ | √ | |
| South West (SW) | Bainskloof | -34° 39’ | 19° 31’ | Munyai -7B | | | √ |
| South West (SW) | Bainskloof | -34° 39’ | 19° 31’ | Munyai -7C | √ | √ | √ |
| South West (SW) | Bainskloof | -34° 39’ | 19° 31’ | Munyai -8B | √ | | √ |
| South West (SW) | Bainskloof | -34° 39’ | 19° 31’ | Munyai -9A | √ | | |

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| Phytogeographic region | Locality | Latitude | Longitude | Collector/ sample no. | *rpl32-trnL* | *rps16* | ISSR |
|---|---|---|---|---|---|---|---|
| South West (SW) | Bainskloof | -34 º 39’ | 19 º 31’ | Munyai -9F | √ | | √ |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5420B | √ | √ | √ |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5420C | √ | √ | |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5420D | | | √ |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5423A | √ | √ | √ |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5423C | √ | √ | |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5427A | √ | | |
| South West (SW) | Hermanus | -33 º 57’ | 19 º 13’ | Munyai -5427A | | | √ |
| South West (SW) | Jonkershoek | -33 º 93’ | 18 º 47’ | Munyai -5408A | √ | | √ |
| South West (SW) | Jonkerhoek | -33 º 93’ | 18 º 47’ | Munyai -5408B | √ | | |
| South West (SW) | Jonkerhoek | -33 º 93’ | 18 º 47’ | Munyai -5408C | √ | √ | |
| South West (SW) | Jonkerhoek | -33 º 93’ | 18 º 47’ | Muasya-5363A | √ | | |
| South West (SW) | Jonkerhoek | -33 º 93’ | 18 º 47’ | Muasya-5373A | √ | | |
| South West (SW) | Liesbeeck | -33 º 64’ | 19 º 44’ | Munyai -16B | √ | | |
| South West (SW) | Liesbeeck | -33 º 64’ | 19 º 44’ | Munyai -16D | √ | √ | √ |
| South West (SW) | Kirstenbosch | -33 º 64’ | 19 º 44’ | Muasya-5606A | √ | √ | √ |
| South West (SW) | Kirstenbosch | -33 º 64’ | 19 º 44’ | Muasya-5606B | √ | √ | √ |
| South West (SW) | Kirstenbosch | -33 º 64’ | 19 º 44’ | Muasya-5606C | √ | √ | √ |
| South West (SW) | Worcester | -33 º 98’ | 18 º 94’ | Munyai -17G | √ | | √ |
| South West (SW) | Worcester | -33 º 98’ | 18 º 94’ | Munyai -17H | √ | | |
| South West (SW) | Worcester | -33 º 98’ | 18 º 94’ | Munyai -17P | √ | | √ |
| South West (SW) | Worcester | -33 º 98’ | 18 º 94’ | Munyai -17R | | | √ |

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| **Phytogeographic region** | **Locality** | **Latitude** | **Longitude** | **Collector/ sample no.** | ***rpl32-trnL*** | ***rps16*** | **ISSR** |
|---|---|---|---|---|---|---|---|
| South West (SW) | Worcester | -33° 98’ | 18° 94’ | Munyai -17S | √ | √ | √ |
| South West (SW) | Worcester | -33° 98’ | 18° 94’ | Munyai -17T | √ | | |
| Langeberg (LB) | Goukou | -34° 02’ | 21° 37’ | Job-G01 | √ | √ | |
| Langeberg (LB) | Goukou | -34° 02’ | 21° 37’ | Job-G02 | √ | | √ |
| Langeberg (LB) | Goukou | -34° 02’ | 21° 37’ | Job-G03 | √ | | √ |
| Langeberg (LB) | Goukou | -34° 02’ | 21° 37’ | Job-G04 | √ | | √ |
| Langeberg (LB) | Goukou | -34° 02’ | 21° 37’ | Job-G05 | √ | | √ |
| Langeberg (LB) | Veldmans | -34° 03’ | 21° 22’ | Job-Vd01 | √ | √ | √ |
| Langeberg (LB) | Veldmans | -34° 03’ | 21° 22’ | Job-Vd 02 | √ | | |
| Langeberg (LB) | Veldmans | -34° 03’ | 21° 22’ | Job-Vd03 | √ | | √ |
| Langeberg (LB) | Veldmans | -34° 03’ | 21° 22’ | Job-Vd04 | √ | | |
| Langeberg (LB) | Veldmans | -34° 03’ | 21° 22’ | Job-Vd05 | √ | | √ |
| Langeberg (LB) | Vietmans | -34° 02’ | 21° 38’ | Job-Vet1 | √ | √ | √ |
| Langeberg (LB) | Vietmans | -34° 02’ | 21° 38’ | Job-Vet2 | √ | | √ |
| Langeberg (LB) | Vietmans | -34° 02’ | 21° 38’ | Job-Vet3 | √ | | |
| Langeberg (LB) | Vietmans | -34° 02’ | 21° 38’ | Job-Vet4 | √ | | √ |
| Langeberg (LB) | Vietmans | -34° 02’ | 21° 38’ | Job-Vet5 | √ | | √ |
| Agulhas Plain (AP) | Baarderskerdos | -34° 58’ | 19° 57’ | Munyai-27A | √ | | |
| Agulhas Plain (AP) | Baarderskerdos | -34° 58’ | 19° 57’ | Munyai-27B | √ | | |
| Agulhas Plain (AP) | Baarderskerdos | -34° 58’ | 19° 57’ | Munyai-27C | √ | | |
| Agulhas Plain (AP) | Baarderskerdos | -34° 58’ | 19° 57’ | Munyai-27D | √ | | |

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| **Phytogeographic region** | **Locality** | **Latitude** | **Longitude** | **Collector/ sample no.** | ***rpl32-trnL*** | ***rps16*** | **ISSR** |
|---|---|---|---|---|---|---|---|
| Agulhas Plain (AP) | Baarderskerdos | -34 ° 58’ | 19 ° 57’ | Munyai-27A | | | √ |
| Agulhas Plain (AP) | Baarderskerdos | -34 ° 58’ | 19 ° 57’ | Munyai-27B | | | √ |
| Agulhas Plain (AP) | Baarderskerdos | -34 ° 58’ | 19 ° 57’ | Munyai-27D | | | √ |
| Agulhas Plain (AP) | Baarderskerdos | -34 ° 58’ | 19 ° 57’ | Munyai-27E | √ | | |
| Agulhas Plain (AP) | Napier | -34 ° 59’ | 19 ° 75’ | Munyai-28A | √ | | |
| Agulhas Plain (AP) | Napier | -34 ° 59’ | 19 ° 75’ | Munyai-28B | √ | √ | √ |
| Agulhas Plain (AP) | Napier | -34 ° 59’ | 19 ° 75’ | Munyai-28C | √ | | |
| Agulhas Plain (AP) | Napier | -34 ° 59’ | 19 ° 75’ | Munyai-28D | √ | | √ |
| Agulhas Plain (AP) | Napier | -34 ° 59’ | 19 ° 75’ | Munyai-28E | √ | | √ |
| Agulhas Plain (AP) | Elim | -34 ° 46’ | 19 ° 90’ | Munyai-29A | √ | | |
| Agulhas Plain (AP) | Elim | -34 ° 46’ | 19 ° 90’ | Munyai-29B | √ | | |
| Agulhas Plain (AP) | Elim | -34 ° 46’ | 19 ° 90’ | Munyai-29C | √ | | √ |
| Agulhas Plain (AP) | Elim | -34 ° 46’ | 19 ° 90’ | Munyai-29D | √ | √ | |
| Agulhas Plain (AP) | Elim | -34 ° 46’ | 19 ° 90’ | Munyai-29E | √ | | √ |
| South East (SE) | Tsitsikama | -33 ° 77’ | 23 ° 14’ | Munyai-23A | √ | | √ |
| South East (SE) | Tsitsikama | -33 ° 77’ | 23 ° 14’ | Munyai-23C | √ | | |
| South Eas (SE) | Tsitsikama | -33 ° 77’ | 23 ° 14’ | Munyai-23D | √ | | |
| South Eas (SE) | Tsitsikama | -33 ° 77’ | 23 ° 14’ | Munyai-23N | √ | | √ |
| South Eas (SE) | Tsitsikama | -33 ° 77’ | 23 ° 14’ | Munyai-23P | √ | √ | |
| South Eas (SE) | Prince Alfred Pass | -33 ° 34’ | 26 ° 49’ | Munyai-24J | √ | | |

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| Phytogeographic region | Locality | Latitude | Longitude | Collector/ sample no. | *rpl32-trnL* | *rps16* | ISSR |
|---|---|---|---|---|---|---|---|
| South East (SE) | Prince Alfred Pass | -33 º 34’ | 26 º 49’ | Munyai-24P | √ | | √ |
| South East (SE) | Prince Alfred Pass | -33 º 34’ | 26 º 49’ | Munyai-24R | √ | | √ |
| South East (SE) | Prince Alfred Pass | -33 º 34’ | 26 º 49’ | Munyai-24S | √ | | |
| South East (SE) | Prince Alfred Pass | -33 º 34’ | 26 º 49’ | Munyai-24V | √ | √ | √ |
| South East (SE) | Grahamstown | -33 º 98’ | 24 º 04’ | Munyai-22Gr8 | √ | √ | |
| South East (SE) | Grahamstown | -33 º 98’ | 24 º 04’ | Munyai-22Gr9 | √ | | √ |
| South East (SE)) | Grahamstown | -33 º 98’ | 24 º 04’ | Munyai-22Gr10 | √ | | |
| South East (SE) | Grahamstown | -33 º 98’ | 24 º 04’ | Munyai-22Gr11 | √ | | √ |
| South East (SE) | Grahamstown | -33 º 98’ | 24 º 04’ | Munyai-22Gr12 | √ | | √ |
| KwaZulu Natal (KZN) | Mpenjati Nature Reserve | -31 º 03’ | 30 º 16’ | Munyai-18mp3 | √ | √ | |
| KwaZulu Natal (KZN) | Mpenjati Nature Reserve | -31 º 03’ | 30 º 16’ | Munyai-18mp5 | √ | √ | |
| KwaZulu Natal (KZN) | Mpenjati Nature Reserve | -31 º 03’ | 30 º 16’ | Munyai-18mp7 | √ | | |
| KwaZulu Natal (KZN) | Mpenjati Nature Reserve | -31 º 03’ | 30 º 16’ | Munyai-18mp8 | √ | | |
| KwaZulu Natal (KZN) | Mpenjati Nature Reserve | -31 º 03’ | 30 º 16’ | Munyai-18mp11 | √ | √ | √ |
| KwaZulu Natal (KZN) | Frasser falls | -30 º 97’ | 30 º 27’ | Munyai-20f3 | √ | | √ |
| KwaZulu Natal (KZN) | Frasser falls | -30 º 97’ | 30 º 27’ | Munyai-20f4 | √ | | √ |
| KwaZulu Natal (KZN) | Frasser falls | -30 º 97’ | 30 º 27’ | Munyai-20f5 | √ | | |
| KwaZulu Natal (KZN) | Frasser falls | -30 º 97’ | 30 º 27’ | Munyai-20f6 | √ | | |
| KwaZulu Natal (KZN) | Frasser falls | -30 º 97’ | 30 º 27’ | Munyai-20f12 | √ | √ | |
| KwaZulu Natal (KZN) | Mkambati Nature Reserve | -31 º 30’ | 29 º 96’ | Munyai-25mk1 | | | √ |

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| Phytogeographic region | Locality | Latitude | Longitude | Collector/ sample no. | *rpl32-trnL* | *rps16* | ISSR |
|---|---|---|---|---|---|---|---|
| KwaZulu Natal (KZN) | Mkambati Nature Reserve | -31 º 30’ | 29 º 96’ | Munyai-25mk2 | √ | | √ |
| KwaZulu Natal (KZN) | Mkambati Nature Reserve | -31 º 30’ | 29 º 96’ | Munyai-25mk3 | √ | | |
| KwaZulu Natal (KZN) | Mkambati Nature Reserve | -31 º 30’ | 29 º 96’ | Munyai-25mk4 | √ | | √ |
| KwaZulu Natal (KZN) | Mkambati Nature Reserve | -31 º 30’ | 29 º 96’ | Munyai-25mk5 | √ | √ | √ |
| KwaZulu Natal (KZN) | Mkambati Nature Reserve | -31 º 30’ | 29 º 96’ | Munyai-25mk 6 | √ | √ | |
| KwaZulu Natal (KZN) | Umtamvuna | -31 º 01’ | 30 º 13’ | John-26mt1 | √ | | |
| KwaZulu Natal (KZN) | Umtamvuna | -31 º 01’ | 30 º 13’ | John-26mt2 | √ | √ | |
| KwaZulu Natal (KZN) | Umtamvuna | -31 º 01’ | 30 º 13’ | John-26mt3 | √ | | √ |

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### 2.2.2. Morphological diversity

#### (a) Morphometric measurements

A comparative morphological study was undertaken using dried *P. serratum* specimens. Individuals from six phylogeographic regions within 21 localities are presented in Figure 2.1 and Table 2.1. The 18 quantitative morphological variables were measured using a STEMI SR dissecting microscope with measuring eyepiece, ruler and digital caliper (see appendix 2.1). A number of qualitative and quantitative morphological characters were measured from *P. serratum* specimens bearing floral structures (see Figure 2.2). All the 18 morphological characters were measured from three specimens from the same locality between the phytogeographic groups. After measuring each character, a data matrix was captured into Excel. Additionally, a number of other potentially informative characters were also included in the floral developmental pattern study on Chapter four.

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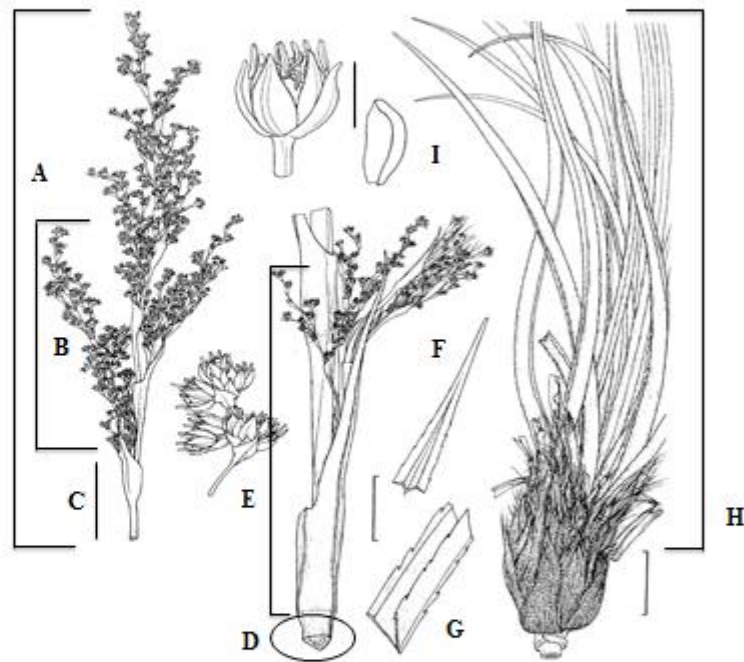


**Figure 2.2** Morphological characters measured for *Prionium serratum*. **A**, inflorescence length; **B**, lowest branching of an inflorescence; **C**, lowest branch inflorescence sheath; **D**, inflorescence base; **E**, inflorescence internode; **F**, leaf apex; **G**, a section of the middle part of leaf; **H**, leaf; **I**, perianth (from Munro *et al*., 2001).

**(b) Morphological character data transformation and analysis**

Before performing data analyses, data for each of the variables or characters measured (in 2.2.2.a) was $Log_{10}$ transformed. Data transformation was performed using STATISTICA version 10 (Stat Soft Inc, 2011). It was done to adjust the characters for normality and linearity which were the assumptions of the discriminant function analysis used for our study. Univariate analysis of variance (ANOVA) was performed to test the null hypothesis of equality of means between phytogeographic groups for each of the morphological characters.

**(c) Cluster analysis**

Before testing how well the data were able to discriminate between the populations, cluster analysis (CA) was used to investigate if there were any clustering patterns in the data. This analysis is an exploratory tool for classifying objects, which has no statistical assumptions about the data (Henderson, 2006). This technique places similar objects in groups and these groups are in turn placed in groups that are more inclusive in a hierarchical manner. In other words, it brings together individuals or populations that are closely associated into a cluster (Blackith & Reyment, 1971). Such a cluster is then considered to be discriminated from other associations that form separate clusters, thus dividing a data set into a *priori* unknown subgroups (Flury & Riedwyl, 1988). The technique involves defining a clustering algorithm and a measure of distance between individuals. The Unweighted Pair Group Method with Arithmetic mean (UPGMA) was used as the clustering algorithm and Euclidean distances were used as a measure of distance. The UPGMA was favoured because it computes the average similarity or dissimilarity of a candidate operational taxonomic unit (OTU) to an extant cluster, weighting each OTU in that cluster equally, regardless of its structural subdivision (Sneath & Sokal, 1973). The Euclidean distance was chosen because unlike other distance measures, (e.g. squared Euclidean, Manhattan, power or percent disagreement distance) it represents the actual geometric distance in the multidimensional space and the distance between any two objects is not affected by the addition of new objects to the analysis (Sneath & Sokal, 1973).

**(d) Discriminant function analysis**

For discriminant function analysis (DFA) the data were processed STATISTICA version 10 (StatSoft Inc, 2011). DFA was used to examine multivariate morphometric differences between the phytogeographic groups. This technique generates a linear combination of variables that maximizes betweem group differences and therefore the probability of

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correctly assigning observations to their predetermined groups and can also be used to classify new observations into one of the groups (Krzanowski, 1990; Quinn & Keough, 2002). The goal was to evaluate overall morphometric differences between putative groups. In populations were characters values were missing, the samples that were excluded in the analysis. The basic principle behind the discriminant function analysis is to find a suitable linear combination of several variables, i.e. canonical variates (CVs) in such a way as to maximise the correlations between the CVs and membership, and the ratio of between to within group variance (Sneath & Sokal, 1973; Krzanowski, 1990). Correlations between the original variables and the derived CVs, as well as the patterns of vector loadings for the original measurements allow reification of the CVs in terms of shape and size differences among groups (Compton & Hedderson, 1997). The DFA result in a classification matrix for each specimen is classified according to the classification functions correctly, either according to the original grouping or into another group. The percentage of correct classifications is given and this gives an indication of the validity of the original grouping. DFA also has the advantage of being able to show which variables are the most discriminatory in classifying specimens and to identify unknown specimens (Henderson, 2006). This information is useful in the development of identification keys in taxonomy.

**2.2.3. Evolutionary history**

**2.2.3. DNA extraction, amplification and sequencing**

**(a) DNA extraction**

*Prionium serratum* total DNA was extracted from the silica gel dried material using a modified version of the Cetyltrimethylammonium Bromide (CTAB) technique from Doyle & Doyle (1987) and Gawel & Jarret (1991) or the new straight-to-PCR procedure of Bellstedt *et al.* (2010). The CTAB protocol was modified as follows: 0.02–0.04 g silica-dried leaf material were placed into labelled 1.5 ml Eppendorf tube with silver balls and macerated using a Retsch MM 301 Mixer Mill for 25 minutes at 30 Hz, into a fine powder.

To each sample, 700 µl of pre-heated CTAB extraction buffer contain ß-mercaptoethanol was added and samples were immediately mixed by inversion or a vortex mixer for 10 minutes and incubated in the 65 °C water bath for at least an hour. After every 15-20 minutes, the samples were shaken gently (by inversion), to make sure that the top and bottom material are mixed. After incubation, 600 µl of chloroform: isoamylalcohol (24:1 v/v) was added into the Eppendorfs, mixed by inversion (or vortex mixer) for 5-10 minutes, and centrifuged at 12 000

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rpm for 10 minutes. 600 µl of the supernant volume was carefully pipetted out and placed into a clean, labelled, 1.5 ml tube, to which an equal volume of ice-cold isopropanol (600 µl) was added and mixed by inversion for 5 minutes. The samples were then placed in freezer a (-20 °C) for a minimum of two days to allow DNA to precipitate.

The chilled samples from the freezer were centrifuged at 12 000 rpm for 10 minutes to recover the DNA pellet, which was visible as a white or brownish pellet at the base of the tube. Isopropanol was carefully discarded and the open tubes were then inverted onto tissue paper to allow residual liquid to drain out, but making sure of not losing the pellet. After 15-20 minutes residual droplets were wiped off from the rim of the tube and DNA pellets were washed by adding 500 µl of 75 % ethanol to each tube, followed by agitating the tube to dislodge the pellet and inverting the tube so as to rinse away any remaining isopropanol from the sides of the tube, and samples were centrifuged at 12 000 rpm for 8 minutes. The ethanol was then discarded and tube was left open on the bench top for the sample to dry, after which the DNA pellet was suspended in 80 µl of sterile distilled water and stored in the fridge. Before running the polymerase chain reaction (PCR), the quality of DNA on samples was verified by running preliminary test at 100 V in 1% agarose gels stained with 0.005% Goldview (Guangzhou Geneshun Biotech, Ltd., Guangdong, China) and viewed under a UV light camera. Preliminary investigations showed that samples could not amplify well unless the DNA was purified. Hence, every extracted DNA was purified using a Zymo research DNA *Clean and Concentrator* ™ kit and this was done following the manufacturer's protocols. DNA quantifications was performed by visualizing under UV light camera, after electrophoresis on 1% agarose gel at 80 V for 15 minutes and compared with a known amount of lambda DNA markers.

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**(b) Screening of DNA sequence markers**

Several markers were screened for successful amplification, good sequencing and sequence variation between samples of *P. serratum*. These include *ITS* (White *et al*., 1990); *trnL-F, ropB-trnC*, *trnQ- rps16*, *rpl*32-*trn*L, trnV-*ndh*C, *rps16*, *ndhF-rp132* and *trnD-trnT* (Shaw *et al*., 2007); *ETS* (Chandler *et al*., 2001). The screening was done by performing PCR reactions (details are discussed below) with five to seven samples of *P. serratum* for each of the different markers, visualising the PCR products on agarose gel and taking a photo of the gel under UV light. Amplified products were then sent to the University of Stellenbosch DNA Sequencing Facility (Stellenbosch, South Africa) for sequencing. The sequences were aligned

and variation was assessed by manual inspection. The markers that were eventually used for this study were the *rpl*32-*trn*L and the *rps16* intron, and details of the primer sequences and the corresponding references are shown in Table 2.2. These DNA sequence markers are employed to infer phylogeographic patterns between populations of *Prionium* and evolutionary history between *Prionium* and selected families of monocotyledons.

**(c) DNA amplification and sequencing**

For the *rpl32-trnL* and *rps16*, PCR was carried out in 30 µl volume that contained 3 µl buffer; 3 µl $MgCl_2$; 1.2 dNTPs; 1 µl forward primer (*rp132 R; rps16 F*); 1 µl reverse primer (*trnL; rps16R*); 0.2 µl *Taq* polymerase; 2 µl of template DNA and 18.6 µl of sterile distilled water. The PCR was run on an Applied Biosystems Gene Amp 2700 thermal cycler (Amplied Biosystems, Foster City, CA, USA). For *rpl32-trnL*, the process involve an initial denaturation phase of 5 minutes at 80 °C followed by 30 cycles of 1 minute at 95 °C; 1 minute at 52 °C (annealing temperature); 4 minutes at 65 °C (extension temperature); and a final extension phase of 5 minutes at 65 °C. For *rps16*, the process involve an initial denaturation phase of 2 minutes at 94 °C; followed by 33 cycles of 1 minute at 94 °C; 1 minute at 52 °C (annealing); 2 minutes at 72 °C (extension) and a final extension of 7 minutes at 72 °C. For both markers (*rpl32-trnL* and *rps16*) the success PCR products were loaded (3 µl of each sample) into wells on a 1% agarose gel (that was stained with ethidium bromide) and ran in an electrophoresis tank containing 0.5 X TBE at 100 V for 15 minutes. The gel was then visualised under UV light and a photo of the gel was taken. Amplified PCR products were sequenced on ABI3730XL cycle sequencers at Macrogen, Inc. (Seoul, South Korea) or at the University of Stellenbosch DNA Sequencing Facility (Stellenbosch, South Africa) using the same primers that were used in the PCR. For *rpl23-trnL* and *rps16* intron, the first batches of samples were sequenced, and then the sequences were aligned and visually inspected. The same process was done to the rest of the samples for both primers.

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**Table 2.2** Name and sequence of primers used in this study. F/R= forward and reverse respectively.

| Primer name | Primer sequence | Reference |
|---|---|---|
| *rps16-F* | GTG GTA GAA AGC AAC GTG CGA CTT | (Shaw *et al*. 2007) |
| *rps16-R* | GTG GTA GAA AGC AAC GTG CGA CTT | (Shaw *et al*. 2007) |
| *rpl32-F* | CAG TTC CAA AAA AAC GTA CTT C | (Shaw *et al*. 2007) |
| *trnL*$^{UAG}$ | CTG CTT CCT AAG AGC ACG GT | (Shaw *et al*. 2007) |

**2.2.4. Sequence alignment and phylogenetic analysis**

**(a) Sequence alignment and gap coding**

Sequences were manually edited and contiguous sequences generated using the SEQMAN v.70.0 (DNAS-TAR, inc. program of DNA*Laser gene (Madison, 1991) and aligned in MegAlign using the CLUSTAL W algorithm (Thompson *et al*., 1994). For certainty, multiple sequence alignment was done for *rpl32-trnL* and *rps16* intron using the Muscle and CLUSTAL W alignment. Insertions and deletions were coded using simple indel coding in Gap Coder (Young & Healy, 2003). For both *rps16* (Table 2.3) and *rpl32-trnL* Typha *latifolia*: HQ913817 and *Scirpus holoschinodes*: HQ705837 sequences obtained from Genbank were used as out-groups in *Prionium* analyses. For evolutionary history studies, *rps16* sequences for monocots which include other members of Poales were downloaded from GenBank (see Table 2.3).

**(b) Haplotype network construction**

Genealogical relationships between *P. serratum* population haplotypes were analysed by haplotype networks reconstructed with TCS version 1.13 (TCS: estimating gene genealogies, Clement *et al*., 2000) from concatenated DNA sequence data (*rpl32-trnL* and *rps16* intron) utilizing the statistical parsimony procedure (Templeton *et al*., 1992). Small gaps in a sequence due to an indel (insertion/deletion) were coded as a single mutation to avoid theoretical intermediate haplotypes that are created by the program, which interprets each gap as independent mutation event. Gaps were treated as a fifth character state. The TCS program was also used to compute the out-group weights of haplotypes. This method estimates the unrooted tree and provides a 95% plausible set for all sequence type linkages within the unrooted tree.

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**(c) Phylogenetic reconstruction**

The *rpl32-trnL* and *rps16* matrices were analysed using Maximum Parsimony (MP) and Bayesian Inference (BI) approaches. Each chloroplast region was analysed separately and both regions together in a combined dataset. An unweighted MP analysis was conducted using PAUP* 4.0d102 (Swofford, 2002). To evaluate the possibility of multiple islands of equally most parsimonious trees (Maddison, 1991), heuristic searches were run seeded with 100000 random addition sequences, employing tree bisection-reconnection (TBR) swapping while retaining up to five trees per replication. Branch lengths were computed for all trees and the strict consensus of all most parsimonious trees was calculated for each analysis. Support for groups was evaluated by estimating 1000 bootstrap replicates using random additional sequence and TBR, but saving one tree per replicate. From the resulting trees, the majority rule consensus was calculated and nodes of the consensus with less than 50 were collapsed. Topology of the trees was evaluated using consistency indices, (CI) and retention indices (RI).

Phylogenetic reconstruction using Bayesian inference analyses (BI) was done separately for both individual and combined *rpl32-trnL* and *rps16* data sets. The nucleotide substitution model was selected using the Akaike information criterion (AIC; Akaike, 1974) as implemented by Modeltest version 3.7 (Posada & Crandall, 1998). Two models were selected for *rpl32-trnL* and *rps16* gene regions. The GRT+I+G (general time reversible gamma-shaped rate variation with a proportion of invariable site), and Hasegawa, Kishino and Yano model (HKY+G) were the best selected models respectively. The Bayesian inference analysis was performed in MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2003), using the Markov chain Monte Carlo (MCMC) with four chains of 3.5 million generations, sampling every 100 generation. Plots of likelihood scores, tree length and average standard deviation (SD) of split frequencies against number of generations showed that the analysis reached stationarity well within the first 10% of trees generated. Thus the first 10% of trees generated were discarded, ensuring that only trees generated at stationary were used to calculate the BI posterior probabilities (PP).The phylogenetic reconstruction for monocots, which *rps16* sequences for Poales was conducted following the same protocol used above for *Prionium* analysis. The GTR+G (general time reversible model with gamma distribution rate variation) was the best model selected. A 50% majority-rule consensus tree was created from the post-burn-in parameter estimates in MRBAYES, with posterior probabilities (PP) of nodes.

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**Table 2.3** GenBank accession details of monocots rps16 sequences analysed.

| Family | Species name | GenBank # | Family | Species name | GenBank # |
|---|---|---|---|---|---|
| Asparagaceae | *Anthericum corymbosum* Baker | EU128959 | Strelitziaceae | *Strelitzia alba* Skeels | JQ027268 |
| Bromeliaceae | *Brocchinia reducta* Baker | EU128981 | Commelinaceae | *Weldenia candida* Schult. f. | EU434227 |
| Bromeliaceae | *Brocchinia_reducta* | AY614140 | Poaceae | *Alopecurus pratensis* L. | EU434229 |
| Bromeliaceae | *Lindmania longipes* (L.B. Sm.) L.B. Sm. | HQ913866 | Poaceae | *Arthrostylidium merostachyoides* R.W. Pohl | EU434200 |
| Bromeliaceae | *Puya humilis* Mez | AF537912 | Poaceae | *Bambusa chungii* McClure | HM448949 |
| Bromeliaceae | *Tillandsia espinosae* L.B. Sm. | HQ913895 | Poaceae | *Bambusa pachinensis* Hayata | HM448951 |
| Cyperaceae | *Capeobolus brevicaulis* Browning | DQ058324 | Poaceae | *Calamovilfa longifolia* (Hook.) | AY508668 |
| Cyperaceae | *Carex flava* L. | JN627835 | Poaceae | *Cephalostachyum pergracile* Munro | EU434210 |
| Cyperaceae | *Carex hostiana* DC. | JN627781 | Poaceae | *Chimonocalamus pallens* J.R. Xue & T.P. Yi | EU434174 |
| Cyperaceae | *Carpha glomerata* (Nees) Nees | AY725941 | Poaceae | *Chloris barbata* Sw. | DQ242043 |
| Cyperaceae | *Chrysitrix capensis* L. | AJ419938 | Poaceae | *Chloris virgata* Sw. | DQ242044 |
| Cyperaceae | *Cyperus papyrus* L | AF449531 | Poaceae | *Cynodon dactylon* (L.) Pers. | DQ242038 |
| Cyperaceae | *Eriophorum angustifolium* Honck. | AY344154 | Poaceae | *Chusquea patens* L.G. Clark | EU434175 |
| Cyperaceae | *Eriophorum vaginatum* L. | AF449553 | Poaceae | *Cryptochloa strictiflora* (E. Fourn.) Swallen | EU434217 |
| Cyperaceae | *Ficinia bergiana* Kunth | EF078974 | Poaceae | *Dactyloctenium aegyptium* (L.) Willd. | AY508682 |
| Cyperaceae | *Ficinia oligantha* J. Raynal | GU012366 | Poaceae | *Dactyloctenium radulans* (R. Br.) P. Beauv. | AY508680 |
| Cyperaceae | *Hellmuthia membranacea* (Thunb.) R.W. Haines | EF174389 | Poaceae | *Dendrocalamus hamiltonii* Nees & Arn. | HM448957 |
| Cyperaceae | *Isolepis inyangensis* Muasya & Goetgh | AJ297506 | Poaceae | *Dendrocalamopsis valida* Q.H. Dai | EU434191 |
| Cyperaceae | *Isolepis levynsiana* Muasya & D.A. Simpson | AF449514 | Poaceae | *Diarrhena obovata* (Gleason) Brandenburg | AJ578777 |
| Cyperaceae | *Kobresia myosuroides* (Vill.) Fiori | GU17626 | Poaceae | *Eleusine coracana* (L.) Gaertn. | DQ242041 |

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|---|---|---|---|---|---|
| Cyperaceae | *Kyllinga brevifolia* Rottb. | AF449543 | Poaceae | *Eleusine indica* (L.) Gaertn. | EU434224 |
| Cyperaceae | *Neesenbeckia punctoria* (Vahl) Levyns | DQ058327 | Poaceae | *Eragrostis cylindrica* (Roxb.) Nees ex Hook. | DQ242047 |
| Cyperaceae | *Pycreus flavescens* (L.) P. Beauv. ex Rchb. | AF449547 | Poaceae | *Eragrostis obtusiflora* (E. Fourn.) Scribn. | HM152787 |
| Cyperaceae | *Rhynchospora brownii* Roem. & Schult. | DQ058336 | Poaceae | *Fingerhuthia s esleriiformis* Nees | AY508669 |
| Cyperaceae | *Schoenoplectus lacustris* (L.) Palla | AF449554 | Poaceae | *Gigantochloa atroviolacea* Widjaja | HM448962 |
| Cyperaceae | *Schoenus nigricans* L. | AY344150 | Poaceae | *Gigantochloa scortechinii* Gamble | GU063065 |
| Cyperaceae | *Scirpoides thunbergii* (Schrad.) Soják | AF449551 | Poaceae | *Leersia hexandra* Sw. | EU434221 |
| Cyperaceae | *Scirpoides holoschoenus* (L.) Soják | AY344153 | Poaceae | *Lolium perenne* L. | EU434227 |
| Cyperaceae | *Scirpus sylvaticus* L. | EF174396 | Poaceae | *Lithachne pauciflora* (Sw.) P. Beauv. | EU434218 |
| Cyperaceae | *Tetraria brachyphylla* Levyns | DQ419895 | Poaceae | *Lygeum spartum* L. | EU434226 |
| Cyperaceae | *Tetraria capillacea* C.B. Clarke | DQ419892 | Poaceae | *Menstruocalamus sichuanensis* (T.P. Yi) T.P. Yi | EU434196 |
| Iridaceae | *Aristea glauca* Klatt | AJ578766 | Poaceae | *Microchloa indica* (L. f.) P. Beauv. | DQ242046 |
| Iridaceae | *Babiana mucronata* Ker Gawl. | GQ925507 | Poaceae | *Microchloa kunthii* Desv. | DQ242045 |
| Iridaceae | *Geissorhiza heterostyla* L. Bolus | AJ578786 | Poaceae | *Miscanthus sinensis* Andersson | EU434232 |
| Iridaceae | *Gladiolus papilio* Hook. f. | AJ578789 | Poaceae | *Mullerochloa moreheadiana* F.M. Bailey | EU434207 |
| Iridaceae | *Iris ensata* Thunb. | AJ578793 | Poaceae | *Nardus stricta* L. | EU434225 |
| Iridaceae | *Ixia latifolia* D. Delaroche | AJ578797 | Poaceae | *Nassella filiculmis* (Delile) Barkworth | HM152787 |
| Iridaceae | *Klattia flava* Goldblatt | AJ578799 | Poaceae | *Neohouzeaua fimbriata* A. Camus | EU434211 |
| Iridaceae | *Micranthus junceus* (Baker) N.E. Br. | AJ578803 | Poaceae | *Neohouzeaua kerriana* A. Camus | EU434212 |
| Iridaceae | *Moraea vespertina* Goldblatt & J.C. Manning | GQ294208 | Poaceae | *Neololeba atra* (Lindl.) Widjaja | EU434206 |
| Iridaceae | *Nassella filiculmis* (Delile) Barkworth | JF698297 | Poaceae | *Neosinocalamus affinis* (Rendle) Keng f. | EU434192 |
| Iridaceae | *Romulea monadelpha* (Sweet) Baker | AJ578813 | Poaceae | *Oligostachyum glabrescens* (T.H.Wen) Keng f. | EU434170 |
| Iridaceae | *Sparaxis variegata* (Sweet) Goldblatt | AJ578817 | Poaceae | *Oreobambos buchwaldii* K. Schum. | EU434208 |

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|---|---|---|---|---|---|
| Iridaceae | *Syringodea unifolia* Goldblatt | AM941387 | Poaceae | *Panicum virgatum* L. | EU434230 |
| Iridaceae | *Thereianthus racemosus* (Klatt) G.J. Lewis | AJ578819 | Poaceae | *Pappophorum mucronulatum* Nees | AY508676 |
| Iridaceae | *Witsenia maura* (L.) Thunb. | AJ578825 | Poaceae | *Phyllostachys nigra* (Lodd. ex Lindl.) Munro | HQ154129 |
| Iridaceae | *Xenoscapa fistulosa* (Spreng. ex Klatt) Goldblatt | AJ578826 | Poaceae | *Pseudostachyum polymorphum* Munro | EU434213 |
| Juncaceae | *Juncus effusus* L. | AY344133 | Poaceae | *Rhipidocladum racemiflorum* (Steud.) McClure | EU434201 |
| Juncaceae | *Juncus gerardii* Loisel. | AY344134 | Poaceae | *Saccharum officinarum* L. | EU434231 |
| Juncaceae | *Luzula multiflora* (Ehrh.) Lej. | AY344135 | Poaceae | *Schizostachyum jaculans* Holttum | EU434215 |
| Juncaceae | *Luzula sylvatica* Gaudin | AY344136 | Poaceae | *Temochloa liliana* S. Dransf. | EU434204 |
| Marantaceae | *Afrocalathea rhizantha* K. Schum. | EF382847 | Poaceae | *Thyrsostachys siamensis* Gamble | EU434197 |
| Marantaceae | *Phacelophrynium maximum* K. Schum. | AY914627 | Poaceae | *Vietnamosasa ciliata* (A.Camus) T.Q. Nguyen | EU434202 |
| Marantaceae | *Phrynium fissifolium* Ridl. | EF382851 | Rapataceae | *Rapatea paludosa* Aubl. | HQ943889 |
| Typhaceae | *Typha angustifolia* L. | AM116858 | Restionaceae | *Restio tetraphyllus* Labill. | AJ404963 |
| Typhaceae | *Typha latifolia* L. | HQ913894 | Ruppiaceae | *Ruppia maritima* L. | JQ034333 |

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### 2.2.5. ISSR DNA amplification, genetic data scoring and analysis

**(a) ISSR-PCR amplification**

ISSR markers have become widely used (Zietkiewicz *et al*., 1994; Li & Jin, 2008; Sheeja *et al*., 2009) in population genetic studies because they require less money and labour than other methods and are highly variable. ISSR markers were also used to elucidate genetic relationships within and between populations of *P. serratum*. Ten primers (UBC primer set, Biotechnology Laboratory, University of British Columbia) UBC810 $(GA)_8T$, UBC812 $(GA)_8A$, UBC834 $(AG)_8YT$, UBC835 $(AG)_8YC$, UBC842 $(GA)_8YG$, UBC846 $(CA)_8RT$, UBC848 $(CA)_8RG$, UBC857 $(AC)_8YG$, UBC868 $(GAA)_6$ and UBC874 $(CCCT)_4$ were screened initially to identify well amplified, polymorphic bands between populations. Out of Ten primers tested, two gave the strongest, clearest and most reproducible band sequences. The two selected ISSR primers are UBC812 $(GA)_8A$ and UBC834 $(AG)_8YT$. These were selected for further study of 105 individuals from six regions.

PCR-amplification was run on an Applied Biosystems Gene Amp 2700 thermal cycler (Amplied Biosystems, Foster City, CA, USA). ISSR-PCR amplifications and analyses were those described by Bornet & Branchard (2001) with the following modifications. PCR reaction volumes were made up to 25µ with PCR-grade autoclaved water. Reaction mixtures consisted of 1X reaction buffer, $MgCl_2$ at 3mM, 0.2 mM of each dNTP, 0.4µM of primer and 0.03 units of Biotaq$^{TM}$. The amplification process involve an initial denaturation phase of 5 minutes at 80 °C; followed by 30 cycles of 1 minute at 95 °C; 1 minute at 52 °C (annealing temperature); 4 minutes at 65 °C (extension temperature); and a final extension phase of 5 minutes at 65 °C.

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**(b) Genetic data scoring and analysis**

The ISSR bands were analysed to estimate the genetic variations between and within *P. serratum* populations. ISSR bands were scored as 1 (present) or 0 (absent) binary characters using GeneMarker v1.97 (SoftGenetics, LLC, USA). For genetic diversity analyses, POPGENE32 Version 1.32 (Yeh, 1999) was used, implementing underlying assumption that populations are in Hardy-Weinberg equilibrium (HWE). POPGENE v.1.32 (Yeh, 1999) was used to estimate genetic diversity parameters such as the percentage of polymorphic bands (PPB), the effective number of alleles per locus (*Ne*), observed number of alleles per locus (*Na*), gene diversity (*H*), mean Shannon's (Lewontin, 1972) information index (*I*). The

Shannon's index of phenotypic diversity was used to partition diversity into components within and between populations.

To examine population genetic structure total population gene diversity (Ht); coefficient of gene differentiation ($G_{ST)}$ and the level of gene flow (Nm) were measured using Nei’s (1973) gene diversity statistics. Nei’s unbiased genetic identity (I) and genetic distance (D) between populations were computed using POPGENE v.1.32 (Yeh, 1999) and GenAlEx6 v6.41 (Peakall & Smouse, 2006). An analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) using $\Phi_{ST}$ (analogous to Wright's Fixation index or $F_{ST}$) as the measure of genetic distance was done to examine the distribution of population genetic variation within and between populations using GenAlEx6 v6.41 (Peakall & Smouse, 2006). Two individual-based analyses were carried out to examine the genetic relationships between different populations. It was first explored by means of a principal coordinate analysis (PCA) and cluster analysis. PCA analysis was done to illustrate the genetic distances and relationships between populations on the basis of Nei’s pairwise genetic distances ($\Phi_{ST}$) matrix of studied populations (Nei, 1973), implemented in the software GenAlEx v6.41 (Peakall & Smouse, 2006). Genetic identity and distances matrices between individuals were obtained using Nei (1978) and then employed to build a dendrogram using Unweighted Pair Group Method (UPGMA), then an unrooted neighbour-joining dendrogram was constructed in PHYLIP version 3.65 (Felsenstein, 2005). In addition, in order to investigate presence of a correlation between genetic and geographical distances in kilometres between populations, a Mantel test was performed using GenAlEx6 v6.41 (Peakall & Smouse, 2006). Significance tests were based on 999 permutations.

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## 2.3. Results

### 2.3.1. Morphological diversity

#### (a) Basic statistics

A summary of the morphometric results for the 18 characters is shown in appendix 2.1. There is considerable overlap in some of the characters between some of the groups for the 18 characters, but the univariate analysis of variance (ANOVA) showed that the means of five (marked with *) of the variables differ significantly ($p<0.05$) between the studied groups in Table 2.4 below.

**(b) Cluster analysis**

The cluster analysis yielded the phenogram showed in Figure 2.3. At a Euclidean distance of 15 two clusters were evident, cluster I and II. Looking at both clusters, there is no clustering on morphology of all operational taxonomic units OTU's from all the phytogeographic groupsat the Euclidean distance 1.5 (Figure 2.3). This implies that none of the populations studied is morphologically distinct for the variable studied.

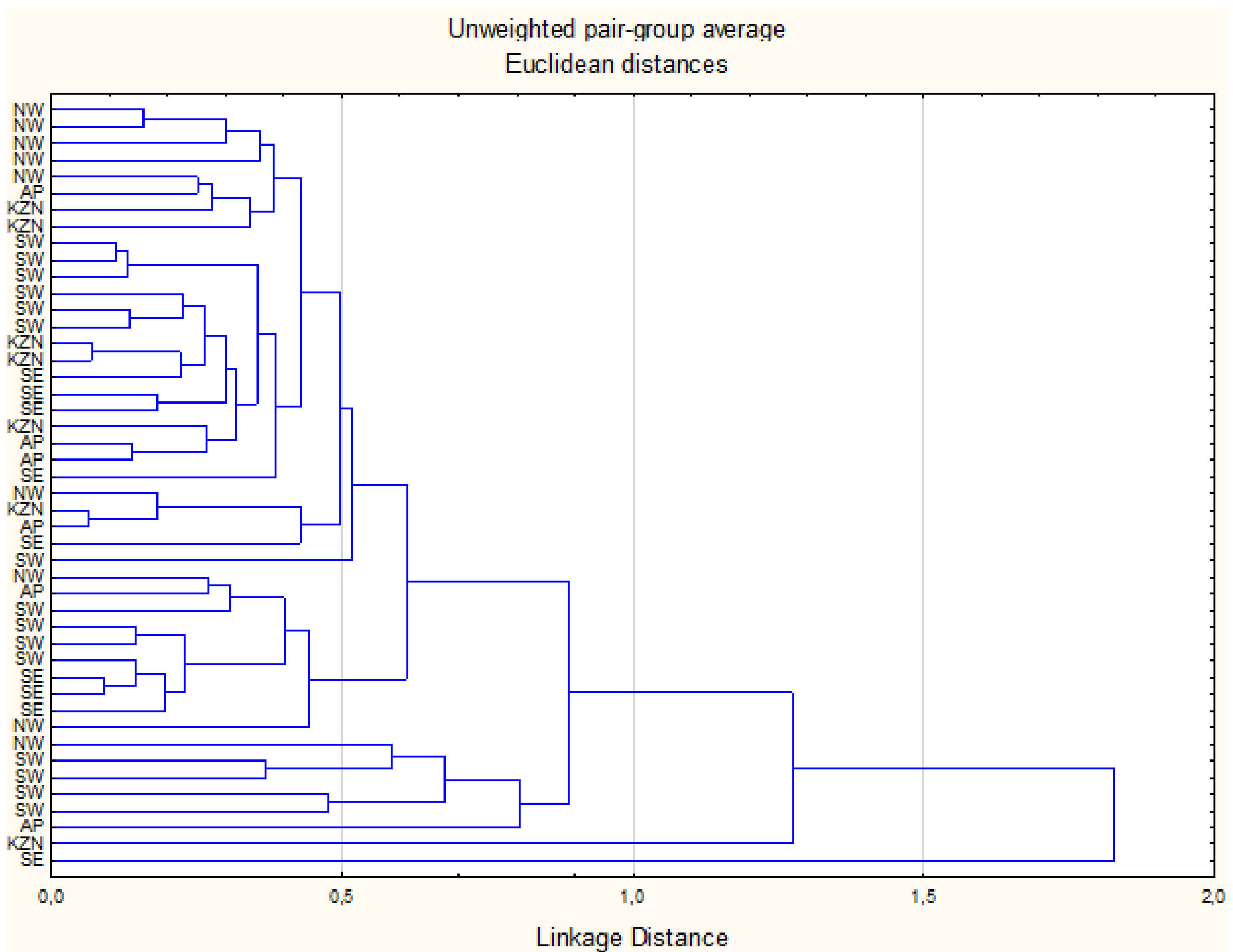


**Figure 2.3** Phenogram from cluster analysis of morphological data for *Prionium serratum* showing difference between phytogeographic regions.

**Table 2.4** Morphological characters for *P. serratum* between phytogeographic groups: Mean±SE and range. Units = mm

| Morphological characters | NW | | SW | | KZN | | SE | | AP | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean±SE | Range | Mean±SE | Range | Mean±SE | Range | Mean±SE | Range | Mean±SE | Range |
| Leaf *L* | 60.5±13.03 | 40-78 | 60.4.5±14.7 | 31-80 | 70±11.5 | 61-95 | 82±28.35 | 48-138 | 72.25±11 | 54-80 |
| Leaf bottom *W* | 14.2±1.6 | 11-16 | 13.5±3.28 | 6-18 | 14.3±2.35 | 9-17 | 15.4±248.2 | 9-759 | 15.52±3.5 | 10-19 |
| Leaf apex *W* | 7.4±2.01 | 5-12 | 6.58±3.4 | 2.7-13 | 9.24±2.6 | 4-12 | 10.6±3.10 | 4.8-13 | 10.14±3.7 | 4-13 |
| **Inflorescence *L**** | **50±10.63** | **28-64** | **50±11.02** | **35-70** | **55±10.24** | **34-62** | **58±6.6** | **48-67** | **59±12** | **36-70** |
| Inflorescence Base *W* | 7.23±2.73 | 4-14 | 6.7±2.23 | 5-14 | 7.9±3.02 | 5-15 | 7.3±2.10 | 5.6-13 | 9.6±4.1 | 2-14 |
| Inflorescence sheath *L* | 7.2±0.70 | 6-9 | 7.9±0.8 | 6.5-9 | 8.1±0.52 | 7.2-8.3 | 7.9±1.05 | 7-9.5 | 6.9±0.15 | 7-7.2 |
| **Inflorescence sheath *W**** | **3.1±0.41** | **3-4** | **3.6±0.50** | **2.6-4** | **4±0.42** | **3.1-4.3** | **3.6±0.51** | **3-4.2** | **2.9±0.12** | **2.7-3.2** |
| Inflorescence internode *L* | 9.5±1.5 | 8-15 | 9.4±1.2 | 6.5-12 | 12±2.36 | 9-14 | 12±1.73 | 9-13 | 11±3.25 | 7.5-16 |
| **Inflorescence bract *L**** | **5.5±0.4** | **5-6** | **5.5±0.3** | **5.2-6.1** | **5.9±0.3** | **5.5-6.2** | **5.5±0.41** | **5.5-6.3** | **5.15±0.25** | **5-5.4** |
| Inflorescence bract *W* | 2.35±0.2 | 2.3-3 | 2.5±0.22 | 2.1-3 | 2.6±0.2 | 2.3-3 | 2.4±0.3 | 2.2-3 | 2.3±0.06 | 2.2-2.3 |
| **Inner perianth *L**** | **5.2±0.43** | **4-6** | **5.45±0.3** | **5-6** | **5.5±0.1** | **5.5-6** | **5.45±0.40** | **5.2-6.3** | **5.08±0.23** | **5-5.2** |
| Inner perianth *W* | 2.35±0.2 | 2.2-2.5 | 2.3±0.22 | 2-3 | 2.35±0.24 | 2.1-3 | 2.35±2.16 | 2.1-2.3 | 2.15±0.06 | 2.1-2.3 |
| **Lowest branch inflo *L**** | **15±4.24** | **7-22** | **19±4.9** | **12-24** | **16±5.34** | **8-23** | **22±2.33** | **20-25** | **17±6.10** | **11-25** |
| Lowest inflo internode *L* | 6.8±2.0 | 3.8-9 | 5.2±1.40 | 3.5-6 | 6±1.2 | 4-7 | 5.8±1.4 | 3.7-6 | 6.65±1.9 | 4-7 |
| Lowest branch inflo sheath *L* | 10±2.6 | 7.5-13 | 10±3.00 | 5-14 | 12.5±3.15 | 9-18 | 12±4.03 | 9-22 | 13±3.12 | 8-16 |
| Lowest branch inflo sheath *W* | 9.17±2.6 | 6-14 | 11±2.02 | 7-14 | 12±2.44 | 7.6-14 | 11.43±1.0 | 11.5-14 | 11.8±2.4 | 7-13 |
| Lowest branch inflo bract *L* | 3±1.0 | 2-3.5 | 3.7±1.33 | 2.6-4.5 | 3.6±0.50 | 3.5-5.3 | 3.6±0.50 | 2.9-4 | 3.5±1.3 | 2-5.5 |
| Lowest branch inflo bract W | 3.34±1.5 | 1.4-7 | 4.06±1.72 | 0.8-6.7 | 4.8±0.6 | 3.5-6 | 4.8±0.6 | 4-5 | 4±1.5 | 2.3-6-5 |

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**(b) Discriminant function analysis**

In terms of the discriminant function analysis (DFA), the canonical analysis yielded four canonical variates (CVs). Discriminant function analysis of the 18 characters is presented in Table 2.5 below. The two Canonical variates (CV 1 and CV 2) accounted for 80.18 % of the total variation within the phytogeographic studied groups (Figure 2.4). The first CV 1 (DF1) explained 47.99 % and the second (CV 2) which explained 32.19 % of the total morphological variation between groups. CV 3 and CV 4 explain 19.27 % of the total morphological variation between phytogeographic groups. All the characters used for discriminant function analysis which are associated with positive and negative correlations are shown in Table 2.5. A cut-off of 0.4 was used when deciding on the significance of the contribution of each variable within CV's. The characters that mostly influence CV 1 and 2 with highest values were inflorescence sheath L (**-3.79**, **2.04**), inflorescence sheath W (**3.36**, **-1.66**), inflorescence Bract L (**1.10**, **-1.55**), and lowest inflorescence branch L (**-1.55**, **-0.65**). CV 1 is largely correlated to inner perianth (**-1.61),** leaf bottom W **(-0.40),** inflorescence L (**1.16**), lowest inflorescence branch sheath L (0.82), and lowest inflorescence branch bract W (0.90). Whereas CV 2 is largely influenced by inflorescence Bract W (0.78), leaf L (0.94) and leaf apex W (1.60). The characters which largely influence CV 3 and 4 are also presented on the table below in bold numbers. CV 1 and 2 (Figure 2.5) showed that four populations (AP, SW and NW) have no separation except for SE and KZN population which has a weak separation.

These two CVs were highly successful in their ability to assign specimens to the correct group as indicated by the posterior probabilities of the classification matrix (2.6). Of the 46 *P. serratum* specimens between phytogeographic regions examined in this study, all groups obtained above 80% classification scores except only for 4 specimens from NW (SW); KZN (NW); SW (NW) and AP (NW) were misclassified. In terms of distances between phytogeographic groups, all groups were significantly different from each other except for NW/SW, SW/SE and SW/AP: $p > 0.05$ (Table 2.7). A scatter-plot of the CV 1 against the CV 2, showing how the different groups are distributed along the two axes is shown in Figure 2.4. With the exception of SW, SE and and KZN which show some overlap, the rest of the distinct groups correspond to the different phytogeographic regions (Figure 2.4).

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**Table 2.5** Discriminant function analysis showing canonical variates between *Prionium serratum* phytogeographic groups populations.

| Variables | CV 1 | CV 2 | CV 3 | CV 4 |
|---|---|---|---|---|
| Inflorescence sheath L | **-3.79** | **2.04** | 0.33 | **1.95** |
| Inflorescence sheath W | **3.36** | **-1.66** | **-0.78** | **-1.33** |
| Inflorescence Bract L | **1.10** | **-1.55** | **-0.81** | **1.14** |
| Inflorescence Bract W | -0.08 | **0.78** | 0.37 | **-1.20** |
| Inner perianth L | **-1.61** | 0.34 | **1.52** | **-1.60** |
| Inner perianth W | **0.47** | **-0.77** | **-0.85** | **1.66** |
| Leaf L | 0.17 | **-0.94** | 0.66 | -0.24 |
| Leaf bottom W | **-0.40** | -0.28 | 0.11 | 0.24 |
| Leaf apex W | -0.23 | **1.60** | **-0.41** | -0.01 |
| Inflorescence L | **1.16** | 0.33 | 0.18 | 0.17 |
| Inflorescence base W | **0.62** | 0.14 | -1.27 | **-0.44** |
| Lowest inflorescence branch sheath L | **-0.82** | -0.16 | -0.34 | **1.04** |
| Lowest inflorescence branch sheath W | **0.64** | **-0.79** | **1.52** | **-1.00** |
| Inflorescence node L | 0.39 | 0.39 | **0.82** | **-0.51** |
| Lowest inflorescence branch node L | **1.09** | **0.19** | **0.47** | 0.12 |
| Lowest inflorescence branch L | **-1.55** | **-0.65** | **-0.63** | 0.05 |
| Lowest inflorescence branch bract L | 0.15 | 0.26 | -0.26 | **-1.39** |
| Lowest inflorescence branch bract W | **-0.90** | -0.26 | -0.32 | **1.69** |
| **Eigenvalue** | **3.74** | **2.51** | **1.07** | **0.47** |
| **Cumulative proportion** | **47.99 %** | **32.19 %** | **13.19 %** | **6.08 %** |

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**Table 2.6** Classification scores matrix for the five phytogeographic groups of *Prionium serratum* species. Misclassifications are printed in bold.

| Phytogeographic groups | Percentage (%) | NW | KZN | SW | SE | AP |
|---|---|---|---|---|---|---|
| **NW** | 89 | 8 | 0 | **1** | 0 | 0 |
| **KZN** | 86 | **1** | 6 | 0 | 0 | 0 |
| **SW** | 93.3 | 0 | **1** | 14 | 0 | 0 |
| **SE** | 100 | 0 | 0 | 0 | 9 | 0 |
| **AP** | 83.3 | **1** | 0 | 0 | 0 | 5 |
| **Total** | 80.18 | 11 | 7 | 15 | 9 | 5 |

**Table 2.7** p values for distances between phytogeographic groups. Non-significant values are printed in bold.

| Phytogeographic regions | NW | SW | KZN | SE | AP |
|---|---|---|---|---|---|
| **NW** | | 0.20 | **0.01** | **0.01** | 0.42 |
| **SW** | 0.20 | | **0.00** | 0.14 | **0.03** |
| **KZN** | **0.01** | **0.00** | | **0.00** | **0.00** |
| **SE** | **0.01** | 0.14 | **0.00** | | **0.01** |
| **AP** | 0.42 | 0.03 | **0.00** | **0.01** | |

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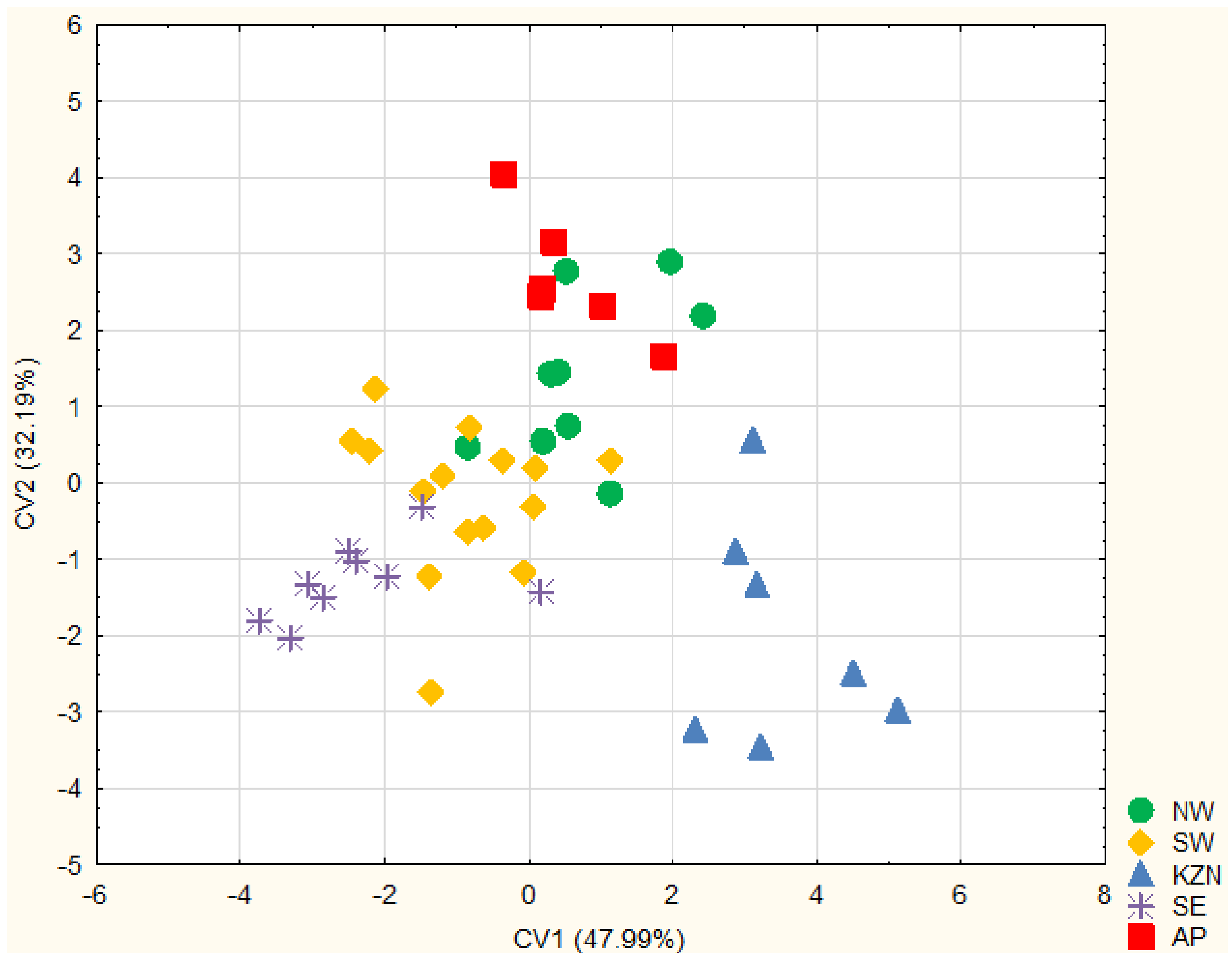


**Figure 2.4** Discriminant function analysis scatter plot of *P. serratum* populations between phytogeographic regions. Abbrevations for sampled localities: Agulhas Plain AP, (red), KwaZulu Natal (KZN, blue), North West (NW, green).South East and Eastern Cape (SE, purple), and South West (SW, orange).

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### 2.3.2. Evolutionary history

#### (a) Haplotype network

Haplotype network based on the concatenated DNA dataset (*rpl32-trnL* and *rps16*) matrices revealed two groups between the haplotypes in the network showing closely and diverged haplotypes. One group (Figure 2.5 A) consists of 23 haplotypes, occurring in all six phytogeographic groups, that were phylogenetically grouped into two haplotypes (KZN; H1 and SE; H7) were identified separated by mutational steps. Of these haplotypes identified, two haplotypes H1 (KZN) and H7 (SE) were the most widespread and shared by all phytogeographic groups. All tip haplotypes (H2-5, H8-10, H13-14, H17-21 and H23-24) in the spinning network were unique to a particular population. On the other group (Figure 2.5

B) 13 haplotypes were detected and these grouped into haplotypes NW (H1) and SW (H4) separated by mutational steps, but none of the haplotypes was detected in KZN populations. Of these detected haplotypes, H2-5, H7-8, H10-12 and H13 were unique haplotypes found on the tip of the minimum spinning network.

**(b) DNA sequence**

*Prionium serratum* aligned matrices contained a total of 904 and 1226 characters for *rpl32-trnL* and *rps16* intron respectively. The combined matrix comprised 2130 characters for 107 taxa, of which one formed out-group and the remainder constituted the in-group for both markers. The descriptive statistics *Prionium* for *rpl32-trnL* and rps16, and *rps16* for the enlarged monocots datasets, which includes dense samples of the Poales, are presented in Table 2.8 below.

**(d) Phylogenetic relationships of *Prionium***

The results for parsimony analyses of individual and combined DNA regions were as follows: *rpl32-trnL Prionium* gave 10 trees with 406 steps, consistency index (CI) = 1.00 and retention index (RI) = 1.00; the *rps16 Prionium* matrix yielded 1428 trees with 248 steps, CI = 0.99, RI = 0.97; combined partition data set gave 1416 tree with 657, CI = 0.99, RI = 0.96; (Table 2.8). Parsimony and Bayesian phylogram showed low posterior probability (PP) and bootstrap support (BS) values for the main clades and also showed similar topology for two analyses approach used. Bayesian posterior probability higher than 70 % and bootstrap support higher than 50 % were considered "supported" and such values were presented (Figure 2.6 A and B). Posterior probability values were indicated above the branches before the backslash symbol and bootstrap values above the branches were indicated after the backslash symbol. DNA region (*rpl32-trnL* and *rps16*) data set trees have been separated for better representation. On Figure 2.6A two well supported clades are observed (A1 and 2), with a Posterior probability = 100%, 70% and Bootstrap = 91%, 64 %, and comprised of individuals from SW and SE (A1) and KZN, AP and LB (A2), whereas in Figure 2.6B, has a Posterior probability of 99% and 85% and Bootstrap of 61% and 53% which include individuals from AP, SW and KZN (B1) and SW and NW (B2). The backbone of *Prionium* phylogram based on *rps16* data (Figure 2.6B) form a polytomy and there is weak support for two large clades (B1 and 2), but these are not observed in *rpl32–trnL* tree (Figure 2.6 A). For the *rps16 P. serratum* tree, several nodes that have posterior probability support are not

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supported with bootstrap values above 50% (Figure 2.6B, marked with *). For combined data set (Figure 2.7) the monophyly of ingroup is supported, but clade 1 is weakly supported (79%/*), comprising three individuals from SW, SE and NW, while the remaining individuals form polytomy. The results for parsimony analyses of the *rps16* monocots which includes other members of Poales datasets had 386 trees with 4040 steps, CI = 0.46, RI = 0.85 (Table 2.8). Parsimony and Bayesian phylogram showed low posterior probability (PP) and bootstrap support (BS) values for clades within *Prionium*. DNA region data set for *rps16* monocots was named according to family names. There is a well-supported stem node for the Cyperid clade (PP = 100% and BS = 92%) showing Thurniaceae (*Prionium*) to be sister to a clade made of Juncaceae and Cyperaceae (Figure 2.8). From their common ancestor, the branch to crown node of Thurniaceae, Juncaceae and Cyperaceae are of similar length, but *Prionium* has shorter terminal branches compared to Cyperaceae and Juncaceae.

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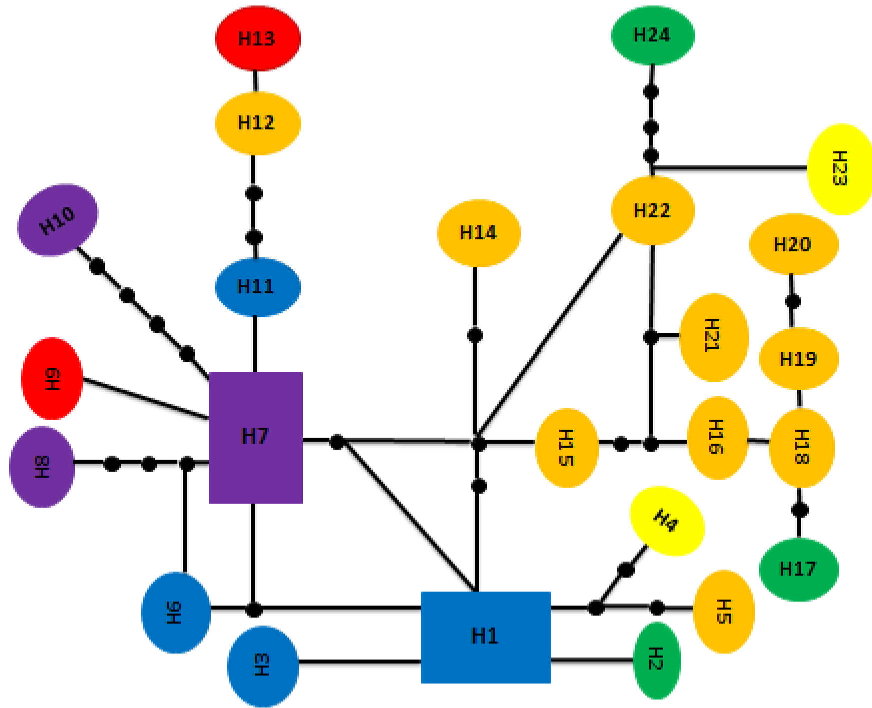
A




B

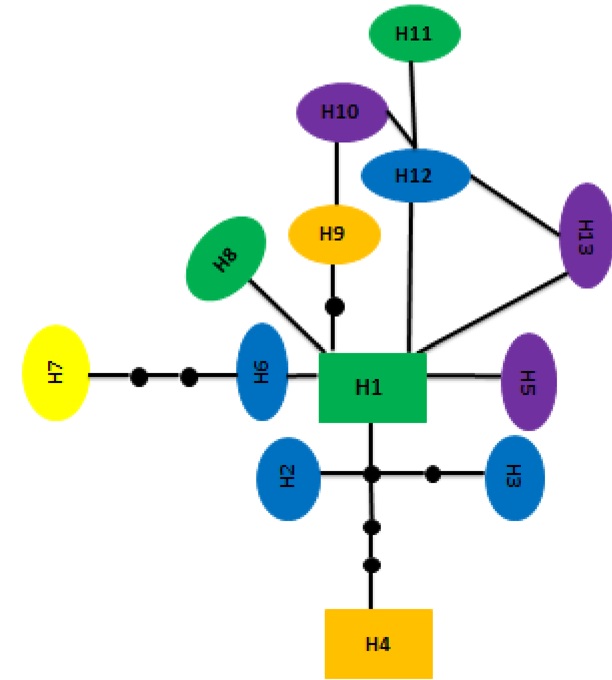



**Figure 2.5** Two unconnected haplotype networks for *Prionium serratum* under the 95% parsimony criterion. The size of ancestral (rectangle) and descended (circle) of each haplotype is proportional to the frequency of the haplotype in the total sample. Mutational step between haplotypes is represented by a line with an intermediate haplotypes indicated by a dot. Haplotypes (H) found within KwaZulu Natal (KZN, blue), South East and Eastern Cape (SE, purple), Agulhas Plain AP, (red), South West (SW, orange), Langeberg (LB, yellow) and North West (NW, green).

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**Table 2.8** Summary of DNA matrixes used in the study of *Prionium* and monocots.

| **Data description** | ***rpl32-trnL* (*Prionium*)** | ***rps16* (*Prionium*)** | **Combined (*Prionium*)** | ***rps16* monocots** |
|---|---|---|---|---|
| Total number of taxa in matrix | 104 | 33 | 107 | 156 |
| Total number of characters in the matrix | 1120 | 904 | 1226 | 1570 |
| Number of uninformative characters | 312 | 175 | 487 | 312 |
| Number of parsimony informative characters | 57 | 47 | 104 | 735 |
| Number of maximum parsimony trees | 10 | 1428 | 1416 | 386 |
| Length of maximum parsimony tree | 406 | 248 | 657 | 4040 |
| Parsimony consistency index | 1.00 | 0.99 | 0.99 | 0.46 |
| Parsimony retention index | 1.00 | 0.97 | 0.97 | 0.85 |
| Employed evolutionary model for BI | GTR+I+G | HKY+G | GRT+I+G | GTR+G |
| Bayesian analysis (number of generations run) | 3.2 million | 3.2 million | 3.5 million | 3.2 million |

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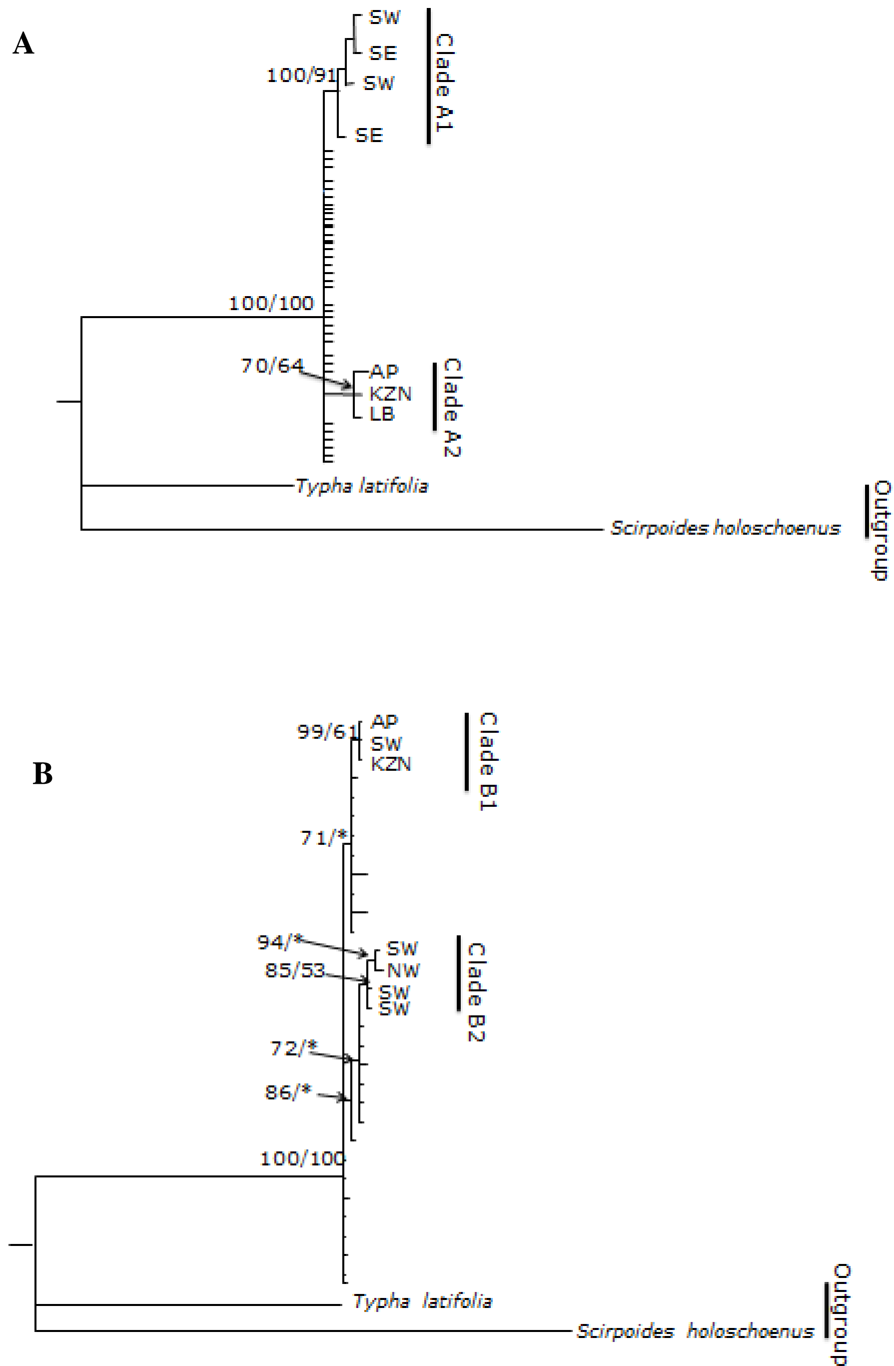


**Figure 2.6** Majority rule consensus phylogram of *rpl32-trnL* gene (A) 10 trees obtained from the parsimony analysis of*rps16* gene (B) 1428 trees obtained from the parsimony analysis for *Prionium serratum*. Posterior probability > 70 % was shown before backslash and Bootstrap values > 50 % was shown after backslash above branches. * indicate values with less than bootstrap values cut-off.

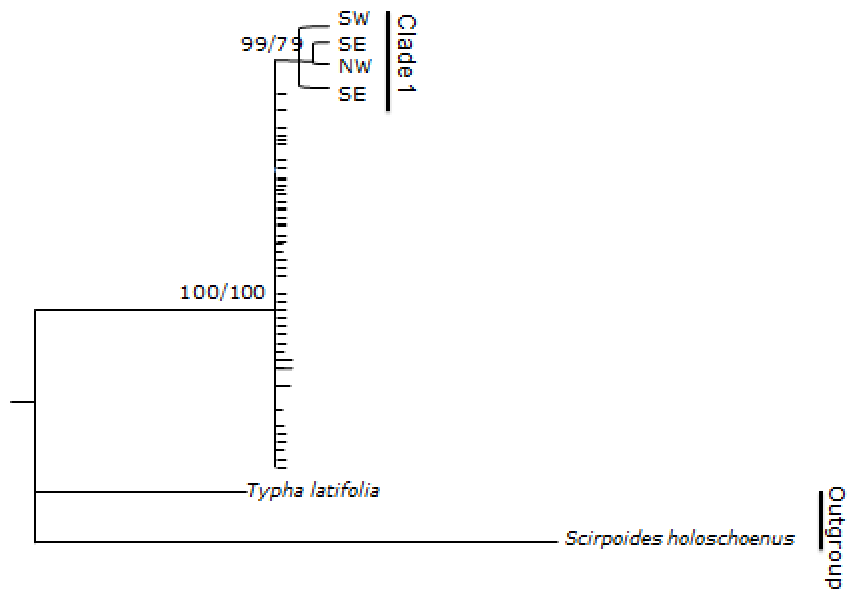


**Figure 2.7** Majority rule consensus phylogram of the 1428 trees obtained from the parsimony analysis of combined DNA regions for *Prionium serratum*. Posterior probability > 70 % was shown before backslash and Bootstrap values > 50 % was shown after backslash above branches.

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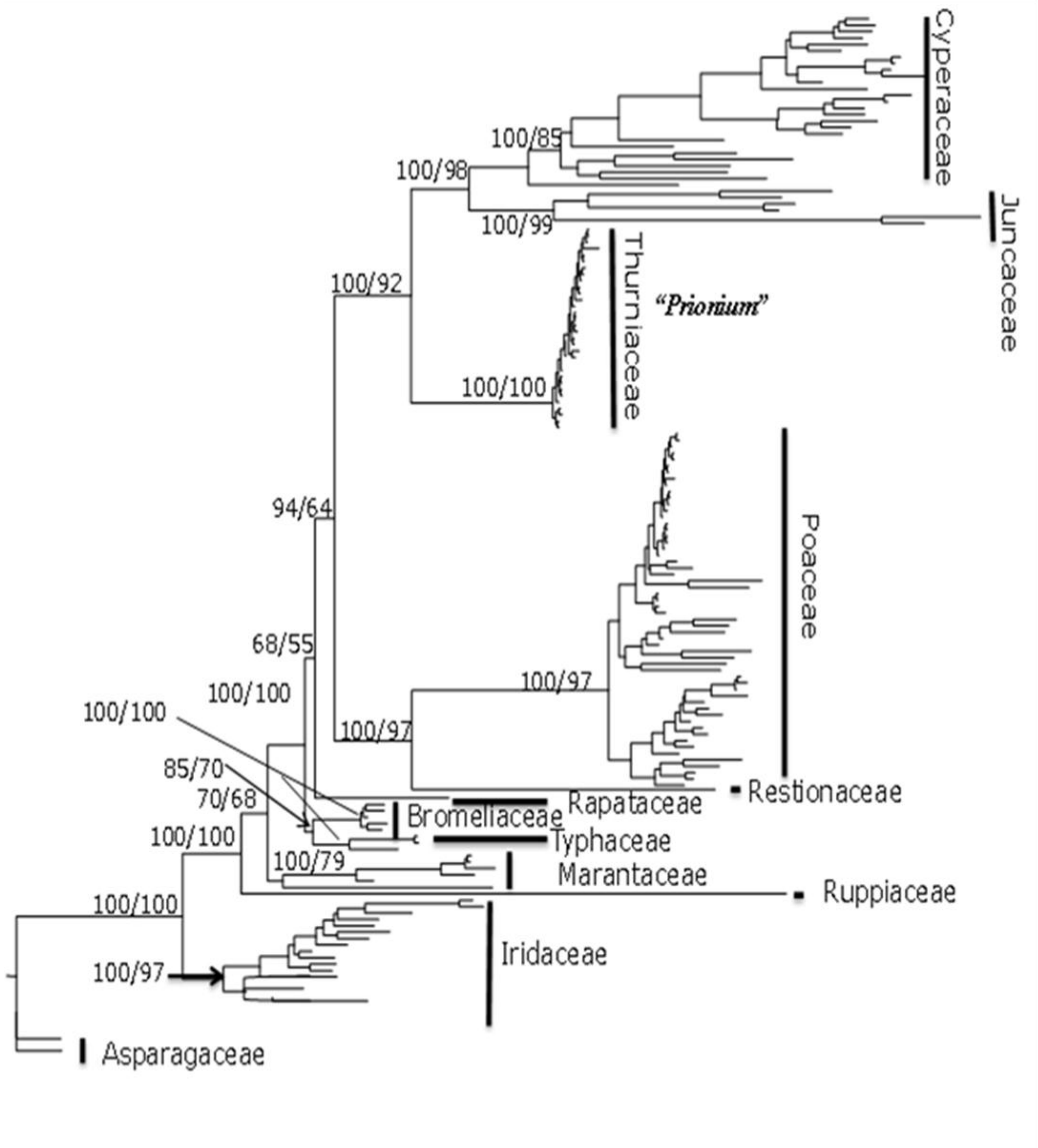


**Figure 2.8** Majority rule consensus phylogram of the 386 trees obtained from the parsimony analysis of *rps16* regions for *Prionium serratum* and Poales. Posterior probability > 1000 % was shown before backslash and Bootstrap values > 50 % was shown after backslash above branches.

### 2.3.3. Genetic diversity

**(a) ISSR polymorphism**

The two ISSR primers generated 47 clear and reproducible bands which ranged from 200 to 2400 base pairs. Among the six populations sampled, the percentage of polymorphic bands (PPB) for single populations ranged from 6.38% (LB) to 34.04% (AP and KZN) with an average percentage of 22.70%, while this percentage polymorphic bands value ascended sharply to 91.41% at the species level. The number of alleles ranged from 1.064 (± 0.25) to 1.340 (± 0.45), while the effective number of alleles ranged from 1.053 (± 0.21) to 1.183 (± 0.34). Assuming Hardy-Weinberg equilibrium, Nei's gene diversity ranged from 0.023 (±0.11) to 0.105 (± 0.180). The Shannon's Information Index ranged from 0.041 ± 0.16 to 0.159 ± 0.26 (see Table 2.9). Shannon's index of phenotypic diversity was used to class diversity into components within and between populations. *Prionium serratum* populations at AP and KZN exhibit the great level of variability (PPB: 34.04% each; H: 0.105± 0.180 and 0.052 ± 0.142; I: 0.159 ± 0.26 and 0.076 ± 0.21, respectively), whereas populations at LB and SW exhibit the lowest level of variability (PPB: 6.38% and 12.8%, H: 0.023±0.112 and 0.052 ± 0.184, I: 0.041 ± 0.16 and 0.076 ± 0.25 respectively), as shown in Table 2.8. Estimates of Shannon's information index showed different numerical values but a similar trend to that of Nei's gene diversity.

**(b) Genetic structure**

The coefficient of genetic differentiation between populations ($G_{ST}$) was 0.743 as estimated by partitioning of the total gene diversity, indicating that about 74.3% of genetic variability existed between *P. serratum* populations, while 25.7% is within populations. In the analysis of molecular variance (AMOVA), there were highly significant ($P < 0.001$) genetic difference between populations in the six phylogeographic areas ($F_{ST}$ analog $\Phi_{ST}$) was 0.529, which indicated that 53 % of total genetic variability occurred between populations and 47 % within populations (Table 2.10). Thus, AMOVA analysis also supported the results of Nei's genetic diversity statistics and Shannon's Information index that there is a high degree of inter population differentiation. The level of gene flow (Nm) was estimated to be 0.173 individuals per generation between the phytogeographic regions.

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**Table 2.9** The genetic variability within six natural populations of *Prionium serratum* detected by ISSR analysis. Abbreviations: Pop ID (Population identity); # (sample number0; Npl (number of populations); PPB (polymorphic percentage band); Na (number of alleles); Ne (effective number of alleles); H (Hardy-Weinberg equilibrium) and I (Shannon's Information Index).

| **Pop ID** | **#** | **Npl** | **PPB** | ***Na*** | ***Ne*** | ***H*** | ***I*** |
|---|---|---|---|---|---|---|---|
| Langeberg | 10 | 3 | 6.38% | 1.064±0.025 | 1.053±0.21 | 0.023±0.112 | 0.041±0.16 |
| South West | 19 | 16 | 12.8% | 1.123±0.048 | 1.092±0.33 | 0.052±0.184 | 0.076±0.25 |
| Agulhas Plain | 11 | 16 | 34.04% | 1.340±0.045 | 1.183±0.34 | 0.105±0.182 | 0.159±0.26 |
| KwaZulu Natal | 8 | 6 | 34.04% | 1.123±0.034 | 1.092±0.26 | 0.052±0.142 | 0.076±0.21 |
| South East | 8 | 12 | 25.53% | 1.255±0.044 | 1.120±0.27 | 0.072±0.150 | 0.111±0.22 |
| North West | 10 | 11 | 23.40% | 1.234±0.043 | 1.183±0.35 | 0.101±0.190 | 0.145±0.23 |
| **Mean±SE** | **66** | **47** | **22.70%** | **0.826±0.046** | **1.140±0.018** | **0.080±0.010** | **0.119±0.014** |

**Table 2.10** AMOVA results of molecular variation in *P. serratum* population found within and between populations. SSD: sum of squared deviations.

| **Source of variation** | **SSD** | **Total variation** | **$\Phi_{ST}$** | **P-value** |
|---|---|---|---|---|
| Between populations | 64.1 | 53 % | 0.529* | < 0.001*** |
| Within populations | 59.0 | 47 % | | |

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**(c) Genetic relationship between phytogeographic groups.**

The genetic distances, based on the allele frequencies were calculated for the 21 natural populations within six phylogeographic regions to estimate the extent of their divergence and population pairs. Population pairwise relationships show that the genetic distance (GD) between populations ranged from 0.2207 for the most closely related populations, to 0.6231 for the most divergent populations (Table 2.11). In addition, genetic relationships between phytogeographic regions were examined by UPGMA cluster analysis and PCA based Nei's pairwise genetic distance ($\Phi_{ST}$). A Neighbor-joining (NJ) dendrogram was constructed by an unweighted paired group method of cluster analysis using UPGMA of PHYLIP Version 3.5 (Felsenstein, 2005; Figure 2.9). UPGMA method of clustering was carried out to estimate relationship between the six natural populations based on $\Phi_{ST}$ genetic distance pairwise

matrix. The dendrogram revealed that AP and KZN populations were the most similar, because they had the lowest value of genetic distance and clustered together. However, unexpectedly low values of $\Phi_{ST}$ genetic distance were found between geographically distant areas (AP and KZN, and KZN and SE).

In addition, PCA results were consistent with the UPGMA dendrogram results based on $\Phi_{ST}$ genetic distance pairwise matrix. The first three coordinates explained 32.37%, 19.76% and 17.94% of the total genetic differentiation between populations (Figure 2.10). Along PCA axis 1, SE, AP and KZN are not separated, and these form a close grouping with NW and SW, but LB is widely separated from rest. Along PCA axis 2 AP, LB, SW and NW are close but SE and KZN are separated widely from one another and also from the other 4 phytogeographic areas. When a Mantel test was applied to geographical distance (Data is not listed) and pairwise GD, no correlation was detected (r = 0.0004, P = 0.6530). This results of the Mantel test show that there is no significant correlation between geographical occurence of the phytogeographic areas and the genetic variability observed in *Prionium*.

**Table 2.11** Genetic relationship of *P. serratum* populations between phytogeographic groups shown by genetic identity (above diagonal) and unbiased genetic distance (below diagonal).

| Pop ID | Pop code | LB | SW | AP | KZN | SE | NW |
|---|---|---|---|---|---|---|---|
| Langeberg | **LB** | **** | 0.5926 | 0.5363 | 0.7156 | 0.6043 | 0.6736 |
| South West | **SW** | 0.5232 | **** | 0.7194 | 0.7658 | 0.6595 | 0.6668 |
| Agulhas Plain | **AP** | 0.6231 | 0.3294 | **** | 0.8020 | 0.7639 | 0.7261 |
| KwaZulu Natal | **KZN** | 0.3346 | 0.2668 | 0.2207 | **** | 0.7980 | 0.7826 |
| South East | **SE** | 0.5036 | 0.4163 | 0.2693 | 0.2256 | **** | 0.7120 |
| North West | **NW** | 0.3951 | 0.4052 | 0.3201 | 0.2451 | 0.3397 | **** |

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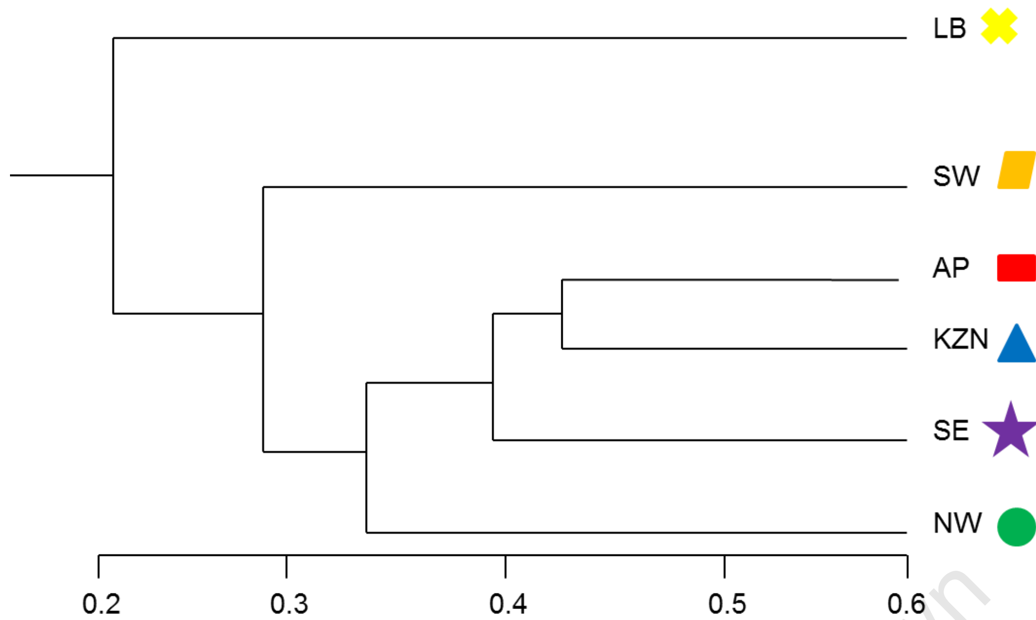


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**Figure 2.9** UPGMA dendrogram of the phytogeographic groups of *Prionium serratum* populations based on $F_{ST}$ genetic distance. Abbreviations are given in Figure 2.1.

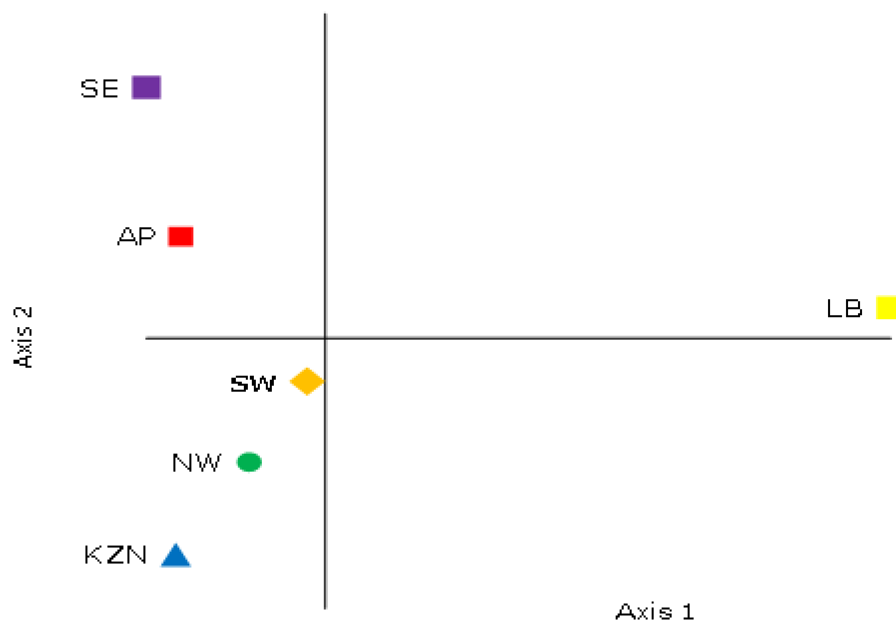


**Figure 2.10** Scatter diagram showing genetic differentiation based on pairwise ΦST values of *Prionium serratum* phytogeographic groups. Phytogeographic group's abbreviations are given in Figure 2.1.

## 2.4. Discussion

The study has investigated morphological and genetic diversity and structure of *Prionium* throughout its entire range. *Prionium* is abundant in sandstone mountain streams below 900 meters, and occurs along such stream to near sea level. Within its range, between VanRhynsDorp (NW, CFR) to Port Shepstone (KZN), *Prionium* does not occur in seasonal streams nor in interior sandstone mountains in the Klein Karoo, and the samples collected in this study (Figure 2.1) are representatives of the known distribution based on previous herbarium collections (http://sibis.sanbi.org/).

The first objective of this study was to investigate the morphological diversity between *P. serratum* populations and predicted that there is high morphological difference between populations. The basic ANOVA statistics of 18 morphometric characters studied revealed five characters (inflorescence sheath *W**, inflorescence sheath L, inner perianth L, inflorescence L and lowest branching length) which differ significantly. Cluster analysis which includes all the 18 characters measured produced only two main clusters (Figure 2.3), but with OTUs from different populations intermixed, implying high similarity between *P. serratum* populations studied across the six phytogeographic regions. Discriminant function analyses demonstrate partial overlap of in morphology of populations (SW/NW), but there is evidence of near separate (AP, SA) to completely separate populations (KZN) (Figure 2.4). Patterns of similarity (or dissimilarity) based on genetic diversity data (Figure 2.9) and phylogenetic results (Figure 2.6 and 2.7) are not congruent with the morphological results, as OTUs from all regions cannot be separated. Furthermore, there is no discrete character to justify separation of any of the populations of *Prionium* into a separate taxon (either at species or infraspecific rank).

The second objective of this study was to investigate the evolutionary history of genus *Prionium* compared to monocots which includes other members of Poales using DNA sequence data, using some of the highly variable plastid markers (Shaw *et al*., 2007; *rpl32-trnL* and *rps16*). There was low number of variable sites between populations of *Prionium* for the two markers, and the resulting phylogeny has weakly supported nodes and formation of numerous polytomies (Figure 2.6A & B, 2.7). The low DNA sequence variation within *Prionium* is puzzling, as the two markers used here are routinely used in reconstruct phylogenies between lineages (genera, families) of plants. Moreover, *rpl32-trnL* is a choice



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marker for reconstructing phylogeny between lineages with recent rapid radiation such as *Cyperus* (Larridon *et al*., 2011), and is too variable within Poales families such that alignment across the order cannot be done with confidence. The phylogram of *Prionium* and monocots which includes other Poales based on *rps16* intron (Figure 2.8), reveals branch length (between stem and crown nodes) similar to sister families Cyperaceae and Juncaceae. However, these sister families have subsequent cladogenesis and long branches leading to their terminal taxa whereas *Prionium* has short branches. While the ancestor of the Poales was a wetland perennial (Linder & Rudall, 2005; Muasya *et al*., unpublished), it is notable that some Cyperaceae and Juncaceae have colonized non-wetland habitats and have varied life history (Balslev, 1996; Goetghebeur, 1998; Kirschner *et al*., 2002a & b; Roalson *et al*., 2005) whereas *Prionium* is a clonal shrubby perennial restricted to permanent wetlands. The lack of DNA sequence variability may be linked to its life form (Smith & Dhonogue, 2008), but is still unusually low for a lineage with a stem age in Oligocene, unless the extant populations have a more recent origin. The phylogeny results support the ISSR results below, which revealed low genetic diversity at population level.

Furthermore, concatenated sequence data were used to better understand genealogical processes underlying *P. serratum* population. The results based on haplotype network analyses (Figure 2.5) indicated that within group A and B, H1 (KZN) and H1 (NW) were the most widespread haplotypes detected. According to the coalescent theory (Schaal *et al*., 1998), H1 (KZN; Figure 2.5A) and H1 (NW; Figure 2.5B) should be the most ancestral haplotype because they showed the largest number of connections with other haplotypes and NW was central in the network. A moderate level of homoplasy is apparent in the network, resulting in convergent evolution. The presence of one common haplotype in all populations suggests that this species may have gone through a bottleneck after which new allelic variants have originated in low frequencies. Similar results on the haplotype network pattern were found between populations of *Hagenia*, a monotypic genus in the Rosaceae family (Ayele *et al*., 2009). *Hagenia* showed six haplotypes that were phylogenetically grouped into two lineages with a clear pattern of congruence between their geographical distribution and genealogical relationships. It was assumed that restricted gene flow through seeds, rare long-distance dispersal, contiguous range expansion and mutation played a primary role on shaping the genetic structure.

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The third objective was to investigate the genetic diversity, population structure and level of gene flow between and between populations of the clonal monotypic species *P. serratum*. Genetic diversity of *P. serratum* was investigated further using ISSRISSR sequence data. Genetic diversity is an important precursor in the study of plant species and has been widely adopted to investigate population genetic processes (Ma *et al*., 2008). Genetic structure is an important feature that indicates gene flow, breeding system, divergence, and reproduction mode of a population (Gui *et al*., 2009). Moreover, isolated marginal populations exhibit low levels of genetic variation because of bottlenecks, environmental stochasticity, small sizes and lower levels of flowering, fruit-set and recruitment (Bauret *et al*., 1998; Landergott *et al*., 2001). Genetic studies of clonal plants species provide reliable information on their population dynamics and detailed demographic data (Ren *et al*., 2005). In this study, prediction was made that the clonal, long-lived, wind pollinated *P. serratum* would have high genetic diversity between populations and low within populations. To the best of our knowledge, this is the first report of genetic diversity in *Prionium* species.

By using ISSR markers, genetic diversity which existed within *P. serratum* populations ranged from 6.38 (LB) to 34.04% (AP and KZN) with an average of 22.70%. This study revealed relatively low genetic variation within *P. serratum* populations. Similar to *P. serratum* results, Qiu *et al*. (2004) and Liu *et al*. (2011) observed a relatively low genetic diversity within populations where PPB values ranged from 29.6 to 36.2% and had an average of 32.6% (*Changium smyrnioides* H. Wolff and *Neopicrorhiza scrophulariiflora* (Pennel) D.Y.Hong). The level of genetic diversity between populations on most of the species was found to be highly dependent on the evolutionary history of that particular species (Booy *et al*., 2000). In addition, similar results was found by Yang *et al*. (2011) on a short-lived annual alpine herb, *Swertia tetraptera* Maxim endemic to the Qinghai-Tibetan Plateau which has low genetic diversity between its population with PPB ranged from 16.2 to 49.5%, with an average of 32.7%.

Analyses of the ISSR markers using different approaches (namely: Nei's genetic diversity analysis, Shannon's diversity index, AMOVA and UPGMA) demonstrated similar interpretations of the genetic structure within *P. serratum* populations. AMOVA revealed that 53 % of total variability is between populations and 47 % within population. Nei's genetic diversity analysis ($G_{ST}$ = 0.743) show high genetic differentiation between *P. serratum*



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populations and level of gene flow (Nm = 0.173) which is lower than one successful migrant per generation. This ineffectual gene flow estimated indicated insufficient genetic exchange between populations contributing towards influencing high genetic differentiation. Gene flow maintains the integrity of species and promotes the dispersion of favourable alleles between populations (Rieseberg & Burke 2001). High to moderate levels of genetic differentiation between populations is a common pattern in endemic or narrowly distributed plant species (Hamrick & Godt, 1989, Nybom, 2004). Similar to this study's findings, Liu *et al*. (2011) observed similar genetic pattern on *Neopicrorhiza scrophulariiflora* with high level of genetic differentiation ($G_{ST}$ = 0.6955) and limited gene flow (Nm = 0.2198) between populations. In contrast to this study, Chen *et al*. (2009) found a low coefficient genetic of differentiation between *Caragana microphylla* populations ($G_{ST}$) was 0.074 as estimated by partitioning of the total gene diversity, indicating that 7.4 % of the total genetic variability with high gene flow (Nm = 10.72) between populations. Several previous surveys of genetic variation in plants have shown that rare, endemic, or narrowly distributed plants tend to maintain low degree of genetic variability due to the impact of genetic drift, the founder effect, and directional selection with high levels of inbreeding (Franklin, 1980; Hamrick & Godt, 1989; Ellstrand & Elam, 1993), action of mutation, selection, and genetic drift, gene flow and mating system which in turn must operate within the historical context of each plant species (Slatkin, 1987; Schaal *et al*., 1998).

There is no correlation between genetic and geographic distance between *P. serratum* populations. Populations in Agulhas Plain (AP) were genetically more similar to populations in KwaZulu Natal (KZN) (Figure 2.9), and more dissimilar from populations in nearby areas such as the Langeberg (LB) and South West (SW) mountains (see figure 2.1) This pattern was confirmed by the Mantel test (r = 0.0004; P = 0.6530), showing no significant correlation was found between genetic and geographic distance. *Prionium serratum* appears to have geographic and ecological constraints (permanent rivers in sandstone mountains), and there is absence of gene flow between nearby areas that do not share drainage systems. If there was widespread gene flow, it would be expected that nearby areas (e.g. NW and SW; SW and AP) would be genetically more similar. The observed absence of correlation between genetic distance and geographical distance imply that other forces may have impacted on the populations of *P. serratum*. Several authors (e.g. Barret & Kohn, 1991 and Fischer *et al*,. 2000) have invoked the role of genetic drift in causing such lack of significant correlation

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between genetic and geographical distance. When populations are isolated from one another, genetic drift shapes genetic structure, leading to genetic distance between populations not to completely correlate with geographic distance between populations. For example, NW, SW and KZN have drainage/ mountain systems which isolate them from one another. The CFR has remained fairly unchanged, especially for the sandstone habitats (NW, SW, LB and parts of SE). On the other hand, AP and KZN occupy areas which may have been under ocean in Pliocene and populations here may be recently recruited (Cowling *et al*., 2009). Biogeography studies indicate that environmental barriers, historical processes, and life history such as mating system may all, to some extent, shape the genetic structure of populations (Donnelly & Townson, 2000; Conzzolino *et al*., 2003).

## 2.5. Conclusion

The morphometric analysis revealed absence of morphology differentiation between *P. serratum* populations within the phytogeographic regions. In the evolutionary history study, the evolutionary rate detected within *Prionium* compared to monocots (includes other members of Poales) revealed an unusually low sequence variation for markers that are routinely used to reconstruct evolutionary history in taxa of comparable age and life form. Also, two haplotypes (found in KZN and NW populations) are highly widespread between populations indicated that *P. serratum* had low mutational change which result from one single mutational event. In addition, the use of ISSR and chloroplast markers reveals low genetic diversity between phytogeographic areas, and limited gene flow and high genetic differentiation between populations. I therefore conclude that *P. serratum* is not genetically poorly differentiated and the distribution of genetic diversity between and within populations might have been shaped by a combination of several genetic factors. Thus, *P. serratum* could be an old species but it’s not known if the populations are young or old.

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## CHAPTER 3

## DISTRIBUTION PATTERNS IN GENUS *PRIONIUM*: EXPLORING EDAPHIC HETEROGENEITY.

### 3.1. Introduction

#### 3.1.1. General distribution patterns of the genus *Prionium*

*Prionium serratum* populations are widespread within the Fynbos biome in the Cape Floristic Region (CFR) and there are some scattered and isolated populations in the Maputaland-Pondoland Region (MPR) (Fig 3.1; Munro & Linder, 1997; Munro *et al*., 2001; Goldblatt & Manning, 2002). The CFR and MPR are generally associated with low nutrient soils, and CFR receives winter rainfall (Cowling *et al*., 1996), while MPR has summer rainfall (Van Wyk & Smith, 2001).

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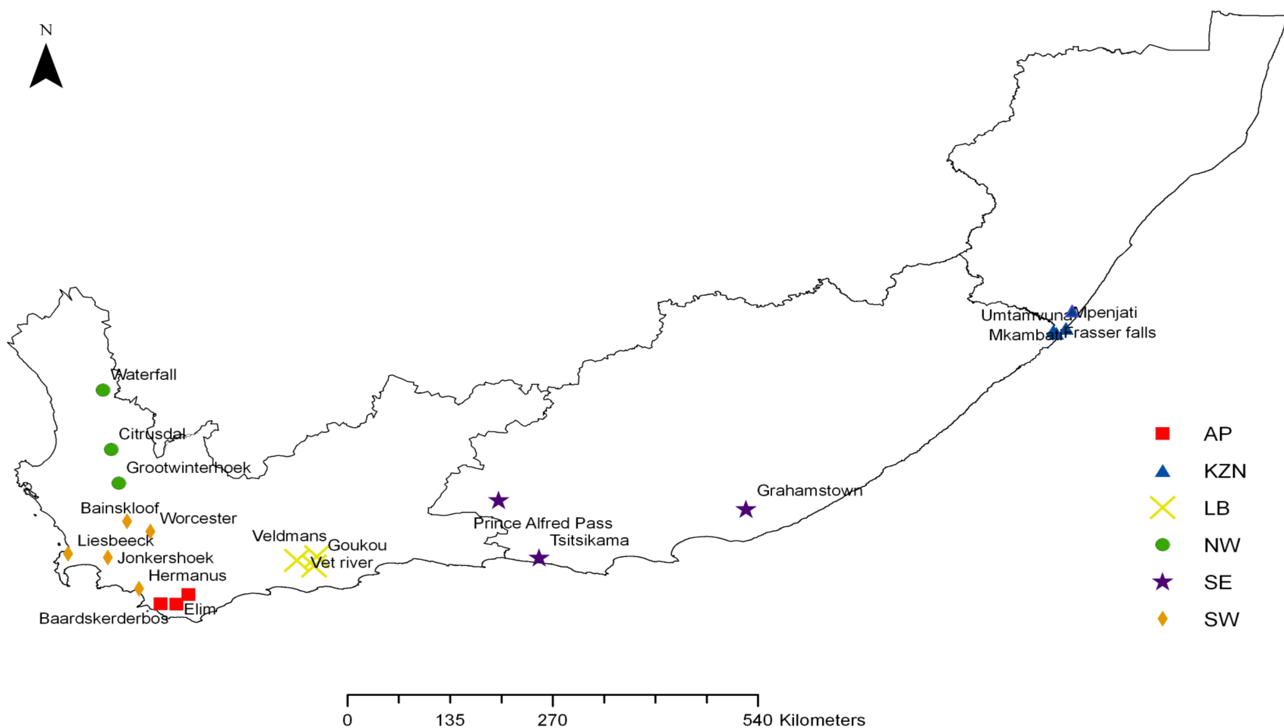


**Figure 3.1** Distribution of *Prionium serratum* within 21 natural sites and four non-*Prionium* sites between the phytogeographic regions included in this study.

Edaphically, the MPR comprises Karoo sediments, interspersed with sandstones of the Natal Group (van Wyk, 1996). King (1982) previously referred to the Natal Group Sandstone as Table Mountain Sandstone. The MPR sandy soils are acidic, nutrient-poor and contribute to high levels of plant endemism, particularly amongst grassland taxa (Scott-Shaw *et al*., 1996). While most of the populations of *P. serratum* occur in the CFR, nearly exclusively within the Fynbos vegetation, outlier populations grow in vegetation islands comprising members of the Fynbos biome. The CFR accommodates a mosaic of soil types, derived from various parent materials, including limestone, granite, sandstone and shale (Cowling *et al*., 1996; Goldblatt & Manning, 2000; Rebelo *et al*., 2006). CFR Mountains are mostly composed of folded quartzites and quartzitic sandstone of the Cape Supergroup's Table Mountain and Witterberg Groups (Deacon *et al*., 1992).

The Fynbos vegetation predominantly occurs on sandstone derived soils (Rebelo *et al*., 2006). These ancient, coarse-grained, sandstone-derived soils have characteristically low nutrient status (oligotrophic) particularly with regard to nitrogen (N) and phosphorus (P) (Witkowski & Mitchell, 1987). P is often the most limiting element for plant growth and development (Vance *et al*., 2003) due to its essential role in genetic, metabolic, structural and regulatory macromolecules (White & Hammond, 2008). Recently, Lambers *et al*. (2007) and Shane *et al*. (2008) reported that low P availability in the soil was a key factor determining nutrient acquisition strategies for plants in the South Western Australia and thus play a role on the distribution of species habitats.

The CFR is characterized by high species turnover along habitat gradients (Beta diversity), of which edaphic diversity has been identified as one of the primary drivers (Cowling, 1990; Cowling *et al*., 1992). The association between species distributions and soil nutrient availability, one of the edaphic factors, may arise through physiological differences in nutrient use and uptake strategies betweenst plant species (Lamont, 1982; Richards *et al*., 1997b; Orians & Milewski, 2007), and a number of strategies which enable plants to grow in these low P soils (Lambers *et al*., 2007). Most plants exhibit strategies that allow them to cope with water shortage and nutrient poor soils (Lambers *et al*., 2007). For example, the distributions of Fynbos species are strongly linked to edaphic factors such as nutrient availability and nutrient acquisition strategies to respond to poor soil nutrition (Cowling, 1990; Cowling *et al*., 1992; Richards *et al*., 1995, 1997a & b). Hawkins *et al*. (2005) found



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that Fabaceae species produce cluster roots, which enhance plant P uptake from poorly available sources through the production of exudates via ligand exchange, thus making it more available to the plants. Other strategies include mycorrhizal symbiotic relationships (e.g. in some Restionaceae and some Cyperaceae) and carnivory (Hawkins *et al*., 2005).

Soil nutrient availability also contributes to the habitat specificity and distributions of legume seeders and resprouters in fynbos. Habitats and distributions of legume seeders and resprouters were linked to fire regime or the availability of resources (Le Maitre & Midgley, 1992; Bond & van Wilgen, 1996). Habitat specificity has been recorded between seeders and resprouters in *Erica* in the CFR (Ojeda, 1998). Fire regime importance was confirmed by Le Maitre & Midgley (1992) in Proteaceae in that the proportion of seeders in a landscape increases with increased fire frequency. Several studies indicated that the distribution of seeders and resprouters is strongly related to resource availability, with seeders occurring more frequently than resprouters in habitats with greater resource such as higher moisture and nutrient availability (Ojeda, 1998; Linder, 2003; Bellingham & Sparrow, 2001; Bond & Midgley, 2001, 2003). Verboom *et al*. (2004) confirmed strong post-fire regenerative strategy in the genus *Ehrharta*. They found that reseeders within *Ehrharta* were associated with high growth rates and invested more resources to seed production, while resprouters were slow growers. Kruger (1983), Bell (2001) and Bellingham & Sparrow (2001) indicated that seeders and resprouters potentially have differing nutritional requirements as a result of their differences in allocation of resources to vegetative growth and reproductive effort.

Plant species distributions are constrained also by local climate, geology, hydrology, disturbance, and interactions with other species, between other environmental gradients (Gottfried *et al*., 1998). The combination of these factors determines an organism's fundamental niche, defined as the range of conditions and resources within which species can persist (Ricklefs, 2001). Moreover, physical and chemical processes that determine soil formation cause variation in soil characteristics at local spatial scales (Brady & Weil, 2002). Southern Africa has an intricate geomorphological history; with remnants of old (Mesozoic) infertile landscapes interspaced with recently exposed (e.g. Pliocene marine sediments) and more nutrient rich substrates (Cowling *et al*., 2009; Hooper, 2009). It is thought that the infertile old landscapes that are buffered from Cenozoic climatic effects (OCBILs sensu Hooper, 2009) harbour relictual lineages of plants, and such lineages tend to be long-lived



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and show adaptations such as clonality (Hooper, 2009). *Prionium* is among the putative relictual genera in the Southern Africa (Warren & Hawkins, 2006), growing along streams in sandstone areas together with *Brabejum* (Proteaceae), raising question on whether such relicts are restricted to nutritionally impoverished habitats. Therefore, this chapter focuses on investigating the nutritional regime of *P. serratum*.

### 3.1.2. Objective and hypotheses of the study

The objective of this chapter was to investigate edaphic heterogeneity in the sites of *P. serratum* in South Africa. This study tests the hypotheses that:

(a) *Prionium serratum* occupy soils with similar nutritional levels between the phytogeographic areas; and

(b) There are soil nutritional difference between *P. serratum* habitats and non-habitats.

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## 3.2. Methods and materials

### 3.2.1. Soil sampling and analysis

Ecological field observations and sample collection were done at representative localities within *P. serratum* entire range (Figure 3.1). Soil samples used to test nutritional levels were collected from 21 *Prionium* sites and four non-*Prionium* sites within the phytogeographic areas. At each site, three replicate samples were collected using a soil auger or a garden trowel taking a slice of soil up to 10-15 cm deep. The samples were placed into plastic bags and labelled according to the voucher specimen number. The samples were air dried, sieved to pass through a 2 mm sieve and sent to BemLab Private Laboratory, Somerset, South Africa for analysis of the concentration of the following soil nutrients: phosphorus (P Bray II), carbon (C), nitrate ($NO_3^-N$), ammonium ($NH_4^+N$), potassium (K), T-value, and pH was also measured.

Soil pH was determined by shaking 2 g of material in 20 mL 1 *M* KCl at 180 rpm for 60 minutes, centrifuging at 10 000 g for 10 minutes, and measuring the pH of the supernatant. Total soil N was determined by combustion method using a LECO FP528 N Analyzer (Leco Corporation, St. Joseph, USA) but using about 20 mg soil per sample. $NH4^+$ and $NO3^-$ were determined by extracting 2 g of soil in 20 mL of 1 *M* KCl, which was analysed colorimetrically according to Keeney & Nelson (1982). Total P was determined in the soil by acid digestion using a mixture of nitric and hydrochloric acids and the extract analysed by

inductively coupled plasma atomic emission spectrometry (ICP-AES; Varian Vista MPX, Mulgrave, Australia). Available P was assessed by extracting 2 g of soil in Bray II solution (Bray & Kurtz, 1945) which was filtered through Whatman No.2 filter paper. The filtrate was analysed colorimetrically using the Malachite Green method (Motomizu *et al*., 1983). Exchangeable cations were displaced from 10 g of sample with 25 mL of 0.2 *M* ammonium acetate. The samples were filtered through Whatman No. 2 filter paper and made to 200 *ml* before concentrations of Ca, K, Mg, and Na were determined using ICP-AES analysis. Fe was extracted with 0.1 *M* HCl and the extract analysed also using ICP-AES.

### 3.2.2. Statistical analysis

For the soil nutrient analysis, the data were normalised by the use of log transformation. Basic statistics (means and standard errors) and one-way ANOVA were performed in STATISTICA v.10 software to test for differences in nutrient concentrations between sites. All the variables were approximately normally distributed and bivariate relationships showed no significant departure from normality after transformation. The analysis of variance (ANOVA) was performed to test the null hypothesis of equality of means amongst the nutrient characteristics of *P. serratum*. Data were analysed using a nested ANOVA to investigate if there were significant differences between regions in different localities where sites were nested within the regions. In addition, One-way ANOVA was used to compare *Prionium* and *non-Prionium* sites at each region. Tukey's pair-wise multiple comparison tests were used to separate significantly different means at $P < 0.05$.

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## 3.3. Results

### 3.3.1. Soil nutrients in *Prionium* sites

The nested ANOVA results of all soil nutrient parameters measured for all the 17 sites and four regions, representing *P. serratum* natural habitats were summarized showing the Mean±SE of the soil characteristics measured (Appendix 3.1 and 3.2). The following eight soil parameters that are presented in this chapter (pH, phosphorus (P Bray II), total P, nitrate ($NO_3^-$), ammonium ($NH_4^+$), potassium (K), carbon (C) and T-value) significant differences ($p<0.05$) between sites in the different regions (Figure 3.2 and 3.3).

*Prionium serratum* occurs in acidic soil (pH 3.27-5.43): Citrusdal, Tsitsikama, Napier and Mpenjati Nature Reserve has the highest pH (4.50; 4.50; 5.43 and 4.60 respectively), while

the Hermanus, Prince Alfred Pass and Grahamstown had the lowest (3.27, 3.50 and 3.63 respectively; Figure 3.2 A). Nitrate levels were highest in Elim (51.53 mg $kg^{-1}$) and lowest in Mkambati Nature Reserve (1.33 mg $kg^{-1}$); Baardskerderbos (1.65 mg $kg^{-1}$); Hermanus (1.76 mg $kg^{-1}$) and Bainskloof (2.34 mg $kg^{-1}$). Ammonium levels were highest at Umtamvuna (35.99 mg $kg^{-1}$) and lowest at Bainskloof (8.75 mg $kg^{-1}$). The highest total P was in Elim (474.35 mg $kg^{-1}$) and lowest in Bainskloof, Hermanus (11.88; 15.06 mg $kg^{-1}$; Figure 3.2A) and Groot Winterhoek (17.38 mg $kg^{-1}$). The lowest K value was recorded from Citrusdal (13.67 mg $kg^{-1}$), while the highest levels were recorded from Napier (234.67 mg $kg^{-1}$). C in soil was highest in Elim and Umtamvuna (3.80 and 4.65% respectively) whereas Bainskloof, Prince Alfred Pass and Groot Winterhoek (0.27, 0.43 and 2.13% respectively) had lowest values. T-value was highest in Baarderskerbos (17.17 cmol $kg^{-1}$) and lowest in Citrusdal (1.07 cmol $kg^{-1}$) and Bainskloof (0.80 cmol $kg^{-1}$).

Comparison of the soil parameters between regions showed significant ($P < 0.05$) variation in the levels of pH, $NO_3^-$, $NH_4^+$, Total P, P Bray II, K, T-value and C (Figure 3.3). The Agulhas plain (AP) region was separated from the rest because it recorded the highest value on pH, $NO_3^-$, total P, P Bray II, K, T-value and C. However, areas in South West, South East and North West showed nearly similar soil levels of pH, $NO_3^-$, $NH_4^+$, K and T-value (Figure 3.3) whereas levels of Bray II and C were similar in the NW and KZN regions. The levels of $NO3^-$, $NH4^+$, T-value, K and total P had similar nutritional values within NW, SE and SW regions and had lowest values compared to KZN and AP regions.

### 3.3.2. Soil nutrients between *Prionium* and non-*Prionium* sites

In addition to the soil nutrient data set from 17 *P. serratum* sites, samples from four sites with similar hydrology but lacking *P. serratum* were sampled in NW (Cederberg vlei); SW (Worcester area); AP (Bredasdorp); and KZN (Oribi George). The Mean±SE of the soil characteristics measured for the various sites grouped into regions are shown in Table 3.1. For all parameters measured, Tukey tests was run in order to determine whether *P. serratum* sites have different nutritional values compared to a site where it does not occur. The following eight soil parameters pH, phosphorus (P Bray II), total P, nitrate ($NO_3^-$), ammonium ($NH_4^+$), potassium (K), carbon (C) and T-value) were significantly different ($p<0.05$) between both the *Prionium* and non-*Prionium* sites (Table 3.1).

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*Prionium serratum* sites have similar pH values compared to non-*Prionium* sites ranging from 3.70 to 4.50. Nitrate was high in Cederberg vlei (NW; 5.97 mg $kg^{-1}$), Bredasdorp (AP; 12.9 mg $kg^{-1}$) and Oribi George (SE; 8.93 mg $kg^{-1}$) and similar to some of the *Prionium* sites. Bainskloof had the lowest $NH4^+$ (8.73 mg $kg^{-1}$) value compared to all the *Prionium* and non-*Prionium* sites in all the regions. Total P (215.1 to 474.3 mg $kg^-$1) and K (101 to 243.7 mg $kg^-$1) values were highest between the remaining sites within AP region except for Bredasdorp which was lowest with 35.31 mg $kg^{-1}$. There was no difference in the C % and T-value (cmol kg $^-$1) between *Prionium* and non-*Prionium* sites.

### 3.2.3. Discriminant function analysis of soil nutrients for *Prionium* and non-*Prionium*

When the soils data were analysed using discriminant function analysis (DFA) yields twenty canonical variates (CVs), of which 90 % of the total variance was explained by the first four variates. These CVs and their corresponding eigenvalues for the different soil characteristics are shown in Table 3.2. The first canonical value (CV 1) accounted for 52.44 % of the total variance, and within this CV 1, the T-value ratio had the highest contribution, followed by the Mg concentration (Table 3.2). CV 2 which explained 27.19 % of the total variance was largely influenced by the calcium concentration (Ca). In the third principal component (CV 3), the variables that had the highest contribution in decreasing order were the exchangable sodium concentration, the Zn concentration and the percentage potassium (% K). Finally, the exchangeable sodium (Exch Na) and T-value concentration was the soil characteristic contributing the most to variation in the fourth principal component (Table 3.2). DFA scatterplot results indicated that *Prionium* and non-*Prionium* sites are comparable (Figure 3.4).

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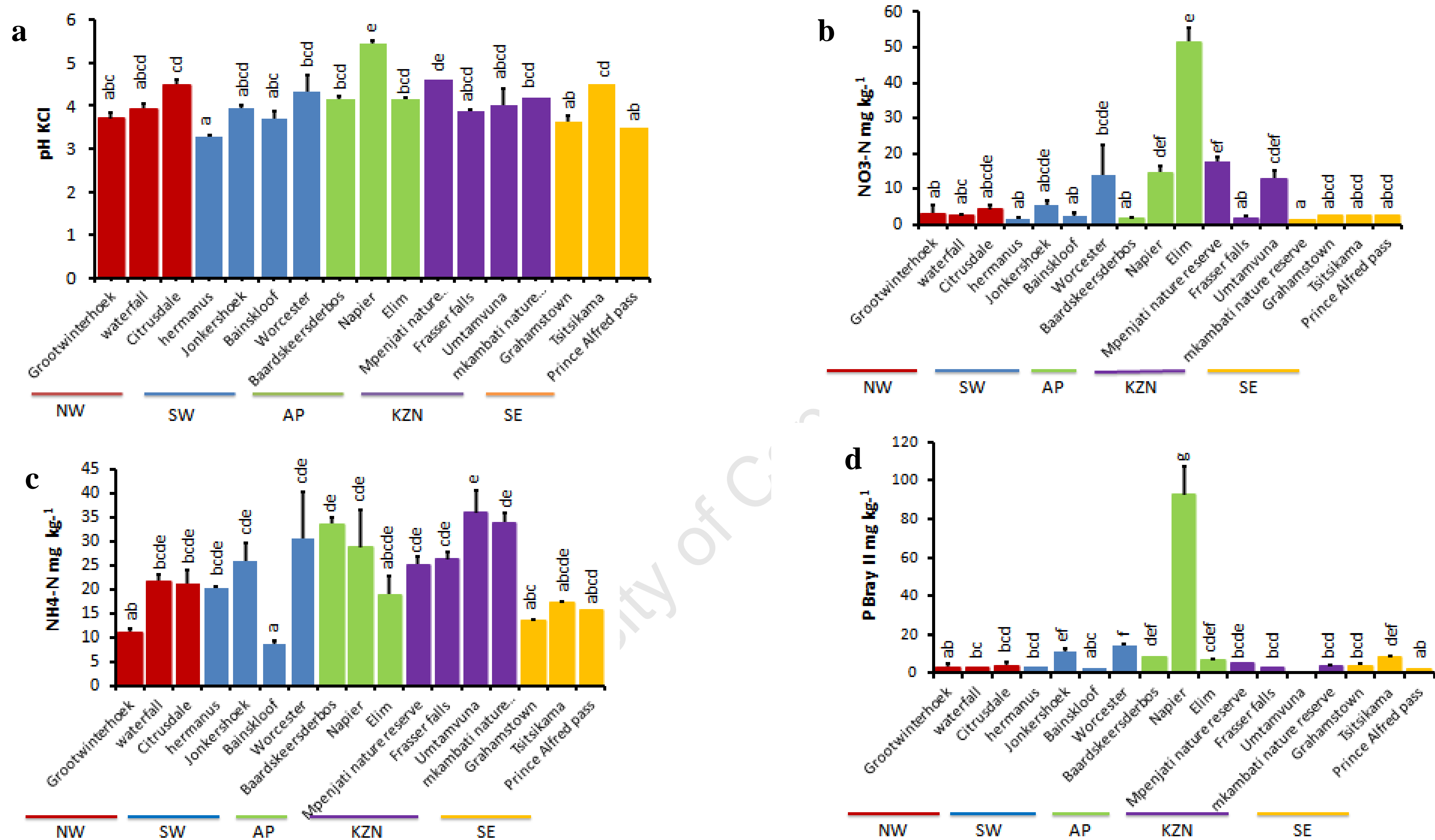


**Figure 3.2** (A) Mean±SE and nested ANOVA results for the nutrient concentrations of the different sites. Only the soil characteristics that were significantly different are shown. Different letters above the values indicate significant differences at p<0.05.

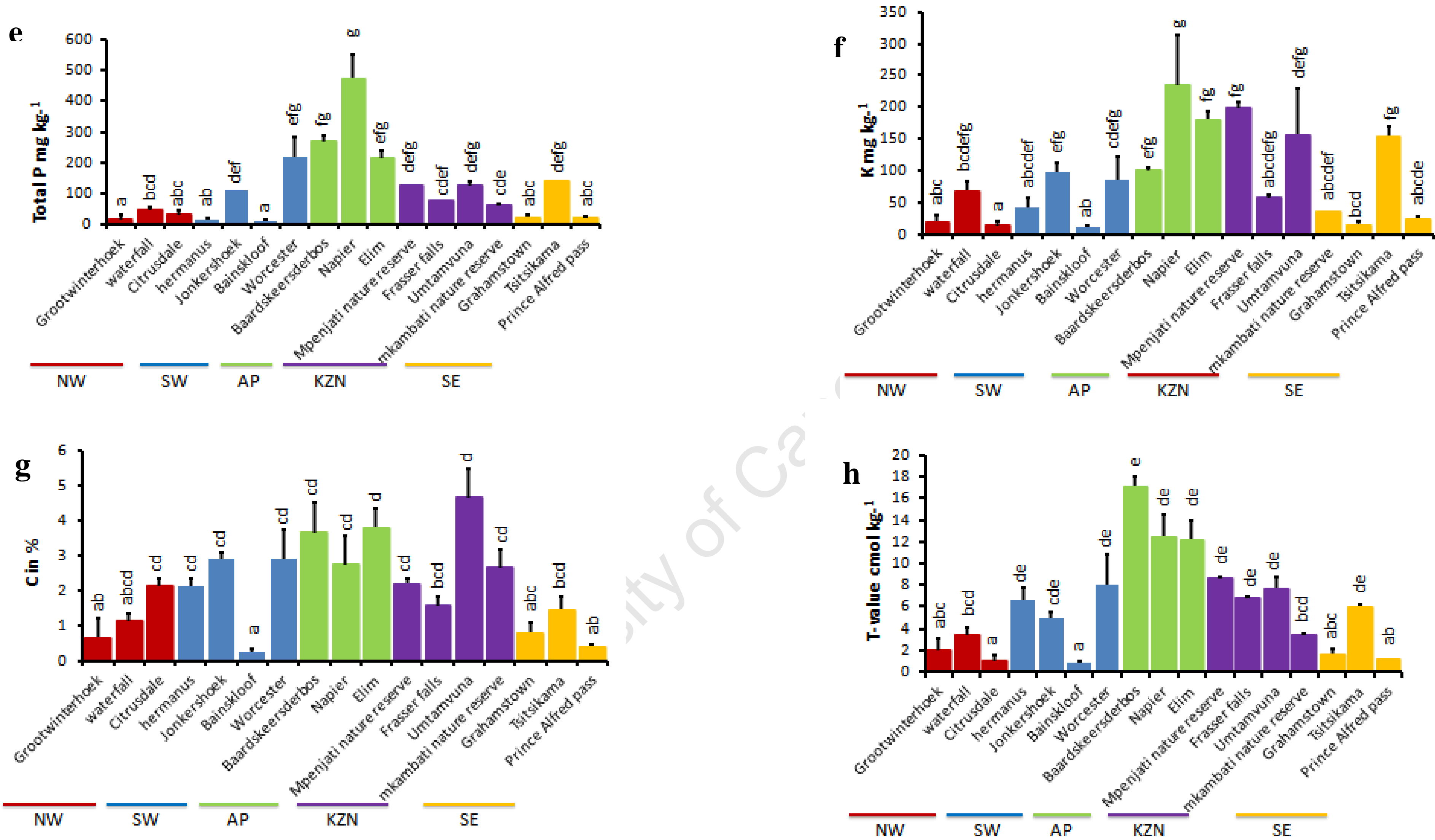


**Figure 3.2** (B) Mean±SE and nested ANOVA results for the nutrient concentrations of the different sites. Only the soil characteristics that were significantly different are shown. Different letters above the values indicate significant differences at $p<0.05$.

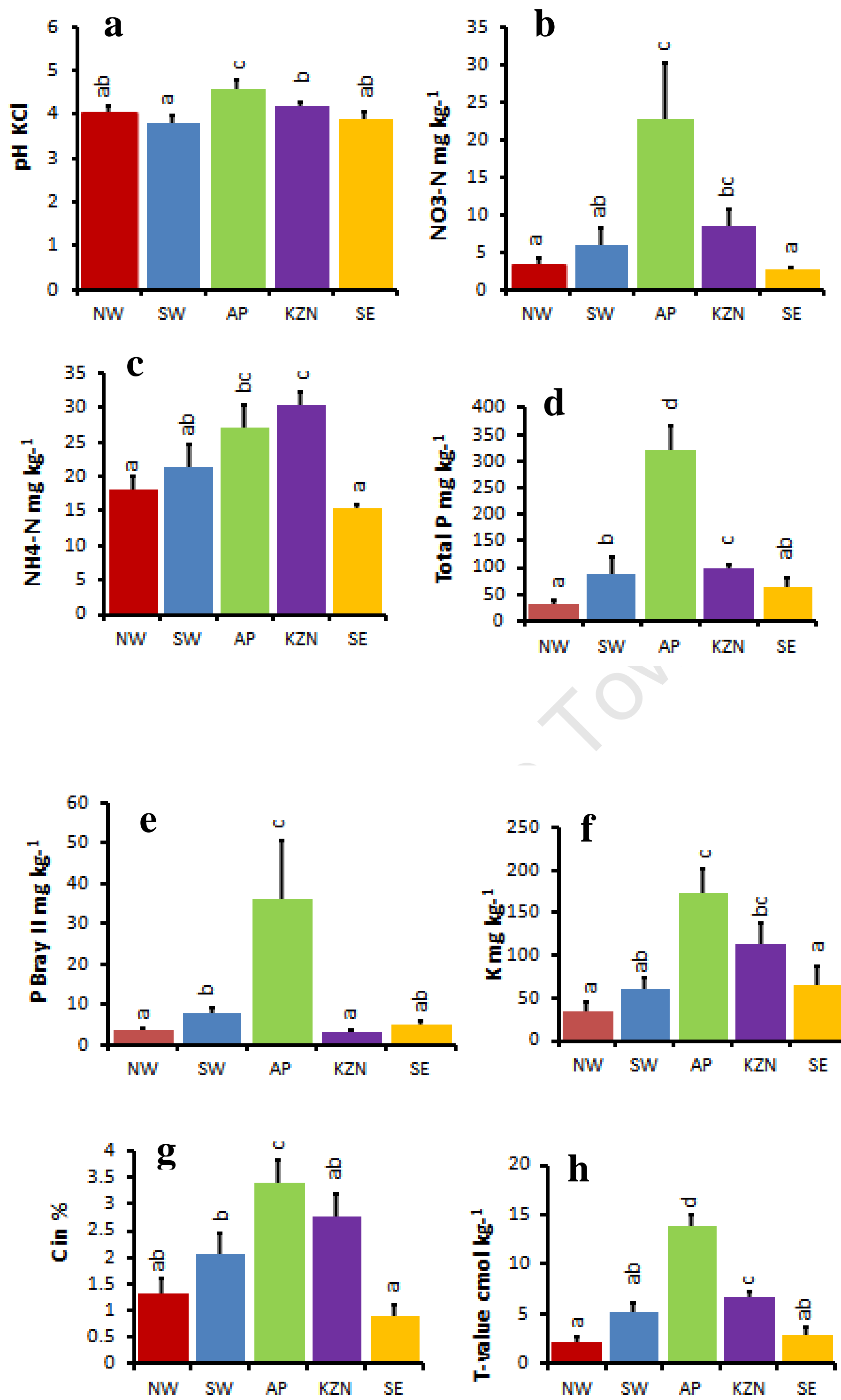


**Figure 3.3** Mean±SE and nested ANOVA results for the nutrient concentrations of the different regions. Only the soil characteristics that were significantly different are shown. Different letters above the values indicate significant differences at $p<0.05$.

**Table 3.1** Mean±SE of different nutrients in soil from different sites. Different letters within a column and a location show that the means differ significantly at P <0.05). # = non-*Prionium* site, - = not measured and NS = not significant.

| | **pH** (KCl) | **NO3-N** (mg/kg$^{-1}$) | **NH4-N** (mg/kg$^{-1}$) | **P Bray II** (mg/kg$^{-1}$) | **Total P** (mg/kg$^{-1}$) | **K** (mg/kg$^{-1}$) | **C** (%) | **T-value** (cmol kg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **NW (North West)** | N=3 | N=3 | N=3 | N=3 | N=3 | N=3 | N=3 | N=3 |
| **Groot Winterhoek** | 3.70±0.1a | 3.26±2.32 | 11.2±0.81a | 3.33±1.85 | 17.38±10.4 | 20.66±8.68 ab | 0.68±0.54a | 2.05±1.08 |
| **Waterfall** | 3.93±0.1ab | 2.47±0.06 | 21.8±1.38b | 3.00±0.00 | 46.38±6.97 | 68.33±14.10b | 1.14±0.1ab | 3.40±0.71 |
| **Citrusdal** | 4.50±0.17b | 4.22±1.38 | 21.1±0.36b | 3.33±0.33 | 30.8±13.26 | 13.66±6.67a | 2.14±0.2ab | 1.07±0.55 |
| #Cederberg vlei | 4.23±0.15ab | 5.97±5.01 | 20.4±2.10b | - | 22.92±1.13 | 16.67±1.67ab | 0.51±0.11a | 1.39±0.12 |
| **F-value** | 7.07* | 0.21$^{NS}$ | 8.63** | 2.70$^{NS}$ | 1.99$^{NS}$ | 5.41* | 3.43$^{***}$ | 1.90$^{NS}$ |
| **SW (South West)** | | | | | | | | |
| **Bainskloof** | 3.70±0.17 | 2.33±1.0ab | 8.73±0.55a | 2.66±0.33a | 11.88±2.54a | 12.33±0.88a | 0.27±0.05a | 0.79±0.15b |
| **Hermanus** | 3.26±0.03 | 1.76±1.70a | 20.4±0.21b | 3.33±0.33a | 15.0±3.34ab | 43±14.0abc | 2.14±0.21b | 6.67±1.14a |
| **Jonkershoek** | 3.93±0.09 | 5.44±1.22b | 25.9±3.83b | 11.0±1.53b | 107.9±3.3cd | 98.66±12.9c | 2.90±0.19b | 5.00±0.43a |
| **Worcester** | 4.33±0.38 | 14.1±8.24a | 30.6±9.97b | 14.0±1.00b | 219.7±64.1d | 85.33±35.3bc | 2.91±0.82b | 8.09±2.71a |
| #Worcester area | 3.93±0.32 | 1.31±0.32a | 28.4±3.25b | - | 38.2±8.24bc | 22.66±6.06ab | 0.83±0.30a | 2.2±0.56bc |
| **F-value** | 2.65$^{NS}$ | 3.20$^{NS}$ | 5.56* | 116.73*** | 25.84*** | 7.75** | 40.94*** | 15.87*** |

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**Table 3.1 Cont.....**

| | **pH** (KCl) | **NO3-N** (mg/kg$^{-1}$) | **NH4-N** (mg/kg$^{-1}$) | **P Bray II** (mg/kg$^{-1}$) | **Total P** (mg/kg$^{-1}$) | **K** (mg/kg$^{-1}$) | **C** (%) | **T-value** (cmol kg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **AP (Agulhas Plain)** | N=3 | N=3 | N=3 | N=3 | N=3 | N=3 | N=3 | N=3 |
| **Baardskerderbos** | 4.16±0.07a | 1.65±0.34c | 33.6±1.30 | 8.67±0.33b | 270.5±19.7b | 101.00±4.5abc | 3.66±0.93a | 17.2±0.81 |
| **Napier** | 5.43±0.09b | 14.7±1.7ab | 28.8±7.68 | 92.6±14.4c | 474.3±77.0d | 243.7±78.21c | 2.75±0.79a | 12.49±1.9 |
| **Elim** | 4.16±0.03a | 51.5±4.20b | 18.9±3.68 | 6.67±0.89b | 215.1±25ab | 181.33±12.4b | 3.80±0.53a | 12.21±1.7 |
| #Bredasdorp | 5.60±0.23b | 12.9±7.10a | 19.8±2.88 | - | 35.31±4.22c | 50.33±10.90a | 0.83±0.21b | 10.25±3.8 |
| **F-value** | 36.72*** | 22.65*** | 2.56 $^{NS}$ | 316.62*** | 84.15*** | 12.43** | 23.8*** | 1.26 $^{NS}$ |
| **SE (South East)** | | | | | | | | |
| **Grahams town** | 3.63±0.13a | 2.70±0.13a | 13.5±0.23a | 4.00±0.57b | 23.79±5.92a | 16.00±3.60a | 0.8±0.26ab | 1.69±0.5ab |
| **Prince Alfred pass** | 3.50±0.00a | 2.78±0.04a | 15.6±0.1ab | 2.00±0.00a | 20.67±3.05a | 25.00±2.31ab | 0.43±0.04a | 1.14±0.04a |
| **Tsitsikama** | 4.50±0.00c | 2.76±0.06a | 17.3±0.18b | 8.33±0.33c | 142.3±2.76b | 154.00±16.4c | 1.45±0.35b | 6.01±0.14c |
| #Oribi George | 4.00±0.00b | 8.93±3.20b | 24.0±2.46c | - | 101.4±11.9b | 39.33±7.42b | 1.52±0.48c | 2.58±1.82b |
| **F-value** | 45.06*** | 7.65** | 22.34*** | 140.80*** | 31.30*** | 33.09*** | 16.46*** | 17.86*** |

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**Table 3.2** Eigenvalues for the first four canonical variates from the DFA of the soil nutrient data set. Eigenvalues that are in bold print are those corresponding to the soil parameters that contribute the most to the variance in the respective discriminant function analysis (DFA).

| **Variables** | **CV 1** | **CV 2** | **CV 3** | **CV 4** |
|---|---|---|---|---|
| pH | -1.04 | 1.23 | -1.45 | 0.03 |
| P BrayII | -1.43 | 0.73 | 0.85 | 0.74 |
| K | -1.42 | 0.69 | 0.09 | -0.08 |
| Exch  Na | 0.16 | -1.03 | **4.87** | **2.70** |
| K | -0.14 | -0.87 | -0.68 | 0.44 |
| Ca | -1.05 | **5.08** | **1.32** | 0.86 |
| Mg | **-3.02** | **-3.45** | **-1.43** | -0.87 |
| Cu | 0.16 | 0.47 | **-1.71** | **-1.19** |
| Zn | 0.75 | 0.93 | **2.54** | -0.25 |
| Mn | 0.33 | -0.30 | **-1.57** | -0.83 |
| B | 1.39 | 0.64 | 0.11 | 0.05 |
| Fe | -0.35 | -0.22 | **-1.90** | **-1.15** |
| C in % | -0.47 | 0.36 | -0.29 | -0.57 |
| NO3-N | 0.40 | 0.42 | -0.04 | -0.21 |
| NH4-N | 0.33 | -0.46 | **1.33** | 0.80 |
| Total P | -1.63 | 0.71 | 0.99 | **1.93** |
| % Na | -1.09 | **2.94** | **-2.11** | 0.07 |
| % K | 0.35 | -0.76 | 0.78 | -0.03 |
| % Ca | 1.89 | **-3.07** | 0.14 | -0.78 |
| %Mg | 0.44 | **1.72** | 0.27 | 0.62 |
| T-Value | **5.93** | **-1.89** | **-3.36** | **-2.31** |
| **Eigenvalue** | **550.94** | **285.72** | **75.60** | **36.49** |
| **Cumulative proportion** | **52.44 %** | **27.19 %** | **7.20 %** | **3.47 %** |

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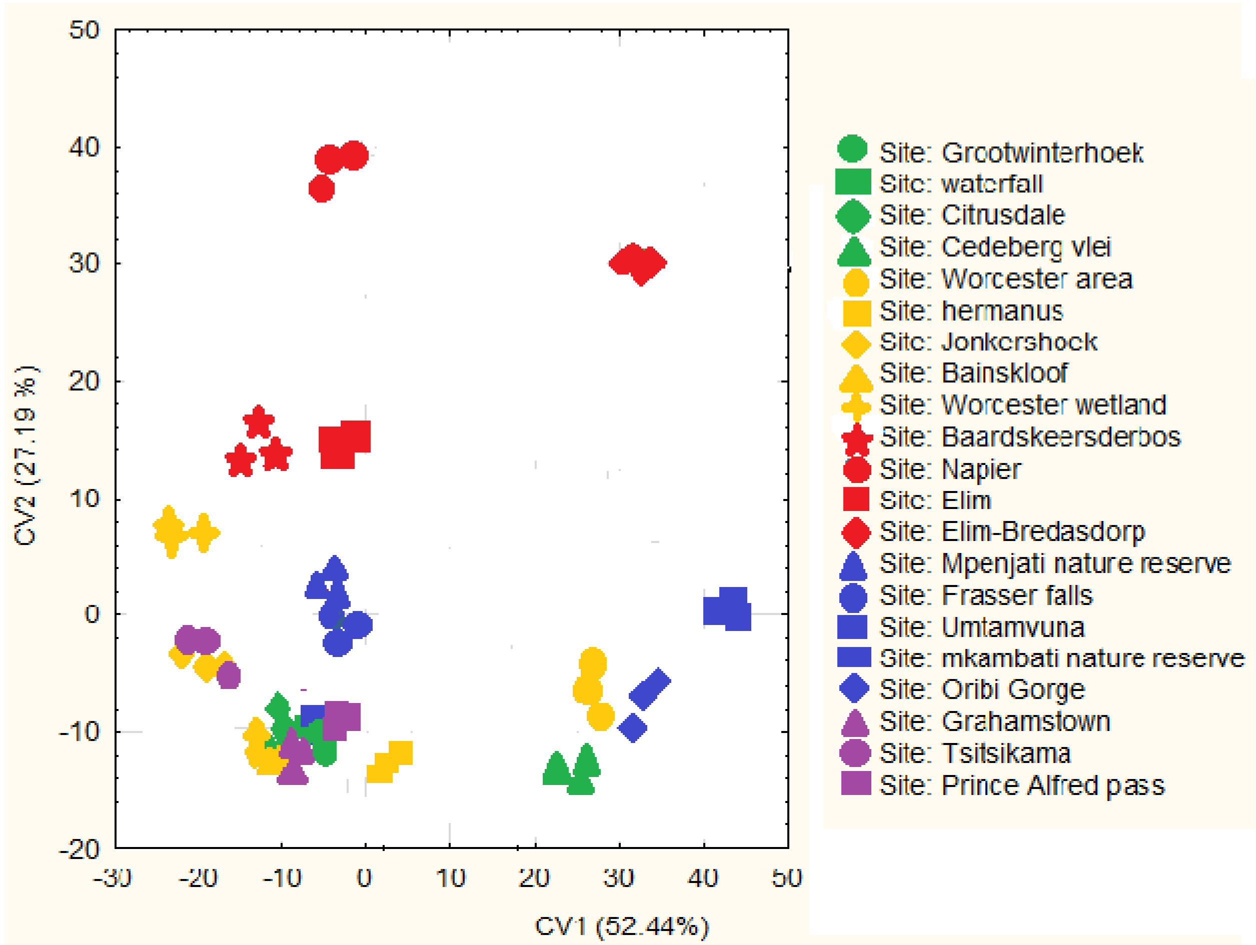


**Figure 3.4** Discriminant function analyses of the first two canonical variates of *Prionium* and non-*Prionium* sites in the phytogeographic areas.

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### 3.4. Discussion

This study was done to test whether *Prionium* occupy soils with similar nutritional levels and if there are nutritional levels difference between *Prionium* and non-*Prionium* sites. The results showed that *P. serratum* populations from different phytogeographic areas occur on the same soil types (sandy soil) but with different proportions of various nutrients analysed. Its populations occurred in habitats that had significantly different ($P < 0.05$) nutrients levels of atleast one of the eight nutrient parameters measured indicating that it tolerates a wide nutritional range. The CFR soil is infertile, with low nutrient levels (nitrogen, potassium and phosphorous). Within *Prionium* sites Elim (AP), Napier (AP), Worcester (SW) and Umtamvuna (KZN) have the highest $NO_3^-$ ranging from 5.44 to 51.4 compared to the other sites. These four sites are within agricultural areas and the increased values are probably due to use of inorganic fertilizers in the area. *Prionium* sites had different total P values across its entire population from 11.8 to 474.3 mg $kg^{-1}$ and such range is similar to the range in the CFR with a high P values within the entire region ranging from 11.9 – 474.3 mg $kg^{-1}$.

*Prionium* sites had different pH levels ranging from 3.26 to 5-60. This implies that it can survive on different pH levels. CFR has has a range of soil types (e.g. Regosol, Podzol and Arenosol), which are typically acid to neutral ranging from pH 4 to 7 and nutrient-poor (Richards *et al*., 1997a). Overall, these results indicate that *P. serratum* can survive acidic soils with different nutritional characteristics. Therefore, the hypothesis that *P. serratum* occupy soils with similar nutritional values within the phytogeographic regions was not supported. However, *P. serratum* sites have nutritional levels comparable to that of the non-*Prionium* sites for all the measured parameters. This implies that the absence of *P. serratum* in the non-*Prionium* sites is not associated with nutritional levels of the soil, but perhaps other ecological parameters (e.g. hydrological regime) may dictate occurrence of *Prionium.* A species may be absent at a particular habitat due to chance or failure to disperse to an otherwise suitable site. For other species, the absence on a particular habitat is due to the past climate and biodiversity dynarnics, spatial locations of refugia under extreme climate conditions, level of extinction (both population level and species level).

In contrast to *P. serratum* results, Proteaceae species were found to be sensitive to P levels in the soil. For example, Shane *et al*. (2008) Showed that *Protea compacta* is restricted to shallow colluvial sands with low P soils and does not occur with *P. obtusifolia* and *L.*

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*meridianum* in adjacent limestone soils with 3.5 times higher level of P. John *et al*. (2007) reported that sites with 2.7, 10.3, and 11.5 times higher of K, Ca and Mg respectively, showed a dominant influence on spatial distributions of 36-51% of tropical trees. Furthermore, soil $NH_4^+$ concentration and $NH_4^+$: $NO_3^-$ ratio of 3.5 and 3 times higher respectively, prevented growth of rare species in some areas compared with common species (Klein *et al*. 2008). The authors further deduced that the rare species had a significantly narrower ecological range than the common species in soil biogeochemical parameters. Therefore, *P. serratum* can be considered to have a wider ecological range in soil biochemical factors because it was able to grow in soils with a wide range of nutrient levels including pH of 3.3 to 5.6, and $NH_4^+$, $NH_4^+$: $NO_3^-$ ratio, Total P, Bray II P and K recording 3.8, 72, 40, 46 and 20 times higher respectively, than the lower sites (Table 3.1).

## 3.5. Conclusion

In conclusion, the objective of this chapter was to investigate whether *P. serratum* is ecologically impoverished by occurring in soil with narrow nutrient regime. The results showed that *P. serratum* occurs on acidic soils with a wide variation of nutritional values for the measured parameters and in both winter (CFR) and summer (MPR) rainfall areas. Both *Prionium* sites and non-*Prionium* sites had soils with similar nutritional levels. I therefore conclude that *P. serratum* is not ecologically impoverished either by the soil nutrients and climatic condition. Further studies, with comprehensive sampling, and incorporating a phylogenetic framework which include sister taxa *Thurnia* is required in order to test whether the sister taxa also occupy soils with similar nutritional levels.

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# CHAPTER 4
# FLORAL DEVELOPMENT IN*PRIONIUM* AND ITS SYSTEMATIC IMPLICATIONS.

## 4.1. Introduction

The phylogenetic relationship of Juncaceae, Cyperaceae and *Prionium* (former Juncaceae) based on molecular data (*rbc*L sequences; Plunkett *et al*., 1995), morphology (Simpson, 1995), or a combination of both (Munro & Linder, 1998) confirm the basal position and close relationship of *Prionium* to Cyperaceae plus the remaining Juncaceae. This genus has a typical juncaceous flower, which allowed inferring that Juncaceae are more similar to the common ancestor than the Cyperaceae (Simpson, 1995). It was suggested to have affinities to family Thurniaceae (Chase *et al*., 2000; Muasya *et al*., 2000), and those affinities were strongly supported by Bremer (2002). Studies using *rbcL* and *atp*B sequence analysis confirmed the position of *Prionium* within Thurniaceae, being sister to *Thurnia*, and Thurniaceae being sister to a clade of Cyperaceae and Juncaceae, jointly forming the 'Cyperid clade' (Bremer, 2002, see Figure 1.1).

### 4.1.1. The 'Cyperid clade'

Dahlgren *et al*. (1985) and Kubitzki (1998) recognized the order Cyperales to group together the three families Cyperaceae, Juncaceae and Thurniaceae, all sharing the presence of pollen grains in tetrads. These families also share the presence of an ovarian obturator, and idioblasts containing phenolic compounds in the floral part (Dahlgren *et al*., 1985). The presence of small flowers with tetrasporangiate anthers, the endothecium with spiral thickenings, tricarpellar gynoecium, tripartite style, and anatropous ovules are other traits shared by Juncaceae, Cyperaceae and Thurniaceae. More recent and robust phylogenetic hypotheses support these three families to form a clade, the so-called Cyperid clade (Bremer, 2002; Linder & Rudall, 2005; Chase *et al*., 2006). The Cyperid clade is sister to a graminoid clade made of Poaceae together with other families. As the Cyperid clade has a fairly uniform vegetative morphology, there is need to investigate floral morphology and development to search for synapomorphies of the three families and to reveal evolutionary patterns.

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### 4.1.2. Vegetative and reproductive morphology

#### (a) Thurniaceae

Thurniaceae is comprised of two genera *Thurnia* and *Prionium* with four species (*T. macrocephala* Schnee, *T. sphaerocephala* (Rudge) Hook.f. *T. jenmanii* Hook.f and *Prionium serratum* (L.f.) Drège ex E. Mey*;* Kubitzki, 1998; Murno & Linder, 1997). *Thurnia* species are centred in the Guayana region of northern South America, extending into the Amazon regions growing in marshy and wet habitats on sandy soil, whereas *Prionium* occurs in South Africa (see Chapter 1 section 1.2.2.).

The vegetative morphology of Thurniaceae was fully described by Kubitzki (1998). All members of Thurniaceae are coarse perennial herbs from an upright roostock, except for *Prionium serratum* which is a basally woody perennial plant with a trunk up to 1 m in length (see Chapter 1 section 1.2.2). The leaves are tristichously arranged, simple, parallel-veined, leathery, flat (or canaliculate) or V-shaped, with a sometimes prickly-margined blade. Leaves have a persistent basal meristem and basipetal development. On the leaves, the stomata are confined to intercostal zones and are paracytic, rarely tetracytic (Cutler, 1969). According to Kubitzki (1998) there is considerable variation in the arrangement of flowers into inflorescences in Thurniaceae, within the two genera *Thurnia* and *Prionium*. The inflorescence consists of much branched panicle and branchlets bearing many flowers. They are sometimes comprised of one or more dense racemose to ellipsoid heads subtended by spreading, bluntly triangular or quadrangular peduncles. Flowers are small with short, swollen, and puberulous pedicels grading into the perigone. Some species within this family have six free perianth parts of 'tepals', two whorled (3+3), isomerous, sepaloid or petaloid (the members are thin, narrow, hyaline, persistent and obtuse). Each flower generally has six diplostemonous stamens are filantherous (the filaments are relatively thin and long, adnate to the base of the tepals). Anthers are basifixed, tetrasporangiate, introrse, opening by longitudinal slits. Pollens are grouped in tetrads, and have scabrate and ulcerate surface. The gynoecium is comprised of three carpels united to form a compound and trilocular pistil with three or more erect, anatropous, crassinucellar, ascending ovules. The ovary tapers distally, scarcely produced into a style with three elongated stigmas.

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**(b) Juncaceae**

Juncaceae is a cosmopolitan family, comprising eight genera and 440 species (Balslev 1996; Kirschner *et al*., 2002a; Drábková *et al*., 2003). Genera are widespread in temperate to subtropics, but rare in the tropics (Kirschner *et al*., 2002a, 2002b). The majority of the morphological and species diversity is found in *Juncus* (315 species) and *Luzula* (115 species) (Kirschner *et al*. 2002a, 2002b, 2002c). Most studies have focused on the phylogeny and evolution of these two large genera. The first taxonomic division of Juncaceae into eight subgenera of *Juncus* and three subgenera of *Luzula* was published by Buchenau (1875). Recently, Kirschner *et al*. (2002a) divided the genus *Juncus* into two subgenera and ten sections, and *Luzula* into three subgenera and seven sections. Drábková *et al*. (2003) were the first to reconstruct molecular phylogeny of the Juncaceae using cpDNA (*rbc*L) sequence data to solve a part of the supraspecific phylogeny, but many sites of backbone remain polytomic. Because of the polytomy, the author then sequenced the non-coding cpDNA regions, *trn*L intron, and *trn*L-*trn*F intergenic spacer, which distinguished genus *Luzula* as monophyletic and *Juncus* as non-monophyletic.

Balslev (1996) and Kirschner *et al*. (2002a, 2002b, 2002c) describe the vegetative morphology of family Juncaceae, particularly *Luzula* and *Juncus*. Members of Juncaceae are perennial or annual, grass-like herb species. The perennials are generally rhizomatous with a long and sympodial rhizome. The rhizome and pith of the stem are often rich in starch and the roots are adventitious along the rhizome. The leaves are either basally concentrated and the cauline stem leafless or variously distributed along the cauline stem. According to Balslev (1996) and Kirschner *et al*. (2002a), there is considerable variation in the arrangement of flowers into inflorescences in the Juncaceae, with most of this variation found in the genera *Juncus* and *Luzula*. The inflorescence is paniculate-racemose, in *Juncus* and *Luzula* sometimes with the appearance of a corymb or anthela, which may be variously contracted but not for smaller genera. For example, *Distichia* and *Patosia* are dioecious with flowers originating in the axils of normal leaves near the shoot apex. Sometimes the flowers are densely aggregated into heads and ebracteolate, and in some taxa the flowers are few. Juncaceae have trimerous, actinomorphic and generally bisexual. Each flower has generally three to six stamens, in most species the inner whorl may be absent. Stamens are free from each other, and sometimes have filaments, with introrse or latrorse anthers. The ovary is superior, globular to oblong and has three or one locular depending on species. Most genera

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have numerous bitegmic, anatropous ovules on central placentae, whereas in *Luzula* the unilocular ovary has three basal ovules only. The pistils have a single style with long and mostly twisted papillose stigmatic branches.

**(c) Cyperaceae**

Cyperaceae has cosmopolitan distribution, comprising 109 genera with approximately 5,500 species (Govaerts *et al*., 2007). About 35% of the genera are monotypic, 26% have two to five species, and there are a seven (6%) genera with over 200 species, the largest being *Cyperus* (686 species) and *Carex* (2,000 species; Goetghebeur, 1998). Most species occur in mesic to hydric habitats, though the family is represented in almost all terrestrial environments. Numerous species host mycorrhizal fungi (Muthukumar *et al*., 2004). Phylogenetic studies in this family have progressed in the last two decades, the relationships at family level have been evaluated using morphological studies (Simpson, 1995; Goetghebeur, 1998), plastid DNA sequences (e.g. *rbc*L, Muasya *et al*., 1998), and combined DNA and morphological studies (Muasya *et al*., 2000). Two subfamilies of Cyperaceae, namely Cyperoideae and Mapanioideae, were recognized in this family (Muasya *et al*., 2009). Mapanioideae comprised tribes Hypolytreae and Chrysitricheae while the Cyperoideae comprised 14 tribes.

The vegetative morphology of Cyperaceae was described by Eiten (1976) and Dahlgren *et al*. (1985). Cyperaceae is comprised of annual and perennial, often grass-like herbs, sometimes with woody scandent stem (e.g. *Gahnia* J.R. Forst. & G. Forst.) or tree-like with trunk (e.g. monotypic genus *Microdracoides* Hua). Perennial Cyperaceae species may be tufted and develop short or elongated rhizomes. In most genera, the stem is unbranched with a basal rossete of leaves and bears terminal or lateral inflorescence. In some genera, such as *Carex* and *Fuirena* cauline leaves are common. The reproductive morphology of Cyperaceae was described in detail by Eiten (1976), Dahlgren *et al*. (1985) and Bruhl (1991). The basic inflorescence unit in Cyperaceae is a spikelet, which is described as a racemosely branched structure consisting of an axis (rachilla) of potentially indefinite growth bearing lateral and terminal true flowers, each subtended by a floral scale. Certainly, the branching pattern or ultimate branching orders of the inflorescence are important characters used to divide the family into subfamilies, tribes, and sub-tribes. A Cyperoid flower usually originates in the axil of a subtending bract, called a glume. They are highly reduced in size and complexity



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and are either unisexual or bisexual. The perianth parts (the calyx and corolla or the tepals) is either absent altogether or represented by up to six (though sometimes one) hair-like to stiff, sometimes barbed bristles. For instance, in *Eriophorum*, the bristles are extremely long and up to more than 10 in number. The androecium in most genera is haplostemonous with usually six, three or less basifixed stamens and introrse anthers. Flowers in Cyperaceae have a superior pistil consisting of a unilocular ovary, long single style and one to three stigmatic branches. Carpels are not distinguishable from the earliest developmental stages, and the ovary contains a single, basal, anatropous ovule.

### 4.1.3. Floral ontogeny within the Cyperid clade

The above mentioned chronicles on floral characters raise many questions and hypothesis as to whether Thurniaceae, Juncaceae and Cyperaceae share similar floral developmental patterns. Little is known about the floral developmental pattern within Juncaceae and Thurniaceae. In the Cyperaceae, studies in the last decade (e.g. Vrijdaghs *et al.*, 2005a, 2005b, 2009) have clarified long-standing queries on floral homologies and evolution. Therefore floral ontogeny has become essential, because it answers questions concerning the patterns of evolutionary change and is also useful in supporting or falsifying hypothesis generated by other data sets. In this study, I focused on species within the three Cyperid families. Within these families, floral developmental data may be useful in identifying new morphological characters as well as apomorphies for clades inferred for phylogenetic studies.

### 4.1.4. Objective and hypotheses tested

The objective of this chapter is to investigate the floral developmental pattern within the Cyperid clade. Hypotheses tested are:

(a) Cyperid species share similar floral developmental pattern; and

(b) Evolutionary patterns can be observed in origin of highly reduced Cyperaceae flower from an ancestral Cyperid flower.

## 4.2. Methods and materials

Within the Cyperid clade families, six species were selected for the study (see Table 4.1). The selection was made to include representatives of the three families Juncaceae, Cyperaceae and Thurniaceae. *Prionium serratum* (Thurniaceae) was selected because of its primitive



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characters such as fusion of carpels and lack of style and the basal position on the Cyperid clade. Munro *et al*. (2001) provide detailed information on its morphology; but no floral ontogenetic study has ever been done. In Juncaceae, species were selected from genera *Luzula* and *Juncus*, the two most wide-spread and morphologically diverse genera within this family. However, an ontogenetic point of view is crucial to support such an argument and little is known about floral development within Juncaceae, therefore *Luzula sylvatica*, *Juncus effusus* and *Juncus bufonius* were selected for this study. In Cyperaceae, two species from the Scirpeae clade, *Scirpus sylvaticus* and *Eriophorum latifolium* were selected. *Scirpus sylvaticus* and *Eriophorum latifolium* are found within the most studied-genera which belong to the Cariaceae--Dulichieae--Scrirpeae clade.

Floral materials were fixed in FAA (70% ethanol, acetic acid, 40% formaldehyde, 90:5:5). The floral buds were dissected in 70% ethanol using an OLYMPUS SZ51 microscope. Each sample was washed twice with 70% ethanol for five minutes and then placed in a mixture (1:1) of 70% ethanol and DMM (dimethoxymethane) for another five minutes. After that, the sample was transferred to 100% DMM for 20 minutes, before it was critical point dried using liquid $CO_2$ with a CPD 040 critical point dryer (BALTEC AG, Balzers, Liechtenstein) critical point dryer. The samples were left in the critical point dryer overnight to dry. The dried samples were mounted on aluminium stubs using Leit-C and coated with gold with a SPI-Module TM Sputter Coater (SPI Supplies, West-Chester, PA, USA). Images were obtained with a JEOL JSM-6460 (JEOL Ltd., Tokyo) at the Laboratory of Plant Systematics (K.U. Leuven).

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**Table 4.1** Taxa studied for floral development.

| Species name | Province | Collector # | Voucher number |
|---|---|---|---|
| *Eriophorum latifolium Hoppe* | UGent (Belgium) | Goetghebeur | PG10185 |
| *Luzula sylvatica L.* | Ptk-KULeuven (Belgium) | Vrijdaghs | AV10 |
| *Juncus bufonius L.* | Ugent (Belgium) | Reynders | UGent1900-5514U |
| *Juncus effuses L.* | Ugent (Belgium) | Reynders | HBUG1984-0126 (w) |
| *Prionium serratum* (L.f.) Drège ex E. Mey | Liesbeeck (S.Africa) | Munyai | 16 |
| *Scirpus sylvaticus L* | Ptk-KULeuven (Belgium) | Vrijdaghs | AV02 |

### 4.3. Results

Floral developmental patterns are presented for the three families in the Cyperid clade. Flowers in Juncaceae and Thurniaceae are borne in singly or in clusters of two to three, whereas in Cyperaceae they are aggregated into spikelets.

#### (a) Thurniaceae

The panicle of *P. serratum* at an early stage consists of flowers with an undifferentiated floral apex (Figure 4.1A) and subfloral bracts. A floral primordium develops with three outer and inner perianth parts. The perianth parts are positioned opposite the stamen primordia. Simultaneously, three abaxial and three adaxial stamen primordia become conspicuous and alternate with inner and outer alternating perianth parts surrounding the gynoecium primordium, which is centrally positioned with three apparent congenitally fused carpels (Figure 4.1B). At this stage, two clusters of flower (Figure 4.1C1-2) are at more or less the same developmental stage, with visible outer perianth parts alternating with less developed inner perianth parts. Stamen primordia surround the gynoecium primordium with complete fused carpels, which are not clearly showing peltation at this stage. Meanwhile, the ovary is trilocular, with the locules separated by septa, but septa not yet fused in the centre (Figure 4.1D). Moreover, at this stage, the gynoecium is still open and forms a bag-like structure; with carpels protruding above the anthers (Figure 4.1E). Simultaneously, each stamen primordium develops into a filament and a basifixed, latrorse anthers. The carpels close when they transform into stigmas, which then grow out above the anther (Figure 4.1F). The surface of the gynoecium for *P. serratum* doesn't have a style (Figure 4.1G). Eventually, the gynoecium opening has numerous bitegmic ovules (Figure 4.1 H).

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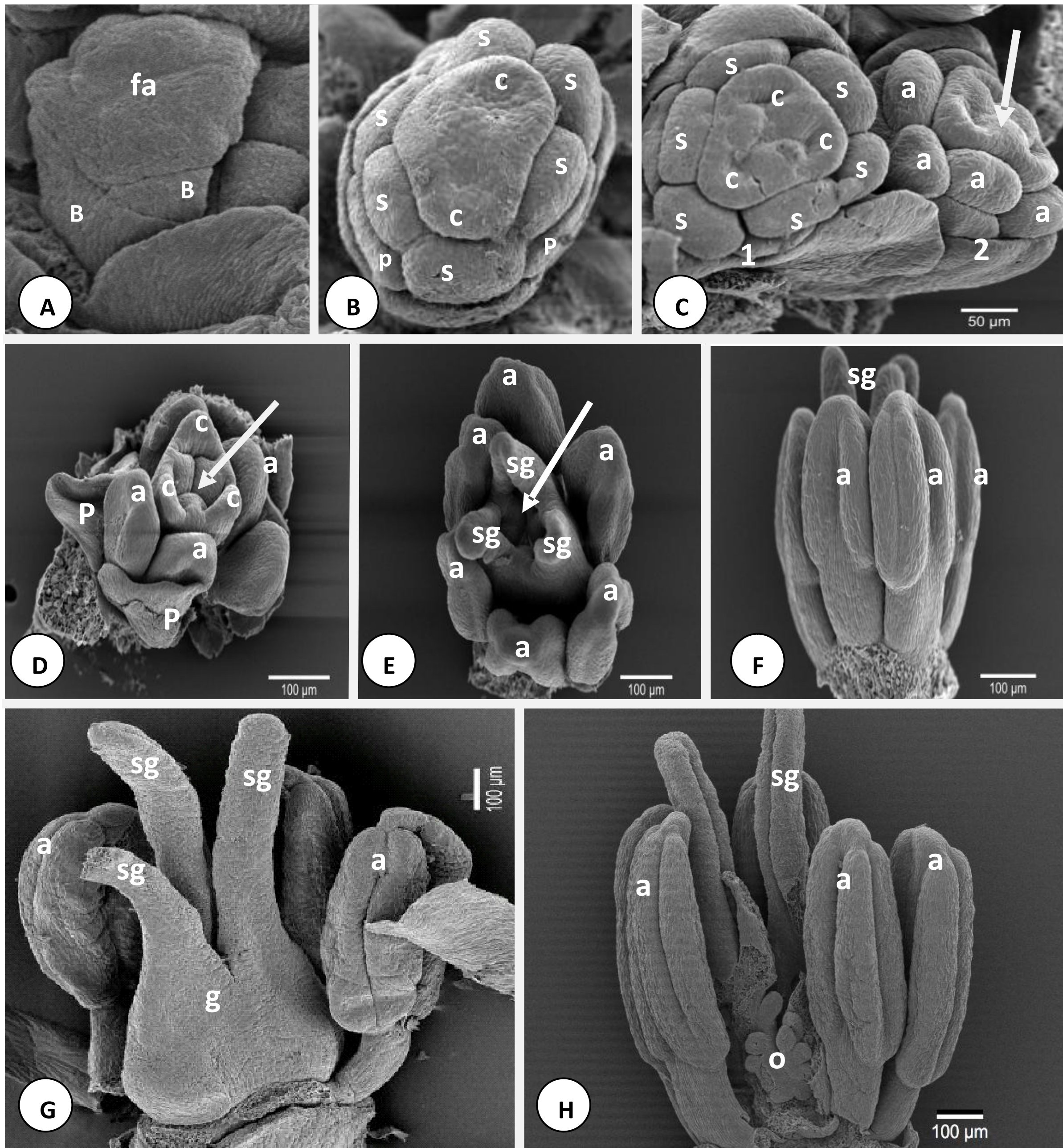


**Figure 4.1** Floral development in Thurniaceae. Abbreviations: a, stamen/anther; B, bract; P, perianth; g, gynoecium; c, carpels; fa, floral apex; ov, ovary, o, ovule; sg, stigma. **(A)** Abaxial view of an early flower primordium **(B)** Apical view of a developing flower primordium. **(C)** Apical and lateral view of two successive developmental stage of B. **(D)** Adaxial view of developing flower with outer stamens positioned opposite the carpels. **(E)** Apical view of a developing flower gynoecium. **(F)** Adaxial view of a flower with stamen developing. **(G)** Adaxial view of gynoecium lacking style. **(H)** Lateral view of an ovary showing numerous bitegmic ovules.

**(b) Juncaceae**

An early stage of *L. sylvaticus* consists of subfloral bract subtending the undifferentiated primordium (Figure 4.2A). Subsequently, a floral primordium (Figure 4.2B) expands laterally, forming six adaxial and abaxial stamen primordia, surrounding an undifferentiated floral apex. The inner and outer perianth parts alternate with the six stamen primordia which encircle the floral apex which differentiates into an annular ovary primordium (Figure 4.2C and D). Meanwhile, the ovary primordium envelops a central ovule primordium (Figure 4.2E). Simultaneously, stamen primordium develops into anthers, the ovary primordium rises, and on top of it two lateral and one abaxial stigma primordium are formed (Figure 4.2F and H). At this stage, anthers are basifixed and introrse and three stigma primordia protrude (Figure 4.2I). Subsequently, a single style and three papillose stigma branches are formed (Figure 4.2J and K). The ovary is unilocular, with ovules attached basally in the ovary (Figure 4.2L), two enveloped by two-layered inner and outer integument forming a micropyle, at the micropylar region an ovule is positioned (Figure 4.2M). Early floral developmental stages in *Juncus effusus* and *Juncus bufonius* are expected to be similar to those observed in *Luzula sylvatica* (Figure 4.2A). A mature flower of *J. effusus* has three visible anthers enveloping the gynoecium, with filaments widened and attached at the base. On top of the ovary, a single style is apparent and stigmas are twisted into spiral-like filaments which have papillae and protrude above anthers (Figure 4.2N, O, and P). A mature flower of *J. bufonius* has bitegmic ovules ascending from the parietal placentation enveloped by two-layered (inner and outer) integuments, and stigma primordia growing out developing papillae (Figure 4.2Q-R).

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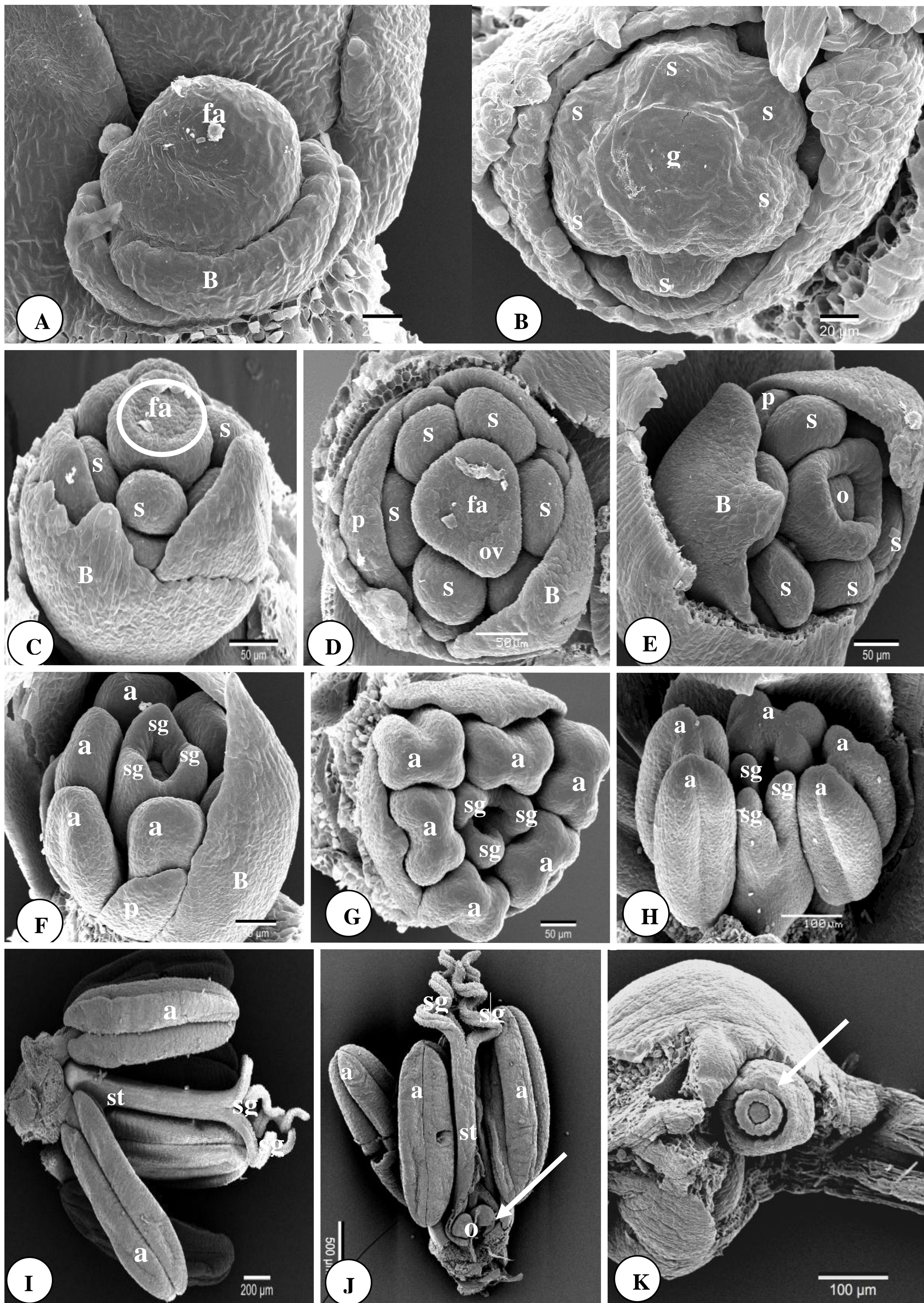


**Figure 4.2** Floral development in Juncaceae. Abbreviations: a, stamen (primordium)/anther; B, bract; P, perianth; g, gynoecium; c, carpels; fa, floral apex; ov, ovary, o, ovule; st, style; sg, stigma. Floral development of *Luzula sylvatica*. (**A-L**). (**A)** Abaxial view of a floral apex surrounded by subfloral bracts**. (B)** Apical view of a developing flower primordium. (**C-D**) Adaxial view of a developing flower. The gynoecium primordium differentiate into an

annular ovary wall. (**E-F**) Lateral view of a developing flower. The ovary wall envelops a single ovule primordium. (**G-I**) Apical view of a developing flower with stamen primordia. **(J)** Lateral view of a developing flower with anthers surround carpels which elongate and transform into stigmas. **(L)** Lateral view of a developing flower the ovary opening with ovules and an ovule with a micropyle bending back**.**

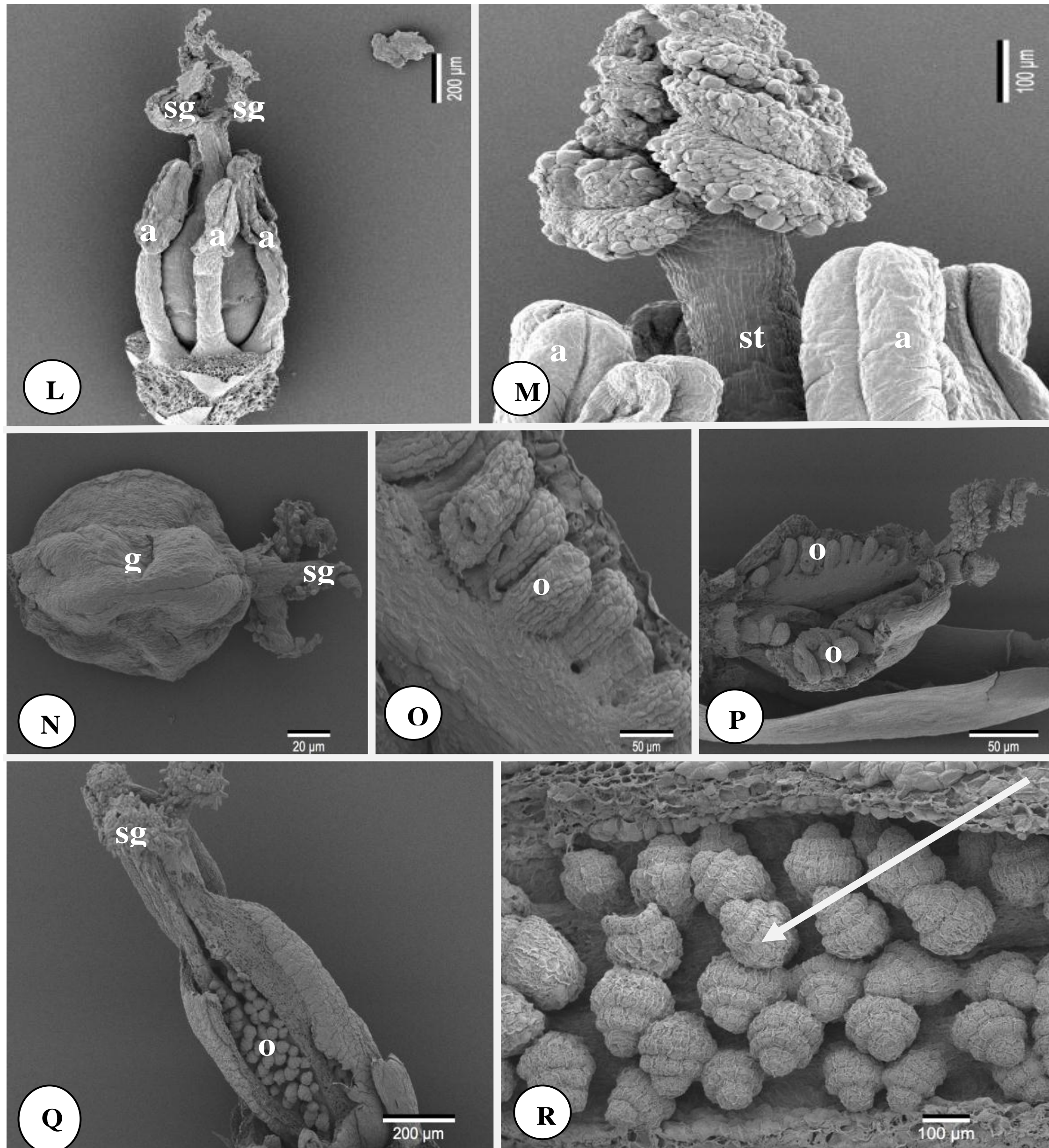


**Figure 4.2** Floral development in *Juncus efflesus* (**L-P**) *and bufonius* (Q-**R**). **(L)** Adaxical view of mature flower. (**N-P**) Lateral view of a complete spiral-like shape of two stigmas with papillae (**Q-R**) Lateral view of an ovary opening with clustered bitegmic ovules.

**(c) Cyperaceae**

The spikelet of *S. sylvaticus L* is indeterminate and consists of many spirally arranged glumes each subtending a bisexual flower (Figure 4.3A and B). Subsequently, a flower primordium develops two lateral stamen primordia (Figure 4.3C). Meanwhile, the subtending glume increases in size and protrudes along the midrib (Figure 4.3D), the two lateral and one abaxial stamen primordium become apparent, the gynoecium is formed as a disc-like structure at the floral apex (Figure 4.3D). At this stage, two tepal whorls (perianth parts) develop, and the gynoecium differentiates into an annular ovary primordium surrounding a central single ovule primordium (Figure 4.3E and F). Subsequently, three perianth parts become visible alternating with stamen primordia. Each stamen primordium develops into filament and basifixed and introrse anther. On top of the rising ovary wall three stigma primordia extend above the anthers (Figure 4.3G and H). Perianth parts develop into long bristles, which become scabrid in appearance at the end of anthesis, when the achene is ripening (Figure 4.3I). The ovary wall rises forming a single style without thickened or distinct style base (Figure 4.3J), meanwhile, the three stigma primordia differentiate into well-developed papillose stigma branches (Figure 4.3J and K). The spikelet of *E. latifolium* has similar gynoecium and androecium ontogeny as *Scirpus*. Flowers of *Eriophorum* are unique in bearing more than 10 perianth parts. On *Eriophorum*, the stage when the stamen and stigmas are differentiated is comparable to stage in Figure 4.3 G. The perianth primordia are arranged in several whorls arising centripetally, the growth of the perianth parts is relatively slow until the stamens are well developed and reaching out above the gynoecium (Figure 4.3L-M). At this stage, the perianth parts reach the base of the anthers with filaments elongating, at adaxial and as well as the abaxial side (Figure 4.9N and O).

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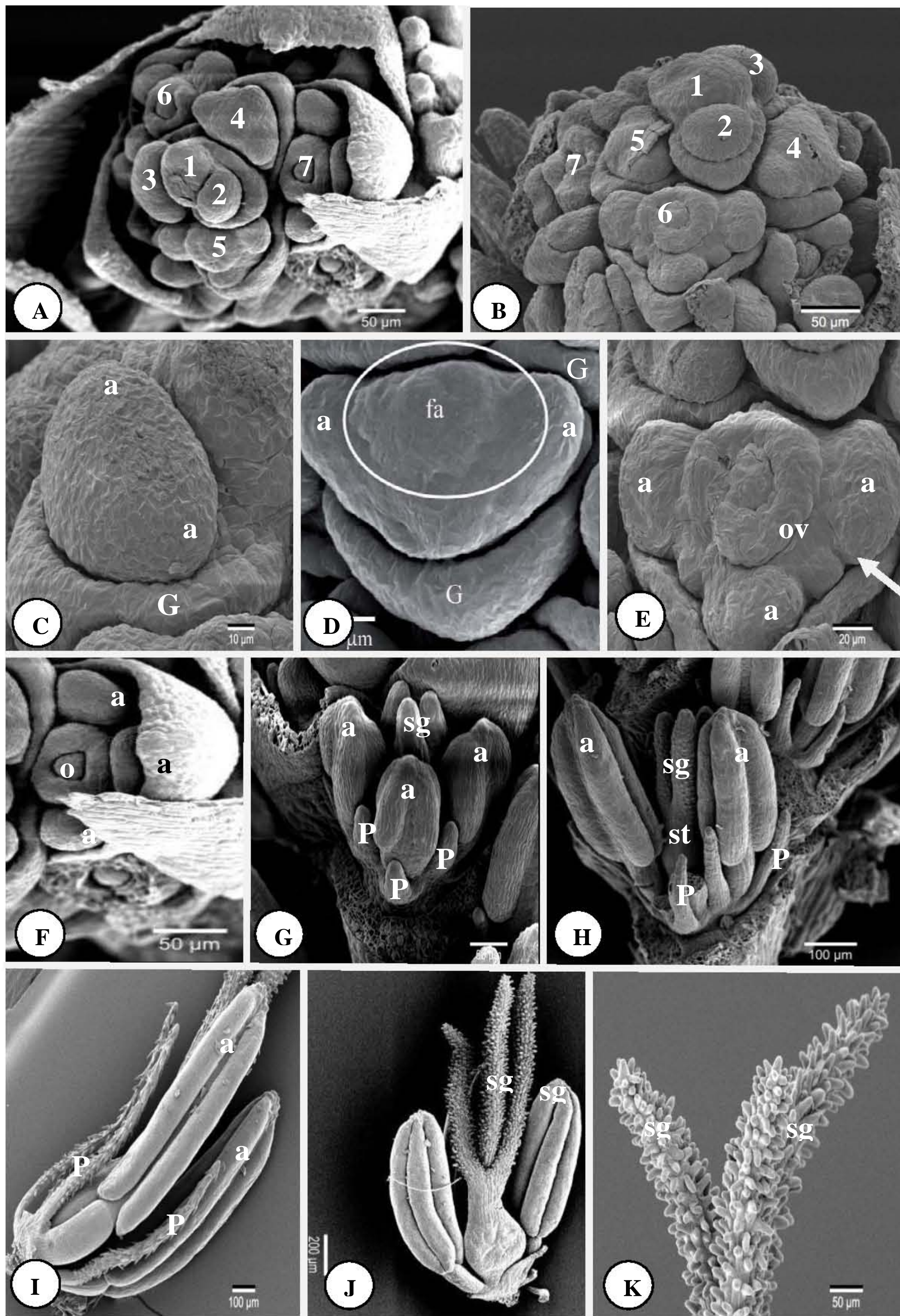


**Figure 4.3A** Floral development in Cyperaceae. Abbreviations: a, stamen (primordium)/anther; G, glume; P, perianth; g, gynoecium; c, carpels; fa, floral apex; ov, ovary, o, ovule; st, style; sg, stigma. Floral development of *Scirpus sylvaticus* (**A-K**). (**A-B)**

Lateral-apical view of a spikelet. (**C-D**) Abaxial view of flower primordium. **(E-F)** Apical view of a developing flower with stamen primordia, the three perianth become visible (*arrow*). (**G-H**) The stamens primordia develop into anthers and grow rapidly above the stigmas. (**I**) Lateral view of a detailed mature bristle. **(J-K)** Lateral view of single style and three detailed stigmas which becomes papillose.

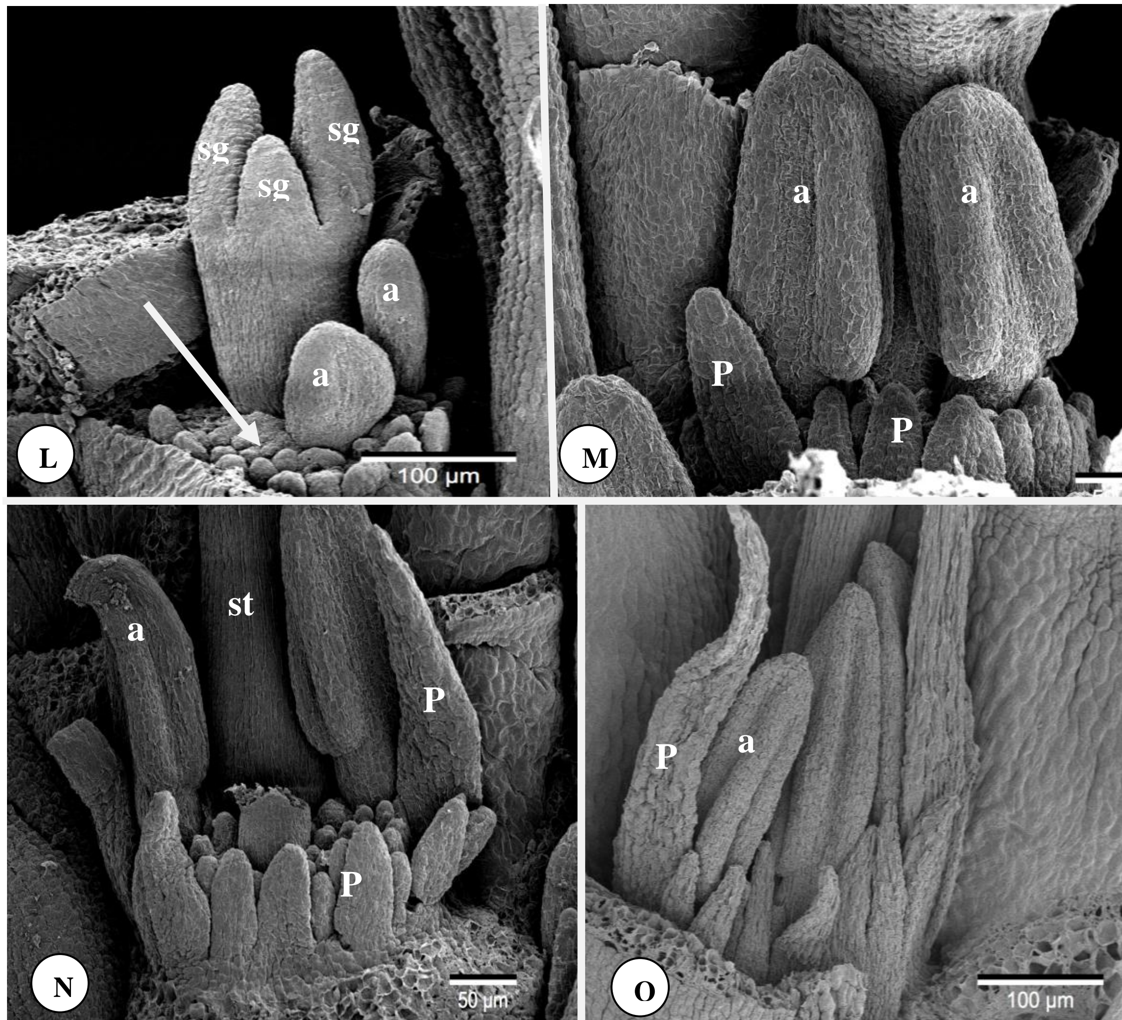


**Figure 4.3B** Floral development in *Eriophorum latifolium*. (**L-M**) Lateral view of a developing flower. (**M-O**) The perianth part opposite the stamens tends to grow faster than other outer ones. (**O**) Lateral view of a developing flower with longest perianth parts reaching the base of anthers at adaxial and abaxial side.

**Table 4.2** Morphological characters used to show floral developmental pattern between Thurniaceae, Juncaceae and Cyperaceae

| **Character** | **Character states** |
|---|---|
| A. Bract | Absent (0) |
| | Present (1) |
| B. Glume | Absent (0) |
| | Present (1) |
| C. Androecium | Latrorse (0) |
| | Introrse (1) |
| D. Ovary shape | Trilocular (0) |
| | Annular (1) |
| E. Ovule numbers | Single (0) |
| | Multiple (1) |
| F. Style | Absent (0) |
| | Present (1) |

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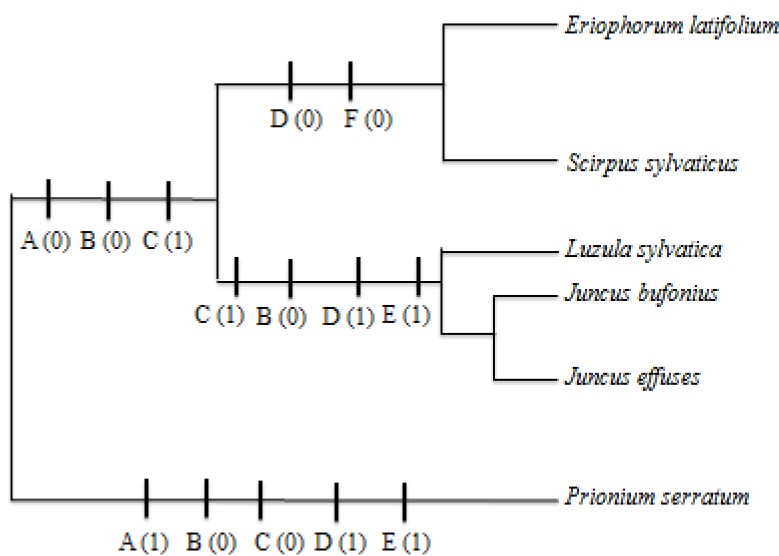


**Figure 4.4** Floral characters of Cyperids optimised onto a hypothetical phylogeny.

### 4.4 Discussion

Our results confirm and substantially extend findings by Plunkett *et al*. (1995), who showed that *Prionium* (formerly placed in Juncaceae) was sister to the group (Cyperid clade) composed of Cyperaceae and the remainder of Juncaceae. In this study, we hypothesise that Cyperid species share similar floral developmental pattern; and that the evolutionary patterns can be observed in origin of highly reduced Cyperaceae flower from an ancestral Cyperid flower. Floral ontogenetic results revealed some variability on the floral developmental patterns, and identify new evolutionary patterns on floral structure such as perianth, androecium, and gynoecium development and therefore the hypotheses were rejected (Table 4.2 and Figure 4.4). There are floral morphological similarities between Juncaceae and Thurniaceae (e.g. flowers borne in singles each subtended by a floral bract aggregated into spikelets, presence of two whorls of tepals alternating with two whorls of stamens, dehiscent fruit), while Floral ontogenetic data show *Prionium* to possess plesiomorphic floral features between the Cyperids (Figure 4.4).

**(a) PERIANTH PARTS**

In monocotyledons there are several descriptions used objectively for the perianth in floral ontogeny studies. For instance the term 'tepals' is sometimes used when all perianth parts cannot be distinguished into sepals and tepals (Vrijdaghs *et al*., 2005a). To avoid any interpretation or suggestion of a particular morphology, Vrijdaghs *et al*. (2005a) used the term perianth part for all perianth members, while Endress (2003) recommended use of the term tepals to encompass both tepals and petals in all basal angiosperms. Balslev (1996) and Kubitzki (1998) used the word 'tepals' for perianth parts on the members of Cyperaceae, Juncaceae and Thurniaceae. In Thurniaceae (Figure 4.1A, B, C, and D) and Juncaceae (Figure 4.2B, C, D and E) the floral primordium develops from the outside to the inside. Six separate perianth parts occur in two whorls of three. The inner and outer whorls of concave perianth parts enclose the floral developmental structures and they are positioned opposite the stamen primordia. The outer perianth parts are often slightly longer, thicker, and more rigid than the inner ones, but Balslev (1996) and Dahlgren *et al*. (1985) indicated that *Juncus effusus* and J. *microcephalus* have soft, flexible perianth parts, whereas J. *arcticus*, J. *ramboi*, and J. *pallescens* have rigid ones. In Cyperaceae, the floral primordium develops from the inside to the outside (Figure 4.3A-E). On some members of Cyperaceae, there is complete absence of perianth parts or modification into bristles (setae) with retrorse prickle hairs or



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scale-like perianth structures referred to by names such as 'perianth parts', 'perianth bristles', 'bristles' or 'scales'. For example, the absence of perianth parts was observed in *Fuirena pubescens* (Vrijdaghs *et al*., 2004).

**(b) ANDROECIUM**

There is variability in the number of stamens in several groups of monocotyledons. Cronquist (1981) reports variation in the number of stamens in petaloid monocots, from one, two, often three to six, except for Orchids. Orchids have one, two or three fertile abaxial stamens (Rudall & Bateman, 2003). The number of stamens is variable, ranging from few to numerous, with stamens often inserted in multiples of three and six. In Thurniaceae and Juncaceae, there is variability of stamens generally from three to six, but in Cyperaceae there are one to three stamens with an exceptional case in *Evandra* where numerous (12-20) stamens are observed (Dahlgren *et al*., 1985). The six stamen primordia in Thurniaceae originate simultaneously (Figure 4.1B and 4.2B), but in the Cyperaceae studied (Figure 4.3E-H and L-O) the two adaxial stamen are formed first followed by a third abaxial stamens.*Mezzetiopsis creaghii* also have stamens reduced to six stamens in one whorl (Ronse De Craene & Smets, 1990).

**(c) GYNOECIUM**

Flowers in the monocotyledons, with the exception of orchids and several smaller families, usually have a superior pistil. The three families studied have a variable gynoecium developmental patterns where the gynoecium primordium originates at the floral apex of the flower but differentiate into various forms. In Thurniaceae, this study confirms observations by Munro & Linder (1998), where the pistil has an elongated trilocular ovary, with each locule containing numerous bitegmic ovules. There is no style in *Prionium*, as has been observed in early diverging lineages of monocots (e.g. Alismatales, Stevens 2001 onwards), whereas other Cyperid clade families and most Poales have well developed styles. This trilocular ovary differentiates into three congenitally fused carpels (Figure 4.1B, C, and D), which remain open till a later stage, and only closes when the the carpels transform into stigma primordia (Figure 4.1H). The ovaries in Cyperaceae are unilocular but in Juncaceae both unilocular and trilocular ovaries occur and *Juncus* quite often are trilocular (Dahlgren *et al*., 1985).

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In the Juncaceae and Cyperaceae studied, the gynoecium differentiates into an annular ovary primordium (Figure 4.2D-E and 4.8 E), of which from the earliest developmental stages, no individual carpels can be distinguished. The ovary wall opens till the ovule is formed. According to Endress (2001), in angiosperms the ovary wall usually closes before the ovules are formed, so that they are not exposed to the open air, but for these two families it closes after the formation of ovules and similar pattern were also observed by Vrijdaghs *et al*. (2006) in other Cyperaceae species.

### 4.5. Conclusion

I conclude that from the phylogenetic analysis and trends in character evolution, it is certain that the Thurniaceae do form a basal group in the Cyperid clade and have some variability on the floral developmental patterns, and evolutionary patterns on floral structure such as perianth, androecium, and gynoecium development. Cyperaceae exhibits more derived floral morphology (e.g. flowers aggregated into spikelets, tepals reduced to perianth bristles or absent, mostly a single whorl of stamens, elongated stigmas, or much higher, indehiscent fruit). Further studies, with broad sampling which include sister taxa *Thurnia* and several Cyperid species is required.

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# CHAPTER 5
# SYNTHESIS AND RECOMMENDATIONS

The study had four major objectives. Firstly, it was to investigate the morphological diversity in *P. serratum* across its entire range. The second objective was to investigate the genetic diversity and structure, and evolutionary history of genus *Prionium*. The remaining objectives were to investigate the edaphic heterogeneity in the sites of *P. serratum* and to investigate the floral developmental pattern within the Cyperid clade.

*Prionium serratum* is a monotypic plant lineage with unique morphology across its entire range between the six phytogeographic regions in the CFR. Except for the anatomy, which has been well documented by Cutler (1969) and morphology description by Munro *et al* (2001), nothing is known about *P. serratum* morphology diversity between the phytogeographic regions. The purpose of evaluating the morphological diversity of *P. serratum* was to address the variety of morphology between phytogeographic regions in the CFR. Evidence from previous studies has shown that the genus *Prionium* occurs on two climatic condition regime (winter to year round (NW, SW, SE, and AP) and summer (KZN) rainfall conditions) which imply that they might have different morphology. The morphometric analysis revealed a slightly separation on the KZN population from the rest *P. serratum* populations between the phytogeographic regions. Furthermore, there was no discrete character to justify separation of any of the populations of *P. serratum* between phytogeographic regions into a separate taxon (either at species or infraspecific rank).

*Prionium serratum* occurs in mountains streams and rivers on sandstone separated by geographical barriers (unsuitable habitats) which are expected to limit gene flow. The distribution of genetic variants in plant populations is strongly affected by patterns of microevolutionary forces, such as gene flow and selection, mating system, method of seed dispersal, by the mode of reproduction of the species, and by the phylogenetic history of populations. The purpose of molecular evolutionary studies was to elucidate the forces governing evolutionary change in species and explore genetic diversity and structure in *P. serratum*. The molecular results indicated that *Prionium* is a strongly supported clade, but produced weakly supported nodes and polytomies between and within *P. serratum* populations. However, AMOVA results showed some genetic differentiation between

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phytogeographic regions, perhaps caused by limited gene flow between regions, but this varied between phytogeographic groups ranging from 6.38 (LB) to 34.04% (AP and KZN). Low genetic diversity between some nearly phytogeographic regions may be linked to life form, but is still unusually low for a lineage with stem age in Oligocene.

The study of the nutritional concentrations, it was found that *Prionium* populations occupy soils with varied nutritional values. Furthermore, it was found that the nutritional values for both *Prionium* and non-*Prionium* sites were comparable. Like some other Cape lineages, the *Prionium* was estimated to have originated before the Miocene climate change and therefore the existence of such species-poor lineage with one species indicate that it is not a product of rapid radiation associated with some lineages in the CFR. *Prionium* E. Mey (*Thurnia*ceae) is an old, species-poor lineage (monotypic lineage) which split from its sister genus *Thurnia* about 33-43 million years ago. Therefore, further studies; with greater sampling of soils and resolution of phylogenetic relationships are required in order to test whether other Thurniaceae and Cyperid clade species occupy soils with similar nutritional value and if their diversification was mainly driven by edaphic factors.

For many years, *Prionium* was included in Juncaceae until it was recognized as a monotypic lineage in Thurniaceae. Floral ontogenetic investigations in Thurniaceae have never been conducted and they are essential to interpret certain morphological features of the flower, such as developmental pattern of perianth parts, androecium and ovary position in the case of *Prionium*. In terms of the floral development and systematic implications, this study was able to show that Thurniaceae (*Prionium*) shared some floral developmental pattern within the Cyperid clade.. Special attention was paid to the sequence of initiation of organ whorls in the perianth, androecium and gynoecium and to provide additional characters for phylogenetic investigation between Thurniaceae, Juncaceae and Cyperaceae. From the phylogenetic analysis and trends in character evolution, it is certain that the Thurniaceae do form a basal group in the Cyperid clade. Ontogenetic observations revealed variability on the floral developmental patterns, and evolutionary patterns on floral structure such as perianth, androecium, and gynoecium development. Floral ontogenetic data show that *Prionium* possess plesiomorphic floral features such as introrse androecium, trilocular ovary shape, multiple ovules and absence of style between the Cyperids.

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**Future recommendations**

I recommend further investigation on the genetic diversity within *Prionium* and *Thurnia* regarding level of gene flow and evolutionary history to give explanation the species distribution pattern of the two genera. Phylogenetic analyses which included other members of Thurniaceae for strong family comparison and other Poales species-poor lineages are desirable and could turn on research the relationships between their variation with other biological traits and these corresponding patterns of evolutionary trends. In floral ontogeny, to clarify many modifications in inflorescence, spikelet and floral structure I recommend studies which include *Thurnia* species and additional information and evolutionary innovations within the Cyperid clade. Finally, further studies on edaphic heterogeneity with greater soils sampling of *Prionium* sites and should include the sister genus *Thurnia* representatives, and other old species-poor lineages, to test whether other species distributions are mainly driven by edaphic factors

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**Appendix 2.1:** Table of A1. Morphological data used for *Prionium serratum*.

| Locality | Sample no. | Leaf length | Leaf bottom | Leaf apex width | Lowest branching length | Lowest branch Inflorescence sheath length | Lowest branch Inflorescence sheath width | Lowest Branch internode | Lowest branch inflorescence bract length | Lowest branch inflorescence bract width |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **LeafL** | **LeafBW** | **LeafAW** | **LowBranL** | **InfloBrL** | **InfloBrW** | **LowBrnodeL** | **LowBrL** | **LowBrW** |
| Waterfall | 10A | 63.5 | 14.26 | 7.29 | 8.39 | 15 | 10 | 8.41 | 8 | 3 |
| Waterfall | 12A | 78 | 15.105 | 11.89 | 14.11 | 15 | 16 | 14.03 | 8 | 5 |
| Waterfall | 13D | 54.5 | 11.89 | 7.395 | 6.22 | 18 | 11 | 12.06 | 6.8 | 4 |
| Waterfall | 14A | 42.5 | 13.45 | 5.8 | 4.27 | 7 | 7.5 | 6.18 | 4 | 2 |
| Waterfall | 15D | 40.5 | 11.685 | 5.15 | 5.95 | 13 | 11 | 9.78 | 3.8 | 2 |
| Liesbeeck | 16A | 82 | 15.67 | 11.09 | 8.59 | 18 | 9 | 11 | 6 | 5 |
| Liesbeeck | 16B | 78 | 15.545 | 11.75 | 9 | 20 | 9.5 | 11.12 | 6 | 5 |
| Liesbeeck | 16C | 79.5 | 15.36 | 11.46 | 9 | 18 | 11 | 11 | 5.8 | 5 |
| Worcester | 17P | 65.5 | 15.525 | 10.48 | 6.66 | 20 | 11 | 10.64 | 4.88 | 3.5 |
| Worcester | 17R | 69 | 17.395 | 13.15 | 10.54 | 24 | 12 | 11.68 | 4.62 | 3.46 |
| Worcester | 17S | 72 | 17.275 | 12.37 | 8.89 | 23 | 11 | 11.8 | 4.74 | 3.5 |
| Mpenjati Nature Reserve | 18mp3 | 75 | 14.37 | 11.89 | 14.26 | 15 | 16 | 14 | 8 | 4.5 |
| Mpenjati Nature Reserve | 18mp5 | 74.5 | 15.115 | 10.27 | 8.45 | 16 | 18 | 13.84 | 6.8 | 4 |
| Magwa falls | 19Mg1 | 70 | 13.37 | 8.05 | 7.89 | 22 | 12.5 | 12 | 5.88 | 3.6 |
| Grootwinterhoek | 1A | 77.5 | 16.39 | 9.68 | 7.15 | 12 | 8.5 | 9.17 | 4.5 | 2.5 |
| Frasser falls | 20F4 | 95 | 16.515 | 11.05 | 11.68 | 23 | 10 | 11.89 | 6.2 | 3.8 |
| Frasser falls | 20F5 | 70 | 13.37 | 8.055 | 7.89 | 22 | 12.5 | 12 | 5.88 | 3.7 |
| Grahamstown | 22Gr10 | 82 | 20.02 | 12.13 | 12.59 | 18 | 22 | 14.16 | 7.5 | 4 |
| Grahamstown | 22Gr9 | 83 | 14.775 | 11.13 | 7.53 | 24 | 12.5 | 12 | 6 | 4 |
| Tsitsikamma | 23D | 138 | 18.13 | 13.20 | 7.76 | 25 | 12 | 12.89 | 6 | 4 |
| Tsitsikamma | 23E | 82 | 15.46 | 10.18 | 7 | 25 | 14 | 11.26 | 5.6 | 3.5 |
| Tsitsikamma | 23H | 112.5 | 16 | 11.12 | 7.3 | 22 | 12 | 11.4 | 5.8 | 3.6 |

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|---|---|---|---|---|---|---|---|---|---|---|
| Prince Alfred pass | 24A | 78 | 759.275 | 10.53 | 7.38 | 23 | 13 | 11.66 | 5.89 | 3.7 |
| Prince Alfred pass | 24D | 59.5 | 11.76 | 5.99 | 5.64 | 22 | 9 | 11.38 | 3.66 | 2.9 |
| Prince Alfred pass | 24H | 56.5 | 10.975 | 5.52 | 5.6 | 21 | 9 | 11.43 | 3.58 | 2.9 |
| Mkambati nature reserve | 25mk1 | 47.5 | 9.045 | 4.87 | 6.02 | 20 | 9 | 11.36 | 3.62 | 2.8 |
| Mkambati nature reserve | 25mk2 | 61 | 9.16 | 4.15 | 5.02 | 8 | 9 | 7.61 | 4 | 0.5 |
| Baardskeerderbos | 27B | 61 | 15.105 | 9.24 | 7.78 | 16 | 12.5 | 15.16 | 6 | 2.6 |
| Baardskeerderbos | 27C | 54 | 11.86 | 7.005 | 5.59 | 12 | 9 | 10.3 | 5 | 2 |
| Napier | 28A | 56.5 | 9.905 | 3.825 | 2.01 | 10.5 | 8 | 7.03 | 4 | 5.5 |
| Napier | 28B | 78 | 15.105 | 11.89 | 14.11 | 15 | 16 | 14.03 | 8 | 5 |
| Elim | 29A | 73.5 | 15.94 | 8.39 | 9.57 | 19 | 14 | 11.81 | 9 | 3.5 |
| Elim | 29C | 80 | 18.945 | 13.22 | 10.01 | 25 | 14 | 12.82 | 6.8 | 3 |
| Grootwinterhoek | 3E | 71 | 17.785 | 12.645 | 9.52 | 24 | 12 | 11.68 | 6.5 | 3.5 |
| Jonkershoek | 5363A | 60 | 15.285 | 7.79 | 8.04 | 18 | 13 | 13.37 | 6 | 3 |
| Jonkershoek | 5363C | 49.5 | 9.945 | 3.885 | 8.31 | 20 | 14 | 8.52 | 6 | 2.5 |
| Jonkershoek | 5373D | 61.5 | 10.955 | 5.335 | 6.43 | 22 | 8 | 6.9 | 5.5 | 2.5 |
| Hermanus | 5420A | 73.5 | 14.035 | 8 | 12.85 | 32 | 18 | 14.07 | 9 | 3.5 |
| Hermanus | 5423A | 64.5 | 12.395 | 6.1 | 6 | 16 | 10 | 8.68 | 3.86 | 3 |
| Hermanus | 5423C | 61 | 12.09 | 5.82 | 5.5 | 18 | 9 | 11 | 3.66 | 3.3 |
| Cederberg | 5716B | 63.5 | 12.4 | 6.445 | 6 | 15 | 9.5 | 11.2 | 3.46 | 2.8 |
| Grootwinterhoek | 6B | 60.5 | 12.76 | 8.515 | 7.23 | 22 | 9 | 8.9 | 9 | 3.5 |
| Bainskloof | 7B | 60.5 | 14.195 | 7.29 | 8.39 | 15 | 10 | 8.41 | 8 | 3 |
| Bainskloof | 8B | 40.5 | 7.885 | 3.895 | 5.25 | 13 | 5 | 7.2 | 3.5 | 2 |
| Bainskloof | 9A | 31 | 6.285 | 2.79 | 5.13 | 12 | 7 | 7.5 | 5.6 | 2.5 |

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| Locality | Sample no. | Inflorescence length | Inflorescence base width | Inflorescence internode length | Infloscence sheath length | Inflorescence sheath width | Inflrescence bract length | Inflorescence bract width | Inner perianth length | Inner perianth width |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **InfloL** | **InfloBW** | **InfloIL** | **InfShL** | **InfShW** | **InfBL** | **InfBW** | **InPL** | **InPW** |
| Waterfall | 10A | 45 | 8.39 | 9.5 | 8.2 | 3.65 | 5.8 | 2.65 | 5.7 | 2.6 |
| Waterfall | 12A | 60 | 14.11 | 10 | 8.5 | 3.85 | 6 | 2.75 | 5.9 | 2.6 |
| Waterfall | 13D | 55 | 6.22 | 12 | 7.2 | 3.05 | 4.95 | 2.3 | 4.65 | 2.2 |
| Waterfall | 14A | 28 | 4.27 | 8.4 | 6.95 | 3.1 | 5.5 | 2.35 | 5.2 | 2.35 |
| Waterfall | 15D | 45 | 5.95 | 8 | 6.7 | 2.8 | 5.35 | 2.3 | 5.2 | 2.25 |
| Liesbeeck | 16A | 50 | 8.59 | 9 | 8.45 | 3.8 | 5.9 | 2.7 | 5.7 | 2.5 |
| Liesbeeck | 16B | 56 | 9 | 10 | 8.75 | 4 | 6 | 2.8 | 5.8 | 2.8 |
| Liesbeeck | 16C | 50 | 9 | 9.5 | 8.55 | 3.9 | 5.55 | 2.45 | 5.5 | 2.35 |
| Worcester | 17P | 58 | 6.66 | 12 | 7.9 | 3.7 | 5.7 | 2.65 | 5.65 | 2.55 |
| Worcester | 17R | 60 | 10.54 | 11 | 8.4 | 4.2 | 6.1 | 2.9 | 5.9 | 2.6 |
| Worcester | 17S | 60 | 8.89 | 12 | 8.15 | 4 | 5.6 | 2.45 | 5.6 | 2.45 |
| Mpenjati Nature Reserve | 18mp3 | 60 | 14.26 | 10 | 7.05 | 3.1 | 5.45 | 2.35 | 5.35 | 2.3 |
| Mpenjati Nature Reserve | 18mp5 | 55 | 8.45 | 12 | 7.2 | 3.35 | 5.5 | 2.35 | 5.45 | 2.35 |
| Magwa falls | 19Mg1 | 52 | 7.89 | 14 | 8.1 | 4 | 5.9 | 2.45 | 5.5 | 2.45 |
| Groot Winterhoek | 1A | 55 | 7.15 | 7.5 | 7.65 | 3.4 | 5.6 | 2.5 | 5.6 | 2.45 |
| Frasser falls | 20F4 | 65 | 11.68 | 14 | 7.9 | 3.7 | 5.8 | 2.65 | 5.55 | 2.55 |
| Frasser falls | 20F5 | 52 | 7.89 | 14 | 8.3 | 4.25 | 6.15 | 2.8 | 5.6 | 2.5 |
| Grahamstown | 22Gr10 | 60 | 12.59 | 13 | 9.4 | 4.2 | 6.25 | 2.9 | 6.15 | 2.75 |
| Grahamstown | 22Gr9 | 66 | 7.76 | 14 | 9.4 | 4.2 | 6.25 | 2.9 | 6.15 | 2.7 |
| Tsitsikamma | 23D | 60 | 7 | 11 | 6.75 | 2.9 | 5.5 | 2.4 | 5.45 | 2.35 |
| Tsitsikamma | 23E | 55 | 7.3 | 12 | 6.95 | 3.05 | 5.45 | 2.4 | 5.25 | 2.3 |
| Tsitsikamma | 23H | 65 | 7.38 | 13 | 7.1 | 3.05 | 5.5 | 2.45 | 5.35 | 2.35 |
| Prince Alfred pass | 24A | 50 | 5.64 | 10 | 7.5 | 3.45 | 5.25 | 2.3 | 5.25 | 2.2 |
| Prince Alfred pass | 24D | 48 | 5.6 | 9 | 7.9 | 3.6 | 5.5 | 2.4 | 5.5 | 2.3 |
| Prince Alfred pass | 24H | 50 | 6.02 | 10 | 8.15 | 3.65 | 5.5 | 2.2 | 5.2 | 2.1 |
| Mkambati nature reserve | 25mk1 | 34 | 5.02 | 9 | 8.25 | 4.05 | 6 | 2.75 | 5.6 | 2.5 |
| Mkambati nature reserve | 25mk2 | 62 | 7.78 | 9 | 8.25 | 4.05 | 6.1 | 2.6 | 5.5 | 2.4 |
| Baardskeerderbos | 27B | 36 | 5.59 | 7.5 | 6.8 | 2.8 | 4.75 | 2.3 | 4.7 | 2.1 |

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|---|---|---|---|---|---|---|---|---|---|---|
| Baardskeerderbos | 27C | 50 | 2.01 | 10 | 6.75 | 2.85 | 4.85 | 2.3 | 4.8 | 2.2 |
| Napier | 28A | 60 | 14.11 | 10 | 6.95 | 2.9 | 5.3 | 2.3 | 5.25 | 2.25 |
| Napier | 28B | 58 | 9.57 | 12 | 6.8 | 2.7 | 5.1 | 2.15 | 5 | 2.1 |
| Elim | 29A | 70 | 10.01 | 16 | 7.05 | 2.95 | 5.2 | 2.25 | 5.15 | 2.2 |
| Elim | 29C | 64 | 9.52 | 15 | 7.1 | 3.05 | 5.4 | 2.3 | 5.25 | 2.1 |
| Groot Winterhoek | 3E | 50 | 8.04 | 11 | 6.8 | 2.7 | 5.05 | 2.25 | 4.8 | 2.25 |
| Jonkershoek | 5363A | 30 | 8.31 | 10.5 | 7.4 | 3.4 | 5.35 | 2.3 | 5.25 | 2.2 |
| Jonkershoek | 5363C | 50 | 6.43 | 6.5 | 8.05 | 3.6 | 5.65 | 2.5 | 5.5 | 2.3 |
| Jonkershoek | 5373D | 70 | 12.85 | 14 | 8.3 | 3.65 | 5.4 | 2.2 | 5.2 | 2.1 |
| Hermanus | 5420A | 45 | 6 | 9.4 | 6.85 | 2.95 | 5.2 | 2.1 | 5.05 | 2 |
| Hermanus | 5423A | 40 | 5.5 | 9 | 7.4 | 3.5 | 5.4 | 2.3 | 5.3 | 2.3 |
| Hermanus | 5423C | 43 | 6 | 9 | 7 | 3.15 | 5.35 | 2.3 | 5.1 | 2 |
| Cederberg | 5716B | 64 | 7.23 | 10.5 | 8.35 | 3.7 | 5.9 | 2.7 | 5.7 | 2.5 |
| Groot Winterhoek | 6B | 45 | 8.39 | 9.5 | 7.05 | 3 | 5.2 | 2.3 | 5.2 | 2.2 |
| Bainskloof | 7B | 35 | 5.25 | 8 | 6.65 | 2.75 | 5.5 | 2.5 | 5.3 | 2.3 |
| Bainskloof | 8B | 35 | 5.13 | 7.8 | 6.5 | 2.8 | 5.25 | 2.25 | 5.2 | 2.25 |
| Bainskloof | 9A | 48 | 5.79 | 8.8 | 6.5 | 2.6 | 5.55 | 2.5 | 5.45 | 2.4 |

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**Appendix 3.1:** Mean±SE of different nutrients in soil from different *Prionium* sites.

| mg/kg$^{-1}$ | **pH KCI** | **$NO_3$-N** | **$NH_4$-N** | **P Bray II** | **Total P** | **K** | **C %** | **T-value** | % Na | **% K** | **% C** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Citrusdal | 4.50±0.10 | 2.47±0.06 | 21.8±1.38 | 4.00±1.52 | 46.38±6.97 | 6.76±13.66 | 1.13±0.19 | 1.07±0.55 | 7.77±3.18 | 3.35±1.10 | 36.02±3.23 |
| Groot Winterhoek | 3.70±0.15 | 8.45±1.77 | 22.8±1.34 | 3.33±1.85 | 17.38±10.45 | 20.66±8.68 | 0.68±0.53 | 2.05±1.07 | 5.12±0.30 | 5.15±0.02 | 2.86±19.63 |
| Waterfall | 3.93±0.12 | 3.26±2.32 | 11.2±0.810 | 3.00±0.00 | 30.81±13.26 | 68.33±14.14 | 2.14±0.21 | 3.40±0.71 | 9.25±1.47 | 4.33±0.93 | 21.59±5.79 |
| Bainskloof | 3.70±0.17 | 5.44±1.22 | 25.97±3.83 | 2.66±0.33 | 11.88±2.54 | 12.33±0.88 | 0.27±0.05 | 0.79±0.15 | 4.14±0.31 | 1.52±0.34 | 24.33±4.70 |
| Hermanus | 3.26±0.03 | 4.22±1.38 | 21.11±0.36 | 3.33±0.33 | 15.05±3.34 | 43.00±14.01 | 2.14±0.21 | 6.67±1.14 | 3.48±0.23 | 5.02±0.38 | 25.48±6.33 |
| Jonkershoek | 3.93±0.09 | 1.76±1.70 | 20.37±0.21 | 11.00±1.5 | 107.98±3.25 | 98.66±12.91 | 2.90±0.19 | 5.00±0.43 | 3.48±0.23 | 5.02±0.38 | 21.93±4.72 |
| Worcester | 4.33±0.38 | 2.33±1.00 | 8.73±0.55 | 14.0±1.00 | 219.68±64.11 | 85.33±35.35 | 2.91±0.82 | 8.09±2.71 | 13.31±6.29 | 2.62±0.26 | 29.43±9.21 |
| Baarderskeerbos | 4.16±0.07 | 14.1±8.24 | 30.56±9.97 | 8.67±0.33 | 474.35±77.05 | 101.00±4.50 | 2.75±0.79 | 17.17±0.81 | 26.94±3.23 | 1.51±0.03 | 21.64±2.56 |
| Elim | 4.16±0.03 | 14.7±1.69 | 33.63±1.30 | 6.67±0.89 | 215.13±25.00 | 0.47±0.03 | 0.52±3.80 | 1.73±12.21 | 10.21±1.15 | 3.87±0.28 | 33.8± 0.98 |
| Napier | 5.43±0.09 | 1.65±0.34 | 28.85±7.59 | 92.6±14.4 | 270.48±19.73 | 181.33±12.38 | 3.66±0.93 | 12.49±1.94 | 2.44±0.35 | 4.61±0.87 | 68.26±2.54 |
| Grahamstown | 3.63±0.13 | 2.70±0.13 | 13.55±0.23 | 4.00±0.57 | 23.79±5.92 | 16.00±3.60 | 0.82±0.26 | 1.69±0.45 | 6.37±1.71 | 2.56±0.22 | 24.87±0.54 |
| Prince Alfred pass | 3.50±0.00 | 2.78±0.04 | 15.64±0.12 | 2.00±0.00 | 20.67±3.05 | 2.00±0.00 | 0.43±0.04 | 1.14±0.04 | 9.58±0.22 | 5.56±0.27 | 21.01±1.24 |
| Tsitsikama | 4.50±0.00 | 2.76±0.06 | 17.28±0.18 | 8.33±0.33 | 142.30±2.76 | 154.00±16.44 | 1.45±0.35 | 6.01±0.14 | 5.62±0.12 | 6.52±0.55 | 34.95±0.50 |
| Frasser falls | 3.86±0.03 | 1.80±0.60 | 26.28±1.43 | 3.33±0.33 | 74.97±1.08 | 57.66±3.58 | 1.56±0.27 | 6.83±0.09 | 6.28±0.28 | 2.17±0.10 | 32.46±0.89 |
| Mkambati nature reserve | 4.20±0.00 | 1.33±0.03 | 33.94±1.89 | 3.66±0.33 | 60.97±4.80 | 37.33±0.88 | 2.66±0.47 | 3.43±0.14 | 4.31±0.20 | 2.78±0.14 | 23.18±0.78 |
| Mpenjati nature reserve | 4.60±0.00 | 17.91±0.16 | 17.91±1.67 | 5.00±0.00 | 124.98±1.48 | 198.33±8.45 | 2.20±0.12 | 8.67±0.12 | 4.75±0.08 | 5.92±0.30 | 43.4±0.42 |

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**Table cont…**

| mg/kg-1 | % Mg | Ex Na | Ex K | Ex Ca | Ex Mg | % K | % C | Cu | Zn | Mn | Br |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Citrusdal | 20.09±3.36 | 0.09±0.02 | 0.03±0.01 | 0.38±0.19 | 0.25±0.16 | 3.35±1.10 | 36.02±3.23 | 0.64±0.21 | 0.08±0.53 | 2.53±1.33 | 0.20±0.01 |
| Groot Winterhoek | 10.56±0.74 | 0.10±0.02 | 0.05±0.02 | 0.44±0.28 | 0.23±0.13 | 5.15±0.02 | 2.86±19.63 | 0.24±0.06 | 0.33±0.06 | 0.33±0.12 | 0.12±0.00 |
| Waterfall | 22.44±5.03 | 0.17±0.03 | 0.17±0.03 | 0.81±0.35 | 0.83±0.31 | 4.33±0.93 | 21.59±5.79 | 0.18±0.08 | 0.43±0.13 | 7.36±3.51 | 0.15±0.00 |
| Bainskloof | 1.26±11.07 | 0.06±0.00 | 0.03±0.00 | 0.19±0.19 | 0.09±0.02 | 1.52±0.34 | 24.33±4.70 | 0.17±0.03 | 0.23±0.03 | 0.56±0.12 | 0.09±0.02 |
| Hermanus | 18.65±1.17 | 0.27±0.03 | 0.11±0.03 | 1.84±0.64 | 1.27±0.27 | 5.02±0.38 | 25.48±6.33 | 0.09±0.01 | 0.40±0.00 | 0.97±0.27 | 0.29±0.04 |
| Jonkershoek | 16.17±2.86 | 0.17±0.02 | 0.25±0.03 | 1.11±0.27 | 0.80±0.14 | 5.02±0.38 | 21.93±4.72 | 0.19±0.02 | 0.83±0.18 | 21.1±6.47 | 0.31±0.04 |
| Worcester | 20.96±2.20 | 1.41±0.98 | 0.22±0.09 | 1.94±0.19 | 1.63±0.52 | 2.62±0.26 | 29.43±9.21 | 1.87±0.59 | 1.63±0.71 | 49.93±38.99 | 0.15±0.02 |
| Baarderskeerbos | 34.91±0.19 | 4.66±0.73 | 0.26±0.01 | 3.70±0.41 | 6.00±0.29 | 1.51±0.03 | 21.64±2.56 | 2.60±0.36 | 54.16±19.61 | 8.53±1.38 | 0.63±0.01 |
| Elim | 31.49±0.81 | 1.23±0.17 | 0.46±0.03 | 4.07±0.63 | 3.87±0.65 | 3.87±0.28 | 33.2± 0.98 | 3.73±0.62 | 3.73±0.41 | 71.63±17.97 | 1.08±0.02 |
| Napier | 19.08±1.47 | 0.29±0.02 | 0.60±0.20 | 8.57±1.45 | 2.37±0.39 | 4.61±0.87 | 68.26±2.54 | 44.2±21.4 | 2.06±0.27 | 8.63±2.09 | 0.10±1.32 |
| Grahamstown | 10.34±1.03 | 0.10±0.01 | 0.04±0.01 | 0.37±0.04 | 0.16±0.03 | 2.56±0.22 | 24.87±0.54 | 0.25±0.01 | 0.66±0.18 | 0.36±0.03 | 0.10±0.02 |
| Prince Alfred pass | 12.00±0.46 | 0.11±0.01 | 0.06±0.01 | 0.24±0.02 | 0.14±0.01 | 5.56±0.27 | 21.01±1.24 | 0.13±0.00 | 0.56±0.03 | 3.40±0.10 | 0.17±0.01 |
| Tsitsikama | 32.57±0.37 | 0.33±0.00 | 0.39±0.04 | 2.10±0.01 | 1.96±0.05 | 6.52±0.55 | 34.95±0.50 | 0.74±0.03 | 2.66±0.12 | 82.4±1.93 | 0.33±0.04 |
| Frasser falls | 28.66±0.15 | 0.42±0.01 | 0.42±0.01 | 0.14±0.01 | 2.21±0.05 | 2.17±0.10 | 32.46±0.89 | 1.24±0.07 | 1.3±0.08 | 12.8±1.00 | 0.53±0.02 |
| Mkambati nature reserve | 19.42±0.49 | 0.15±0.01 | 0.15±0.01 | 0.09±0.00 | 0.79±0.02 | 2.78±0.14 | 23.18±0.78 | 0.45±0.03 | 0.76±0.06 | 6.96±0.42 | 0.22±0.02 |
| Mpenjati nature reserve | 29.49±0.42 | 0.40±0.01 | 0.40±0.01 | 0.50±0.02 | 3.72±0.08 | 5.92±0.30 | 43.4±0.42 | 1.60±0.13 | 3.40±0.33 | 77.3±5.83 | 0.56±0.03 |
| Umtamvuna | 33.73±3.96 | 19.5±0.78 | 0.23±0.04 | 0.23±0.04 | 0.53±0.06 | 2.52±0.06 | 0.23±0.04 | 0.54±0.01 | 1.00±0.17 | 5.06±1.29 | 4.43±0.34 |

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**Appendix 3.2** Mean±SE of different nutrients in soil from different *Prionium* regions.

| (mg/kg$^{-1}$) | pH KCL | P Bray II | $NO_3$.N | $NH_4$-N | Total P | K | Cu | C % | T-value | Zn | Mn |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **NW** | 4.04±0.13 | 3.44±0.71 | 3.32±0.83 | 18.05±1.98 | 31.53±6.74 | 34.22±10.03 | 0.35±0.10 | 1.32±0.28 | 2.17±0.53 | 0.43±0.06 | 3.41±1.50 |
| **SW** | 3.81±0.15 | 7.75±1.52 | 5.91±2.33 | 21.41±3.32 | 88.65±29.05 | 59.83±13.42 | 0.59±0.26 | 2.06±0.37 | 5.14±1.04 | 0.78±0.23 | 18.1±10.39 |
| **AP** | 4.59±0.21 | 36.0±14.77 | 22.6±7.58 | 27.16±3.28 | 319.9±46.18 | 172.33±30.02 | 16.85±9.24 | 3.41±0.40 | 13.96±1.13 | 19.99±10.2 | 29.6±11.7 |
| **KZN** | 4.17±0.12 | 3.00±0.56 | 8.51±2.22 | 30.37±1.81 | 96.74±9.26 | 112.58±25.60 | 0.96±0.15 | 2.77±0.41 | 6.66±0.64 | 1.63±0.32 | 25.55±9.15 |
| **SE** | 3.88±0.16 | 4.78±0.95 | 2.75±0.05 | 15.49±0.55 | 62.26±20.12 | 65.00±22.82 | 0.38±0.09 | 0.90±0.20 | 2.95±0.78 | 1.30±0.35 | 28.72±13.4 |

| (mg/kg$^{-1}$) | Br | Fe | % N | % K | % C | % Mg | Exch N | Exch K | Exch Ca | Exch Mg |
|---|---|---|---|---|---|---|---|---|---|---|
| **NW** | 0.16±0.01 | 72.14±17.3 | 7.83±1.37 | 3.95±0.44 | 25.75±3.32 | 17.70±2.53 | 0.12±0.02 | 0.09±0.03 | 0.55±0.16 | 0.44±0.15 |
| **SW** | 0.22±0.03 | 149.6±60.2 | 7.55±1.84 | 3.38±0.48 | 25.29±2.89 | 16.72±1.40 | 0.48±0.27 | 0.15±0.03 | 1.27±0.26 | 0.95±0.22 |
| **AP** | 1.02±0.11 | 604.5±103.42 | 13.2±3.75 | 3.33±0.54 | 41.06±7.09 | 28.50±2.46 | 2.06±0.70 | 0.44±0.08 | 5.45±0.92 | 4.08±0.58 |
| **KZN** | 1.44±0.53 | 390.45±77.30 | 4.59±0.36 | 4.46±0.62 | 33.20±2.34 | 24.27±1.47 | 0.30±0.04 | 0.32±0.06 | 2.31±0.31 | 1.66±0.21 |
| **SE** | 0.21±0.04 | 390.45±77.30 | 7.19±0.79 | 4.88±0.63 | 26.95±2.72 | 18.30±3.59 | 0.18±0.04 | 0.16±0.06 | 0.91±0.30 | 0.75±0.30 |

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