An Assessment of the Anatomical and Genetic Diversity of *Themeda triandra* Forssk.

By

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Abstract

Themeda triandra Forssk. (Poaceae) is a polymorphic species, with some forms that grow to heights of over 2m and others that grow only up to 30cm. They have leaf colours varying from yellow-green to blue-green and even brown. These morphological differences extend to the differences in basal and culm tiller development patterns observed in different parts of South Africa. These differences are a result of light availability and competition, and frost. Nutrient availability in the soil also has a great influence on the morphology and growth of the species. Due to this variation in morphology and development, studies have been conducted on the ecology, physiology and cytology of the species, in an effort to understand underlying causes of this polymorphism.

Cytological studies done on *T. triandra* have shown ploidy levels varying from 2n=20 to 2n=80. The eastern and southern parts of southern Africa have diploid (2n=20) specimens while the species on the Highveld of South Africa appear to be hexaploids (2n=60), with tetraploids and pentaploids occurring in the intermediate regions. The variation in ploidy levels is a result of possible hybridization between diploids, tetraploids and hexaploids that co-occur. This may also lead to apomixis. Studies have also shown that *T. triandra* is a decreaser, i.e. a species whose abundance decreases with over-grazing and underutilization. *T. triandra* has large seeds compared to other grasses as a result dispersal is slow and has low colonization abilities.

The main aims of the study were to evaluate leaf anatomical diversity using Scanning Electron Microscopy (SEM) and Light Microscopy (LM), to evaluate genetic diversity within the species and to assess the species limits between South African *T. triandra* and the Australian *T. australis* (also considered by some to be *T. triandra*). One hundred and twenty six herbarium specimens were used for SEM, as well as 92 freshly collected specimens which were used for SEM and LM.

The results from leaf anatomy are presented as UPGMA phenograms obtained using Simple Matching (SM) and Euclidean Distance (ED) measures. These showed no geographic pattern among the specimens, and none of the characters showed taxonomic significance.

Genetic diversity was assessed from 85 specimens using DNA sequences from the nuclear Internal Transcribed Spacer Region and 84 specimens using DNA sequences from nuclear External Transcribed Spacer Region. Genetic diversity analyses are presented in TCS networks and Mr Bayes phylogenetic trees. The DNA data also showed no infra-specific groups and therefore no geographic pattern. The inclusion of Australian specimens indicated that there was no clear differentiation within the Australian samples and some differentiation between these samples and those from southern Africa.

All the results (anatomical and genetic) showed no grouping within the species. This lead to the conclusion that *T. triandra* is an ochlospecies, with its morphological diversity driven by the environmental differences in areas where it occurs. These results of this study are motivation for further studying of *Themeda triandra* to further confirm the current findings.

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1 Chapter 1: General Introduction *Themeda triandra*

The grass family (Poaceae), with approximately 800 genera and 12,000 species globally, is one of the most important plant families (Ellis 1981, Clayton and Renvoize 1986, Fish *et al*. 2015, Cabi *et al.* 2017). It is the fifth largest plant family in the world (after Asteraceae, Leguminosae, Orchidaceae and Rubiaceae; (Clayton and Renvoize 1986, Fish *et al*. 2015). In South Africa the family is very important economically and ecologically. Grasses contribute into the economy of the country in terms of land management, especially in ensuring the long-term survival of the land, the ecosystem and those who use it for commercial purposes (Ellis 1981). Grasses also provide cereal crops on which most of the world's population depend for food (Clayton and Renvoize 1986). Grasses occupy over 75% of South African land surface (Ellis 1981). There are about 10,000 grass species in the world (Fish *et al*. 2015) and about 1,050 species and subspecies in southern Africa (Ellis 1981).

Themeda Forssk. is a genus in the grass family, tribe Andropogonae, subtribe Anthistiriinae and contains about 27 species (Veldkamp 2016, Dunning *et al*. 2017). It consists of annual and perennial forms that are bisexual with spikelets that are male, neuter and hermaphrodite on the same plant (Gibbs Russell *et al*. 1991). *T. triandra* Forssk. is commonly known as Red Grass or Rooigras. It is a blue-green tufted perennial grass that can grow to height of about 2m (Fish 2004). This is one of the most complex and least clearly understood species of grasses in southern Africa (Gibbs Russell *et al.* 1991). The species is widely distributed and abundant in Africa (eastern and southern), Asia, India, Middle-East, China and Australia (Liebenberg *et al*. 1993, Dell'Acqua *et al*. 2013, Dunning *et al*. 2017, Godfree *et al.* 2017). Within these regions it is found in grasslands and savannas that receive an annual rainfall of 500-1400mm (O'Connor and Bredenkamp 1997, Godfree *et al.* 2017). It has also been found to grow in a wide range of climates, topography and substrates (O'Connor and Bredenkamp 1997, Godfree *et al.* 2017). A phylogenetic study by Dunning *et al*. (2017) shows that *T. triandra* emerged in Asia about 1.5 million years ago (Ma), and then spread to Australia 1.3 Ma and to Africa 0.5Ma. This shows a recent spread of the species. The success and spread of *T. triandra* cannot only be based on one aspect, but rather a combination of multiple traits that favour dispersal and survival (such as fires, grazing and even polyploidy; (Dunning *et al*. 2017).

1.1 Themeda triandra and T. australis: One species or two

Over the last two decades *T. australis* from Australia has been referred to as a synonym for *T. triandra*. This is mainly as a result of an informally hypothesized colonization of Australia by the species through the importation of camels from Asia and Africa in the 19th century (McCarthy 1980). This is supported by Peart (1979) showing that the seed morphology of *T. triandra* also suggests an epizoochoric dispersal strategy. However, there has not been any formal experimental data to clarify this theory, but studies have been conducted to determine similarities and differences between the two supposed species.

Lock and Milburn (1971) and Groves *et al*. (1973) found a number of similarities between what was considered different species, *T. triandra* and *T. australis* and therefore concluded that it is one species where *T. australis* is a synonym of *T. triandra*. The conditions under which they grow and flower are similar, providing more evidence that the two are conspecific (Groves *et al*. 1973). As a result, the Australian National Herbarium (A. Whelan. Pers. Comm.) and the Food and Agriculture Grassland Index (2005a and b) consider them a single species – *T. triandra*. However, molecular studies are currently being conducted to further compare this species across its distribution range. These studies include the current study.

1.2 The ecological and economic importance of T. triandra

T. triandra is of great importance economically and ecologically. This is because it forms a part of livestock and wildlife diet, as it is highly palatable and nutritious (Snyman *et al*. 2013). It has also been found to be of value for roof thatching in rural areas, and thus is of great importance to rural livelihoods. It is also used as a reference species to measure the quality of grasslands (Baxter 1996) and as an indicator of the quality of the ecosystem function and soil quality (Dunning *et al*. 2017). Because of the species' value to the economy and livelihoods, the management of the species in rangelands is considered to be of great importance. However, a

detailed analysis of the ecological importance of the species and genus (*Themeda*) is still lacking (Dunning *et al*. 2017).

1.3 Morphological variation

Morphologically the structure of *T. triandra* consists of roots, tillers (consisting of phytomers from the base of the tuft) and spikelet inflorescences (Briske 1993). Each of these units has been shown to have varying sizes which may be affected by environmental conditions (Briske 1993). Phytomers comprise the leaf blade, ligule, intercalary meristem, the sheath, the node, internode and the auxiliary bud (Briske 1993). Phytomers are units in plants that are continuously produced by the root and shoot meristems throughout the plant's vegetative life cycle (McMaster 2005). Tillers and leaves are produced in spring and summer, during which the spring and summer rains fall (Danckwerts 1987). This production is commonly initiated after 12mm of rain (Bridgens 1968). *T. triandra* has two types of tillers, i.e. basal and culm tillers (Tainton and Booysen. 1965). Basal tillers are those that develop from closely spaced basal nodes and culm tillers develop from nodes above the internodes (Tainton and Booysen. 1965).

A number of studies have been conducted on the ecology, management, and the taxonomy of the species (Glover *et al*. 1962, Heady 1966, Baxter *et al*. 1994, Morgan 1999, Morgan and Lunt 1999, Van Rensburg 2003). A number of studies have also been conducted to understand the morphological variation on the species. Meredith *et al*. (1955) showed that some forms of *T. triandra* are shorter, grow in temperate regions and grow and flower in spring. The taller forms of the species grow in tropical regions and grow and flower at a later stage, i.e. summer (Meredith *et al*. 1955). Gibbs Russell *et al*. (1991) and Fish (2004) showed that *T.triandra* grows and flowers from spring to mid-winter (September to July), with varying flowering times, differing from region to region. According to Tainton and Booysen (1965) the flowering period can be extended to almost any season depending on the locality and environment of the population.

Van Rensburg (2003) found significant differences in the basal versus culm tiller patterns in different populations of *T. triandra* in South Africa, both in the field and in seedlings germinated under controlled conditions. These differences were attributed to light competition strategies interlinked with the presence or absence of frost. In mesic areas with frost, basal tillering was dominant, with culm tillers only emerging in response to increased light competition through self-shading after two or more seasons of growth. Plants from regions without frost (but that experience periodic drought) displayed an inherent tendency to immediately extend the node and produce culm tillers following rainfall, giving them an early season competitive advantage to access light under favourable rainfall conditions (Van Rensburg 2003).

Elberse and Berendse (1993) found that nutrient availability in the soil had an effect on the morphology of *T. triandra,* particularly the tussock and height size. Plants from nutrient-rich habitats have higher leaf area, but lower root length while nutrient-poor habitats have a higher root length, but lower leaf area. This is mainly because plant roots are responsible for transporting nutrients to the rest of the plant, so if the soil is nutrient-poor the roots penetrate deeper into the ground to get more nutrients. These observed differences in growing and flowering times may create an impression of a change in species composition within an environment (Meredith *et al*. 1955).

1.4 <u>Cytological variation</u>

Cytologically, the species has been found to have varying ploidy levels ranging from 2n=20 to 2n=80 (Brown and Emery 1957, Birari 1981, Fossey and Liebenberg 1992, Liebenberg *et al.* 1993, Godfree *et al.* 2017). Polyploidy is said to have a positive role in the success of the species following dispersal, where the species is viewed as having a number of populations of varying ploidy levels (Hayman 1960, Soltis *et al.* 2009, Linder *et al*. 2014, Godfree *et al.* 2017, Cai *et al.* 2019, Lavania 2020). Polypliody also plays a role in improving the species' ability to colonise and adapt to new and varying environments (Dunning *et al*. 2017, Godfree *et al.* 2017). It has been observed that in the eastern and southern areas of southern Africa diploid (2n=20) species

occur. In the Highveld of South Africa hexaploids (2n=60) occur and the tetraploids and pentaploids occur in the intermediate regions (Fossey and Liebenberg 1992). The diploids, tetraploids and hexaploids can co-occur and thus can hybridize and sexual segregation can occur resulting in apomictic plants (Fossey and Liebenberg 1992). Tetraploids appear more successful in high-stress environments (Godfree *et al.* 2017). According to Liebenberg *et al*. (1993) pentaploids observed in the Magaliesberg mountain range, near Pretoria, undergo chromosome doubling which result in decaploids (Liebenberg *et al*. 1993). In India Birari (1981) showed that *T. triandra* appeared to be tetraploid with 2n=40 chromosomes. Brown and Emery (1957) showed that the species in India can be diploid (2n=20), aneuploid and octoploid, and found that *T. triandra* shows a polyploid chromosome series and irregular meiotic divisions, resulting on the species being of great taxonomic complexity. Similar studies have also been conducted in Australia on *T. australis* (*T. triandra*) (Hayman 1960, Carnahan and Hill 1961). These have shown chromosome numbers ranging from 2n=20 to 2n=60 (Hayman 1960, Carnahan and Hill 1961). The variation in the ploidy levels and increased apomixis of the species have shown no clear geographical and/or other pattern (Liebenberg 1986).

Studies done on *T. triandra* have shown that it forms an agamic complex (Brown and Emery 1957, Liebenberg 1986). An agamic complex refers to a species that can reproduce without the union of male and female cells. This is also termed apomixis. According to Stebbins (1950) terminology, *T. triandra* is referred to as an aposporic apomict. This is the type of apomixis where gametophytes are formed directly from the diploid cells. *T. triandra* has been defined as a polyploid complex and this is a characteristic of most agamic complexes (Gluckmann 1951 and Birari 1981).

1.5 <u>Ecological variation</u>

Most studies on the ecology of *T. triandra* have been based on its management regimes (Everson 1994, Everson and Everson 2016). *T. triandra* is considered to be a decreaser species as its abundance decreases when overgrazed or underutilised (Everson *et al*. 1985, Theunissen 1992).

Smit *et al*. (1992) found it to be a climax or subclimax species. It is therefore important to manage its abundance in grasslands and savannas. Fire and grazing are the two commonly used management tools that have been proposed as important for maintaining healthy *Themeda* grasslands in sourveld mesic grasslands (Everson 1994, Everson and Everson 2016). In areas with high rainfall *T. triandra* cannot thrive successfully in the absence of fire (Tainton 1981). Grazers help in distributing the species and in promoting germination of the seeds, but the greatest impact of grazing is the removal of materials that may cause self-shading of the grass (Tainton 1981). In high rainfall areas, grazing coupled with fire plays an important role in preventing self-shading. Fire is important in maintaining the species in mesic areas through preventing plants from becoming moribund (Everson and Everson 2016). However, burning after growth has begun (in spring) has proven to be detrimental to the species because it reduces the species' ability to produce seeds (Tainton 1981). In contrast, in arid areas in South Africa such as the sweet veld in the Trompsberg area of the Free State, fire is considered by farmers to be detrimental to the *Themeda* veld (Van Rensburg 2003). Fire frequency and fire season are thus important considerations in management and seem to differ in what might be appropriate depending on the local conditions, despite this apparently being a single species. Without efficient and effective management the species abundance is reduced (Everson 1994). However, the management regimes practiced in one area for *Themeda* may not be suitable to populations occurring under different conditions. Proper management also helps protect the environment from soil erosion, improves air and water quality and promotes plant diversity (Tainton 1981). As a result, understanding the diversity of *T. triandra* from both the ecological and taxonomic perspective will aid the application of appropriate management strategies.

1.6 Seed biology

Studies done on the species' seed biology show that *T. triandra* seeds mature from the inflorescence apex downwards (Everson 1994). Seed production is also affected by the occurrence of fire and on the type of fire regime, and seed dispersal appears to be slow in populations from high rainfall mesic grasslands and results in a low probability of disturbed areas being colonised by *T. triandra* (Everson 1994). However, Van Rensburg (2003) found significantly higher seed production, seed weights and

germination success in populations from Zululand, compared to other *T. triandra* populations studied, including the mesic grassland form studied by Everson (1994). High reliance on seed production and germination rates with low dormancy in the Zululand population was attributed to an ecological adaptation to the sub-tropical frost free climate in Zululand combined with high rainfall variability (Everson 1994).

The seeds of *T. triandra* are large and the occurrence of an awn serves as an advantage in seed dispersal (through zoochory) and burying the seed underground (by twisting when it lands on soft ground) due to its hygroscopic movements (Lock and Milburn 1971). Heavy seeds and long awns are said to be advantageous in adapting to arid environments (Godfree *et al.* 2017). Once seeds are dispersed and on the ground, they may be prone to granivory (Everson 1994). Seed germination is influenced by temperature, soil moisture, space between seeds and the amount of time the seeds are stored (Saleem *et al*. 2009). High temperatures promote seed germination and the narrower the space between seeds provides a better rate of soil moisture utilization (Saleem *et al*. 2009). The period over which *T. triandra* seeds can stay dormant is 12 months and any longer than that may result in seeds rotting (Cresswell and Nelson 1972, Baskin and Baskin 1973, Martin 1975, Saleem *et al*. 2009). Once the seeds have germinated, the plumules come out in a vertical orientation due to the awn which orients the seed in a standing position (Peart 1984).

Van Rensburg (2003) found significant differences in germination rates and dormancy levels in seeds from different populations within South Africa, sampled from over a variety of rainfall gradients. She proposed that populations from subtropical regions that experience a high coefficient of variation in rainfall with periodic droughts (Northern KwaZulu-Natal) relied more on seedling recruitment to sustain populations with high seed production, low dominancy and high germination rates. In contrast populations from mesic areas with predictable rainfall relied more on vegetative reproduction as they had lower seed production, high dormancy and low germination rates. Seedling re-establishment is also affected by fire treatments and the time of the fire, as winter burns result in lower seedling establishment as compared to summer burns (Everson 1994).

1.7 Growth physiology

In addition to the above mentioned aspects of *T. triandra*, there have been studies conducted on the effect of inorganic nitrogen ions on photosynthesis and the availability of carbohydrates in the species (Weinmann and Reinhold 1946, Weinmann 1961, Amory and Cresswell 1984). The inorganic nitrogen ions infiltrated into the leaves of *T. triandra* are said to have a great influence on photosynthesis (Amory and Cresswell 1984). Weinmann and Reinhold (1946) found that the most important carbohydrate reserves in grasses are sugar and starch, and in *T. triandra* they are found mainly in the underground parts (roots and rhizomes). There appears to be a gap in the literature on the physiology of the species, and how this might differ with respect to geographical location.

1.8 Variability in Genetic diversity

Forage species have been largely ignored in molecular biology studies and there have not been many published molecular studies on *T. triandra* (Dell'Acqua *et al*. 2013). However, Taylor and Barker (unpublished) assessed the genetic diversity of a few samples using Inter Simple Sequence Repeats (ISSR). However, due to a small number of specimens sampled during the study, the results were not conclusive as they were not representative enough of the wide range and variability of the species. Another study which looked at Kenyan *T. triandra* populations using 366 Amplified Fragment Length Polymorphism (AFLP) markers showed that the African and Australian genotypes to be similar, and that there is sexual recombination which appears to play a major role in the species reproduction (Dell'Acqua *et al*. 2014). However, the study also showed that the environmental characteristics of the collection sites may affect the distribution of different alleles (Dell'Acqua *et al*. 2014).

1.9 Scope of this Study

Despite the range of ecological and cytological studies done on *T. triandra* (Liebenberg 1986, 1990, Fossey and Liebenberg 1992, Liebenberg *et al*. 1993, O'Connor 1993), the question remains as to whether the observed variability is a result of the species consisting of varieties or subspecies, or simply local adaptation. These morphological differences may be due to environmental differences caused by the responses to environmental changes (i.e. phenotypic plasticity) or ecotypes. Phenotypic plasticity is quite common in immobile organisms (plants) as a means of surviving the environmental changes (Schlichting 1986). The ecotype concept can be used to accommodate any genetic differences that may occur within a polymorphic species (Quinn 1978). Another possibility is that *T. triandra* may be an ochlospecies complex. This is a species concept applied to some morphologically variable (polymorphic), but not widely polytypic species (Cronk 1998, Barbosa *et al*. 2012). The variation in ochlospecies is usually correlated to the geography and ecology of the species. Therefore it is important to understand the environmental differences and all other factors that may influence the variation (Cronk 1998). It has also been hypothesized that the ochlospecies concept can be paralleled with phenotypic plasticity (Cronk 1998).

This study thus aims to investigate anatomical and genetic variability within *T. triandra* using samples from a wide range of environmental and geographical locations. The anatomy of the species has not received much attention in the past, and studies have shown that studying the leaf anatomy of the tribe Andropogonae is beneficial in understanding species taxonomy and classifying them (Dávila and Clark 1990).

Specific aims are:

1: Evaluate the leaf anatomical diversity of *T. triandra* from different regions of South Africa by means of Scanning Electron Microscopy (SEM) and Light Microscopy (LM).

2: To use DNA sequence data to evaluate the genetic diversity between the individuals of the species found in different regions of southern Africa.

3: To use DNA sequence data to assess species limits between specimens of *T. triandra* from South Africa and Australia.

1.10 <u>Overview of the dissertation structure</u>

In order to test the above hypotheses, genetic and anatomical analyses were conducted on specimens from a wide distribution range in southern Africa. Each component of the study has a separate chapter. Chapter 2 deals with the topic of leaf anatomical analyses (both scanning electron microscopy and light microscopy). Chapter 3 is genetic diversity study. Chapter 4 is the general discussion (synthesis), taxonomic implications and conclusions.

2 Chapter 2: Assessment of leaf anatomical variation

2.1 Introduction

Plant anatomy is an important tool in the classification/taxonomy and systematics of plants, especially if combined with cellular studies (Cutler *et al*. 2008). Correct/accurate identification and classification of plants has great economic and ecological value and correct classification in the food industry, ecology, medicinal industry (chemists, microbiologists and pharmacologists), genetics (Heywood 1976, Calisher and Mahy 2003, Cutler *et al*. 2008, Bennett and Balick 2014) and conservation is essential. In the past Poaceae taxonomy was only based on reproductive characters, but should also be expanded to anatomical characters (Leandro *et al*. 2016).

The use of microscopic techniques in grass taxonomy is said to provide a different perspective than the traditional morphological view and can be used to quantify morphological and structural changes (Akin 1979, Torre *et al*. 2003). Traditional morphology studies lengths and widths of all the characters that can be seen with a naked eye. These include leaf lengths, inflorescences and roots (Akin 1979). The application of Scanning Electron Microscopy (SEM) in plant taxonomy has grown over time because it is very easy to operate, can be performed quickly and no extensive training is required (Stant 1973, Cole and Behnke 1975). Images obtained from SEM can be viewed on any television or computer screen using a computer display unit (Stant 1973, Cole and Behnke 1975). The images are also said to be more easily understood as opposed to images of sectioned specimens (Stant 1973). Other advantages of using SEM include that the depth of focus is 500 times greater than that of a light microscope, the specimen can be magnified up to 2,000,00X, SEM gives a resolution of about 150Å and the specimen can be viewed in more than one angle on the stage (Stant 1973). However, the number and size of samples examined using SEM is usually limited due to the lengthy preparation periods (Cole and Behnke 1975).

Anatomical features of the leaf blade have been found to be of great value in Poaceae taxonomy (Brown 1958, Ellis 1981, Hilu 1984, Dávila and Clark 1990, Vieira *et al.* 2002, Leandro *et al*. 2016). Leandro *et al.* (2016) showed that leaf blade anatomy is of great importance in grass systematics, and can provide diagnostic characters for some taxa (Soreng *et al*. 2015). Soreng *et al*. (2015) studied a new species of *Dupontiopsis* (Koidz.) Soreng, L.J. Gillespie & Koba where they used Scanning Electron Microscopy and Light Microscopy. Different characters were selected to distinguish between *Dupontia* R. Br. and *Arctophila* Rupr. Ex Andersson. Certain characters of *Dupontia* also appeared to be similar to those of the genus *Poa* L. (Soreng *et al*. 2015).

To study the leaf epidermis, the most commonly used technique is Scanning Electron Microscopy (SEM), which is extremely useful in studying the three-dimensional structure of the leaf epidermis in extant and extinct grasses (Dávila and Clark 1990). A far older but just as important tool in understanding the anatomy of grass leaves is their structure as seen in cross section using a light microscope (Cutler *et al*. 2008). Different characters are observed from the abaxial and adaxial surfaces of the leaf blades as well as cross sections. These characters can be used to identify and distinguish different taxa (Ellis 1981).

According to Dávila and Clark (1990), surveying the leaf epidermis by means of SEM appears to be very useful in understanding the taxonomy of species in the Andropogonae (the tribe in which *T. triandra* is placed). Leaf epidermal anatomical characters such as trichomes and stomata are very useful for identification and classification of plants including grasses at species level, especially for the problematic species such as *Aristida stricta* Michx (Stant 1973, Kesler *et al.* 2003). Brown (1958) found the presence or absence of bicellular (micro) hairs to be a major character in grass systematics. Characters such as size, number and position of stomata can give an idea of the environment where the specimens are found i.e. rainfall, humidity etc. (Torre *et al*. 2003). According to Christin and Osborne (2014), high densities of minor veins (secondary vascular bundles) enable high rates of photosynthesis and are advantageous in productive environments.

T. triandra, as a morphologically variable species, has not received much attention in terms of leaf anatomy (Botha 1992, Botha *et al.* 1993). The studies so far only focused on the kranz-mesophyll bundle sheath, not on the anatomical diversity within the species leaves.

Considering the importance of leaf anatomy in Poaceae taxonomy, this chapter describes the leaf anatomical diversity within *T. triandra.* Abaxial and adaxial leaf anatomy was examined with Scanning Electron Microscopy (SEM) and transverse leaf blade sections examined using Light Microscopy (LM). The diversity of anatomical features were then coded and analysed using numerical taxonomic methods ("phenetics"; Sokal (1963)) to determine if the specimens can be grouped according to anatomical differences and similarities and to see if there are any geographical patterns in the anatomical variation.

2.2 Materials and Methods

2.2.1 Selection of samples

One hundred and twenty six specimens from two herbaria were surveyed: 66 from the Selmar Schonland herbarium (GRA) in Grahamstown and 60 from H.G.R.J. Schweickerdt herbarium (PRU) at the University of Pretoria. The specimens are from a wide range of regions in South Africa as well as countries outside of South Africa.

In addition 92 freshly collected specimens from southern Africa were also used. According to Ellis (1980) when sampling plant material one needs to standardise the leaf samples for all plants and which part of the leaf is being sampled. For the current study, the most mature leaves were sampled mid-blade. The fresh material was fixed as soon after collection as possible in Formalin-Acetic acid-Alcohol (FAA), [50% ethanol: 36% Formaldehyde: 14% Glacial Acetic Acid]. Where possible, Specimens were also removed from the wild and planted in the greenhouse at the University of Pretoria experimental farm. These were grown and used to make herbarium

specimens once they flowered. For SEM both fresh and herbarium specimens were used, but only fresh specimens were used for LM. This was mainly because for herbarium specimens to be used in light microscopy, re-hydration was required and this process is time consuming and would have delayed the study. The diagram below summarises how the study was structured and the resultant analyses of the data sets.

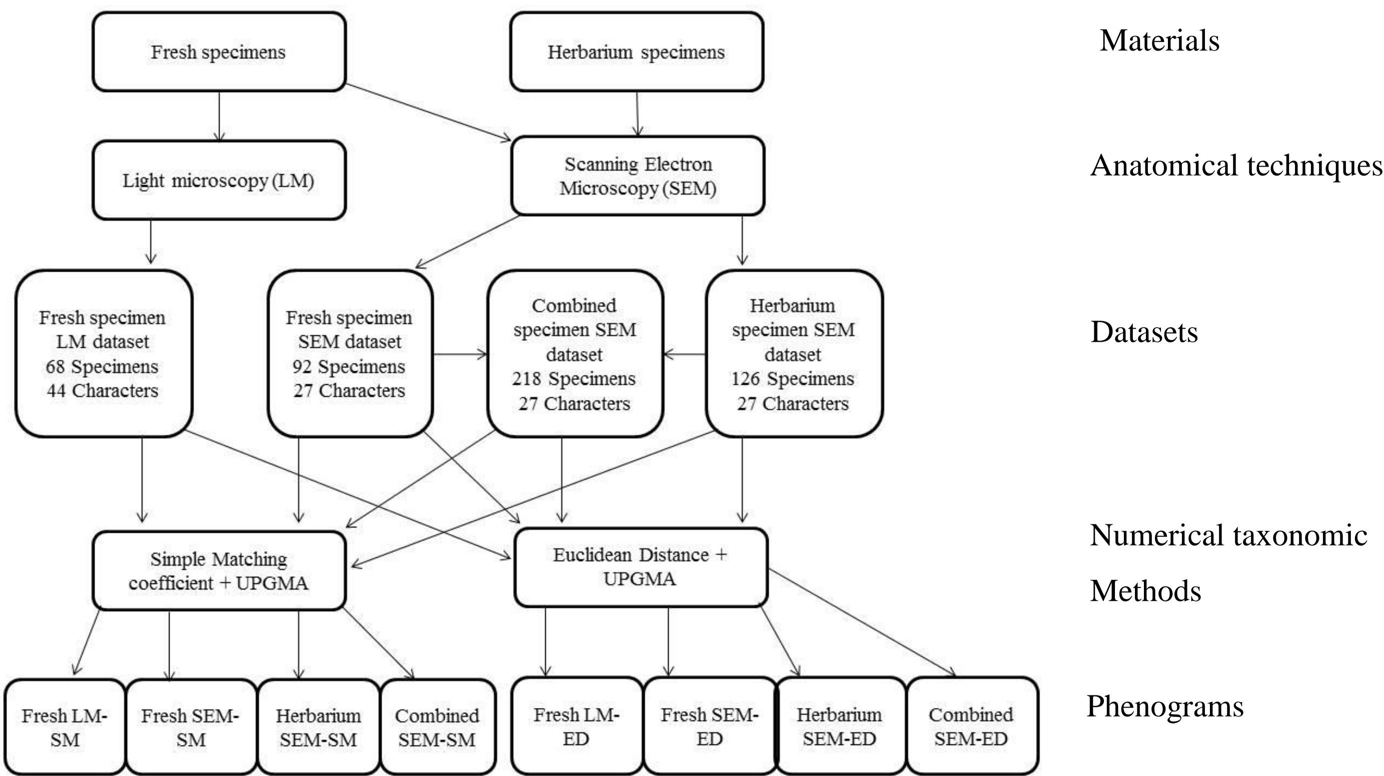


Figure 2.1: A summarised structure of how the anatomical survey was conducted.

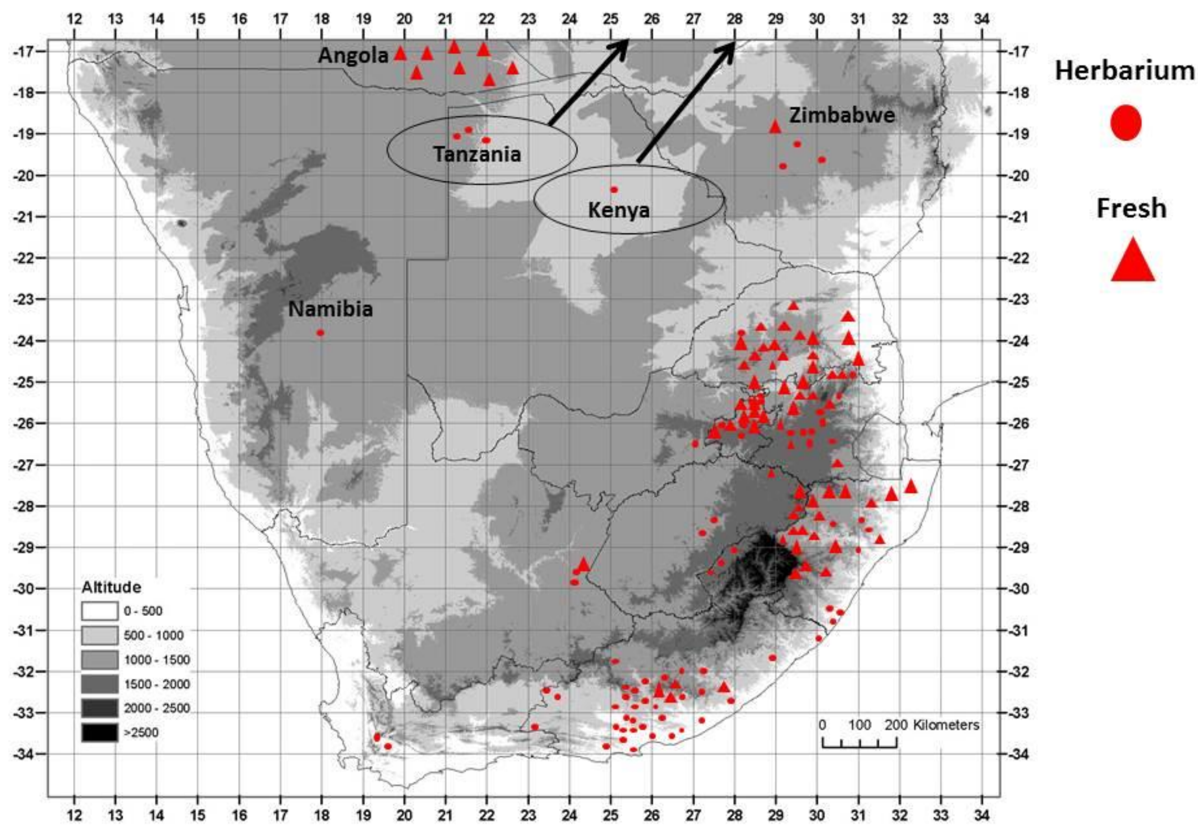


Figure 2.2: Map of the locations of the specimens (both herbarium and fresh specimens) used in the study

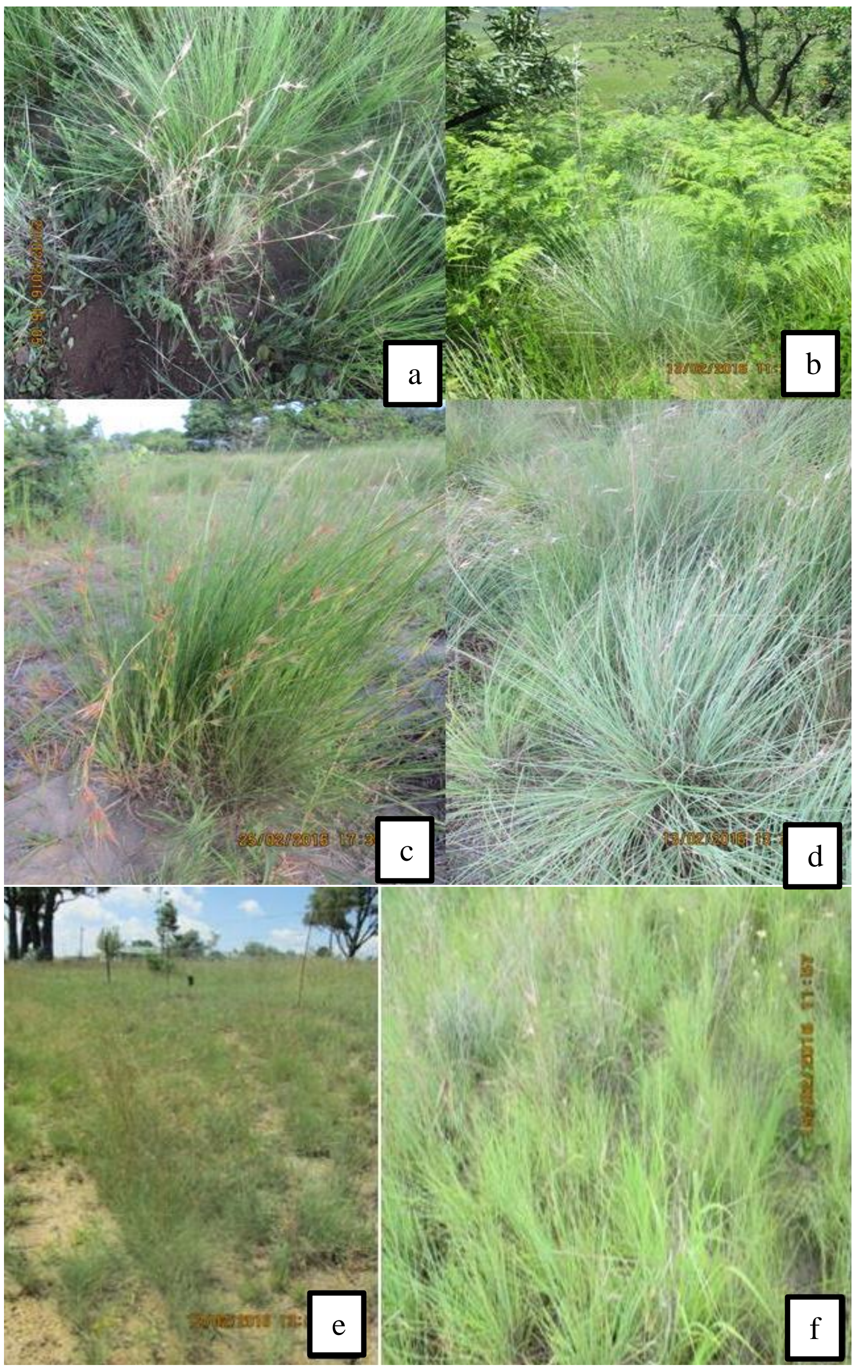


Figure 2.3: Different forms of *T. triandra* collected from KwaZulu-Natal. (a) from the Lowlands (short and thinner tuft, with a yellowish colour); (b) and (d) from the midlands (1.3m tall, thick tuft and bluish-green colour), (c) from the coastal area, near St. Lucia (with a big tuft, about 40cm tall and a bright green colour), (e) from Warden (1.5m tall, brownish-green colour, with a thin tuft) and (f) from Harrismith (35cm tall, yellowish-green colour and a thin tuft).

2.2.1.1 Scanning Electron Microscopy (SEM)

Herbarium material was already dry, and therefore did not require dehydration. However, the fresh material that had been fixed in FAA required dehydration. The procedure used was as follows:

1. The leaf samples were cut small pieces (±5mm long).
2. They were then rinsed in 1M Na_2PO_4 buffer three times for five minutes each time.
3. They were transferred into a dehydration series of ethanol (30%, 50%, 70% and 90%) for 10 minutes in each concentration.
4. They were then transferred into absolute ethanol for 30 minutes.
5. Specimens were transferred into a 50/50 solution of absolute ethanol and 100% Hexamethyldisilazane (HMDS) for an hour.
6. The specimens were then transferred into 100% HMDS overnight then removed and allowed to dry overnight.

Once the specimens were dried, they were coated with carbon (University of Pretoria) or gold (Rhodes University) before viewing using a JEOL JSM-7001F Scanning Electron Microscope at Rhodes University and a Zeiss Field Emission Electron Microscope at the University of Pretoria.

Twelve micrographs were taken per specimen, i.e. six for the abaxial surface and six for the adaxial surface of the leaf. The magnifications used for the micrographs were standardised at 50X, 150X, 250X, 500X, 1000X and 2000X magnification. Specific features were sought out and photographed at the relevant magnification.

2.2.1.2 Light microscopy

As a consequence of their age and the time consuming nature of the rehydration process, herbarium material was not prepared for light microscopy. The following procedure was followed during the preparation and analysis of the transverse sections from fresh material.

1. Dehydration: The dehydration method followed that described by Ellis (1981). The fixed material was cut into small sections (±5mm) and placed into polytops with 70% ethanol for 30 minutes. The specimens were then transferred into 90% ethanol for six hours. This was repeated twice. They were then transferred into absolute ethanol for six hours and this was also repeated twice. They were then transferred into 50/50 ethanol/xylene for three hours, after which the specimens were transferred into 100% xylene for three hours.

2. Infiltration: After dehydration specimens were transferred into 100% xylene and two scoops (±1.5g per scoop) of paraffin wax were added into the polytops. These were put on the slide warmer for overnight. The following day three scoops (±1.5g per scoop) were added and left on the slide warmer overnight again. The following day these were left in the oven set at 60˚C overnight. The following day this wax and xylene mixture was poured out and fresh molten wax was poured into the specimens. The specimens were left in the oven overnight. This was repeated twice.

3. Wax embedding: An embedding mould long enough to hold two to three ±5mm long specimens was made with tough paper. The mould was labelled at the bottom with a pencil. Molten wax was then poured into the mould which had been in the oven at 60˚C. Before the wax could set, two or three leaf pieces were removed from the polytops and placed in the moulds with heated forceps. These were placed at the centre and vertically, to allow for better cutting position. Once the samples had been positioned, the mould was placed in cold water to solidify the wax.

4. Sectioning: The wax blocks were removed from the moulds and trimmed, with care taken to ensure that the material did not get damaged. These trimmed blocks of wax were mounted on wooden microtome discs using wax and a heated spatula. The discs were then left in the fridge overnight. They were placed on the microtome and ribbons of 8µm serial sections were cut. These ribbons were then placed in cold water to allow for debris to be removed. They were then transferred into a warm bath at 60˚C for about 5 minutes and then transferred into cold distilled water to cool. They were then transferred onto the slides to be made into permanent slides. They were dried on the slide warmer for about 30 minutes, and then cooled for 10 minutes.

5. Staining: Once the slides with wax ribbons had cooled, they were put into xylene for 10 minutes (twice). They were then dried on the slide warmer for 30 minutes and again cooled for 10 minutes. The sections on the slides were stained in Safranin for 15 minutes, and then transferred into 70% ethanol for a few seconds, then 90% ethanol for a few seconds. They were then transferred into fast-green for 1-2 minutes, after which they were transferred into 100% ethanol, then into 50/50 ethanol/xylene and lastly 100% xylene. The slides are then dried with a paper towel then a mountant (Entellen) was used to mount the cover slip, making permanent slides. The slides were then observed under the Nikon Eclipse E200 light microscope and photographed using a Nikon digital camera Coolpix 950 at 40X, 100X and 400X magnifications.

2.2.2 Character selection for numerical taxonomic analyses: Scanning Electron Microscopy

From the micrographs 27 characters were selected from the abaxial side of the leaf epidermis and 27 characters from the adaxial side. The characters and their states are described in the table below (descriptions and terminology as per Ellis (1981). Each specimen was coded for each character and the data was recorded in an Excel spread sheet. In instances where a character did not exist and therefore there was no data, a code 999 was used. The codes were chosen using a criteria indicated in Sokal (1963).

Table 2.2: Table of characters for numerical taxonomic analyses derived from Scanning Electron Microscopy survey of the abaxial and adaxial surface of *Themeda triandra* leaves.

Character	**Character number**	**Character states**
Macro-Hairs: These are unicellular, long trichomes (longer than the micro-hairs and prickles).	1	Macro-hairs: Absent = 0; Present = 1
	2	Macro-hair frequency: Dense = 0; Occasional = 1
	3	Macro-hair length: (numerical value in µm)
Micro-hairs: These are bicellular trichomes (sometimes unicellular hairs occur, but are homologous with the bicellular hairs).	4	Micro-hairs: Absent = 0; Present = 1
	5	Micro-hair frequency: Dense = 0; Occasional = 1

	6	Micro-hair length: (numerical value in μm)
Prickle hairs: These are robust, pointed structures of varying sizes and shapes. *Themeda triandra* was found to have two types of these prickle hairs. They are **large prickles** (with bases at least twice as large as the stomata) and **long barb prickles** (thinner than the large prickles and barb as long as, or longer than the base). Large prickles	7	Large prickles: Absent = 0; present = 1
	8	Large prickles density: Dense = 0; Occasional = 1

Long barb prickles	9	Large prickles base structure: with a collar = 0; without a collar = 1
	10	Large prickles length: (numerical value in µm)
	11	Large prickles size uniformity (whether the large prickles on one leaf epidermis are of the same sizes or not): Not uniform = 0; Uniform = 1
	12	Large prickles orientation: Erect = 0; not erect = 1
	13	Large prickles distribution: On all 1° and/or 2° bundles, but not the midrib = 0; on 1°, 2° and midrib = 1
Leaf margin prickles	14	Long barb prickles: Absent = 0

		Present = 1
	15	Long barb prickles length: (numerical value in µm)
	16	Long barb prickles density: Dense = 0; Occasional = 1
	17	Leaf margin prickles: Absent = 0; Present = 1
	18	Leaf margin prickles length: (numerical value in µm)
Silica bodies: These are silica cells found in the grass leaves. They come in different shapes. *T. triandra* consists of the dumbbell-shaped silica bodies	19	Dumbbell-shaped silica bodies (DSSB): Not visible = 0; Visible = 1

<u>Papillae:</u> These are variously shaped protrusions from the leaf epidermis. *T. triandra* has circular-shaped papillae.	20	Papillae: Absent = 0; Present = 1
	21	Papillae diameter: (numerical value in µm)
<u>Stomata-</u>(Singular-stoma): These are structures on the leaf epidermis that are used for gas-exchange. They comprise of two guard cells that form a tiny opening. They are normally found on the abaxial side, but sometimes do occur on the adaxial side. L W	22	Stomata: Absent/not visible = 0; Present/visible = 1
	23	Stomata length (L): (numerical value in µm)
	24	Stomata width (W): (numerical value in µm)

Epidermal cells: These occur on the epidermis of the leaf. They may be intercostal or costal. *Themeda triandra* consists of costal and intercostal cells, mostly visible on the adaxial side of the leaf, with a few occurrences on the abaxial side.	25	Intercostal cells: Absent/not visible = 0; Present/visible = 1
	26	Cell length: (numerical value in µm)
	27	Cell width: (numerical value in µm)
		Cell shape (within one leaf epidermis): Varying shapes = 0; constant shapes = 1

2.2.3 *Selection of characters for Light Microscopy of Themeda triandra leaves*

Forty four characters were derived from the light micrographs (Table 2.3). The specimens were scored for each of the characters and the data recorded in an Excel spread sheet.

Table 2.3: Table of characters for numerical taxonomic analyses derived from Light Microscopy survey of the leaf transverse sections of *Themeda triandra*.

Anatomical feature	Character number	Character states and coding
Outline of open leaves: *T. triandra* leaves are not permanently infolded, but due to different environmental conditions their shapes may differ. V-shaped U-shaped Wide-open Narrow Concave Convex	1	V-shaped (0) U-shaped (1) Wide open – almost 180˚ (2) Folded (3) Concave (4) Convex (5)
Ribs: These are all the longitudinal ribs/ridges and furrows/grooves that commonly occur on the adaxial side and seldom occur on the abaxial side of the leaf. Ribs	2	Ribs on the adaxial side: Absent = 0; Present = 1
	3	Ribs on the abaxial side: Absent = 0; Present = 1
	4	On the adaxial side: Ribs only over the 1˚ and 2˚ bundles = 0; Over only 1˚ or 2˚ bundles = 1
	5	On the abaxial side: Ribs opposite 1˚ bundles = 0; Opposite both 1˚ and 2˚ bundles

		= 1
Vascular bundle arrangement: Measured on one half of the leaf expanding from the midrib. The two halves of the leaf are of the same length and thickness.	6	Total number of 1˚ vascular bundles
	7	Total number of 2˚ vascular bundles
	8	Total number of 3˚ vascular bundles
	9	1˚ bundles fewer than 2˚ and 3˚ bundles = 0; They are an equal number = 1
	10	Two 3˚ bundles occur between the larger bundles = 0; More than two 3˚bundles occur between larger bundles = 1
	11	Two 2˚ bundles occur between 1˚ = 0; More than two 2˚ bundles occur between 1˚ bundles = 1
	12	Vascular bundles: Positioned at the centre of the blade = 0; They are closer to either the abaxial = 1; The adaxial side of the leaf = 2
Vascular bundle description:	13	3˚ bundles: With a circular shape = 0; With an elliptical shape = 1

	14	2° bundles: With an circular shape = 0; With an elliptical shape = 1
	15	1° bundles: With an egg-shape, i.e. broad on the adaxial side = 0; With a circular shape = 1
	16	Phloem of the 1° bundle: Completely surrounded by thick-walled fibres = 0; Divided by the intrusion of small fibres = 1
	17	Lysigenous cavity: Absent on the 1° bundles = 0; Present = 1
Vascular bundle sheath:	18	Vascular bundle sheath: incomplete due to slight interruptions from narrow/ wide girders of 1-3 or more fibres = 0; Sheath complete, i.e. completely surrounds the 1° bundle = 1
	19	Length of extension of sheath: 1-2 cells long = 0; 2-5 cells long = 1
	20	Number of cells comprising the 1° bundle sheath.
	21	Number of cells comprising the

		2° bundle sheath.
	22	Number of cells comprising the 3° bundle sheath.
<u>Sclerenchyma of the leaf:</u> Sclerenchyma	23	Sclerenchyma: Associated with 1° vascular bundles only = 0; Associated with either 2° or 3° bundles = 1
	24	On the adaxial side sclerenchyma: Narrow, with only 2-4 fibres wide = 0; With more than 4 fibres = 1
	25	1° vascular bundle girders on the adaxial side: Well developed, conspicuous and narrowing towards the 1° bundle = 0; They form a straight/horizontal band towards the 1° bundle = 1
	26	2° vascular bundle girders on the adaxial side: Well developed, conspicuous and narrowing towards the 2° bundle = 0; They form a straight/horizontal band towards the 2° bundle = 1
	27	Girder fibres of the adaxial and the abaxial side: In contact with the cells of the single or outer 1° bundle sheath =

		0; Fibres interrupt the cells of the single or outer 1° bundle sheath = 1
	28	Girder fibres of the adaxial and the abaxial side: In contact with the cells of the single or outer 2° bundle sheath = 0; Fibres interrupt the cells of the single or outer 2° bundle sheath = 1
	29	Sclerenchyma of the abaxial side: Deep and wide than that of the adaxial side = 0; Is the same size as that of the adaxial side = 1
	30	Girder on the abaxial side: Narrowing towards the 1° vascular bundle, i.e. triangular/trapezoidal = 0; Forms a straight/horizontal band = 1
	31	Sclerenchyma in leaf margins: Absent = 0; Present, comprising a couple of fibres = 1; or Present, with a width less than 3° bundle = 2
	32	The marginal cap: Pointed = 0; Round = 1;

		Crescent-shaped, i.e. sclerenchyma extends shortly along both the abaxial and adaxial sides of the leaf = 2
Mesophyll of the leaf: Mesophyll	33	Chlorenchyma: Radiate = 0; Irregular = 1
	34	One layer of Chlorenchyma cells around bundles = 0; More than one layers = 1
	35	Radiating mesophyll groups: Separated by irregular Chlorenchyma and intercellular air-spaces = 0; Separated by bulliform and colourless cell groups = 1
	36	Radiating cells: Reduced to two strips of Chlorenchyma by large girders or colourless parenchyma = 0; Completely surrounding the bundles = 1
	37	Colourless cells: Absent = 0; Present = 1
	38	Colourless cells: Closely associated with bulliform cells = 0; Not closely associated with bulliform cells = 1
	39	Colourless cells: Smaller than bulliform cells; are uninflated, often the same size as

		the Chlorenchyma cells = 0; Have similar size or shape as the bulliform cells, and are inflated = 1
	40	Colourless cells: Narrower than the bulliform cells = 0; Are the same width as the bulliform cells = 1
	41	Only one extension of colourless cells from each bulliform cell group = 0; Two extensions, one on either side = 1
<u>Epidermal cells</u>	42	Bulliform cells: Absent = 0; Present in groups = 1; Present, but not in groups = 2
	43	Bulliform cells occupy less than ¼ of the leaf thickness = 0; Occupy more than ¼ of the leaf thickness = 1
	44	Outer walls of typical epidermal cells: Slightly thickened or with a thin cuticle = 0; Not thickened with a very thin cuticle = 1

2.2.4 Numerical taxonomic analyses

To analyse the data, Numerical Taxonomy and Multivariate Analysis System (NTSys) V. 2.2 (Rohlf 2009) was used. This software was used to create Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phenograms and Principal Component Analysis (PCA). The data consists of a combination of quantitative and qualitative characters, therefore both Simple Matching (SM) and Euclidean Distance (ED) measures of similarity were used when creating the UPGMAs. SM is suited for qualitative data and Euclidean distance suited for quantitative data. As the data consists mostly of qualitative characters, the best option was considered to be SM. Phenograms from both SM and Euclidean distance measures were compared to see if there are specimens from similar localities on the same clusters. Visual comparisons of SM and ED phenograms were undertaken to observe similarities in the results. Similarly, PCA data was analysed and the Eigen values were recorded along with percentage and cumulative percentages.

Data obtained from the SEM survey was divided into three datasets, i.e. data from fresh specimens, herbarium specimens and combined fresh and herbarium specimens. Phenograms were created for each of the data sets. For light microscopy, phenograms were only created for fresh specimens, because only fresh specimens were used in light microscopy analysis. Finally scanning electron microscopy and light microscopy characters were combined to create phenograms that consisted of specimens common to both analyses, but this was only done for fresh specimens. Cophenetic Correlation Values (CCVs) were obtained for each analysis. The CCVs were used to compare the fit of the phenogram to the triangular matrix of similarities. CCV were also used to compare the similarities between the two triangular matrices from the phenograms obtained from the SM and ED analyses of the data. In addition, CCV’s were obtained from direct comparison of the two matrices of similarity (i.e. SM and ED).

2.3 Results and Discussion

2.3.1 Scanning Electron Microscopy Data Sets

UPGMA phenograms were created using two different distance measures, i.e. Simple Matching (clusters labelled with letters) and Euclidean Distance (clusters labelled with roman numerals). These are presented below (Figures 2.7, 2.9 and 2.11), separately, with distribution maps showing where specimens from each cluster are located (Figures 2.8, 2.10 and 2.12). Furthermore, the two phenograms were compared and the results of the comparison are shown below. Figure 2.7 presents the results obtained from the analysis of SEM of fresh specimens data set, Figure 2.9 is the results from the analysis of SEM of herbarium specimens data set and Figure 2.11 shows results from the analysis of SEM of the combined fresh and herbarium specimens data sets.

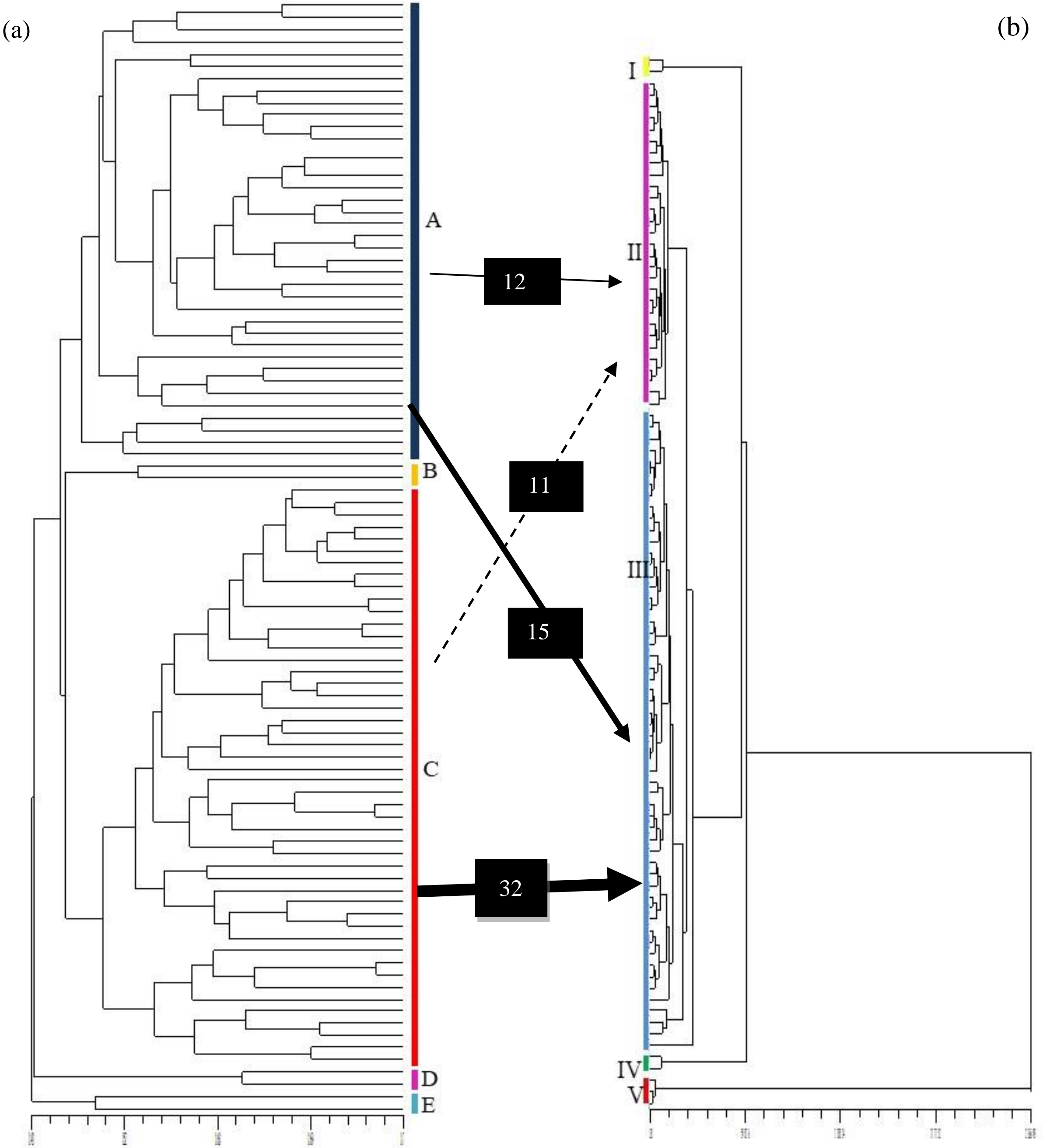
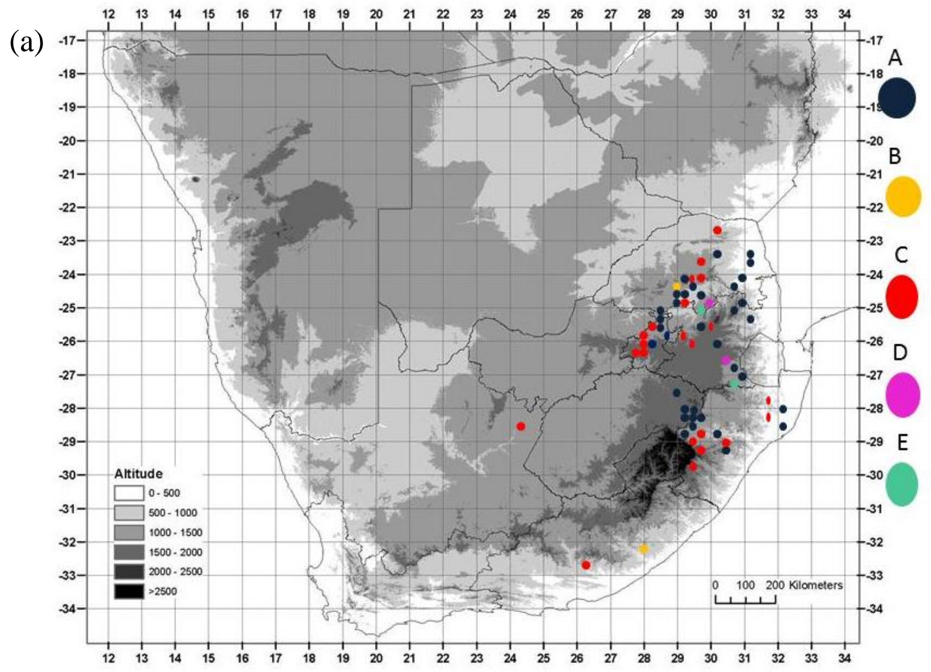


Figure 2.7: UPGMA phenograms from (a) SM coefficient (CCV = 0.61) and (b) ED coefficient (CCV = 0.43) applied to data obtained from SEM survey of fresh specimens. The number of specimens shared between the clusters is indicated by the lines inbetween the phenograms.

(a)



(b)

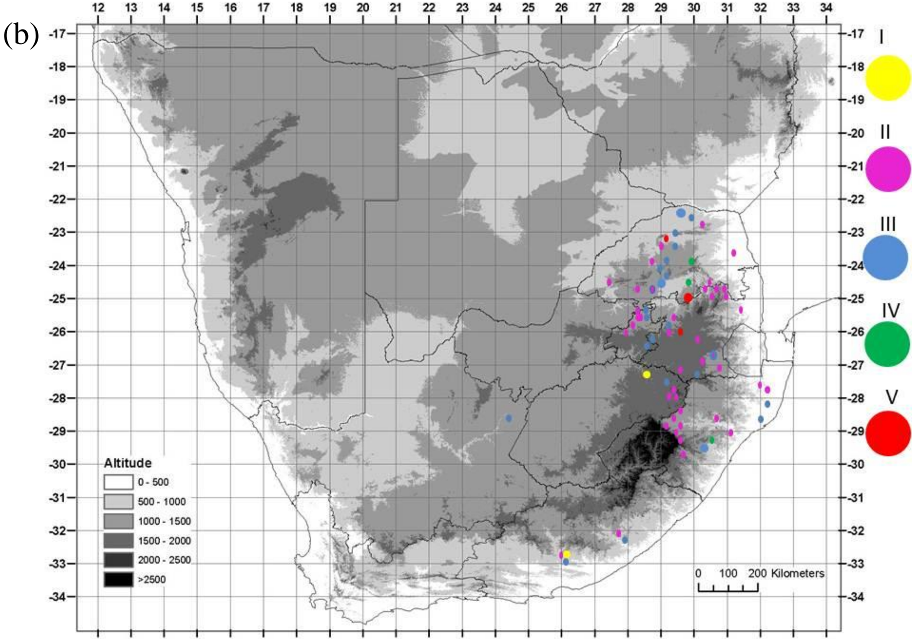


Figure 2.8: Maps showing the distribution of specimens in the clusters obtained from the SEM data of fresh material (a) UPGMA-SM and (b) UPGMA-ED analyses.

Table 2.4: The CCVs obtained from tree to matrix comparison, tree-tree comparison and matrix to matrix comparison for both SM and ED analyses of SEM - fresh specimens

	Simple Matching	Euclidean Distance
Tree – Matrix	0.61	0.43
SM Tree – ED Tree	0.023	
SM Matrix – ED Matrix	-0.068	

The anatomical diversity of the leaf abaxial and adaxial surfaces does not show any correlation of clusters with the specimens' geography.

SM and ED phenograms are shown in Figure 2.7. SM has five clusters (labelled A to E) with two big clusters (A and C), and ED also has five clusters (labelled I to V) with two big clusters (II and III). Table 2.4 shows the CCV values. The value obtained from the comparison of SM matrix to tree (0.61) shows that the phenogram is a fair representation of the data matrix. The value obtained from ED matrix to tree (0.43), indicates that the tree does not represent the matrix of similarity very well. The value obtained from comparing the CCVs of the tree matrices (0.023) show that there is no correspondence between the trees. Lastly, the value obtained from comparing the CCVs of the two matrices (-0.068) shows no correspondence between the SM and ED matrices. These poor correlations between SM and ED are probably because SM is suited to qualitative data and ED is suited to quantitative data. The data analysed in the current study is mainly qualitative with only 11 quantitative characters out of 27 characters.

In order to determine which characters and states were specific to each cluster, the data sets were examined to assess the character state distribution per cluster observed. The analysis of the clusters (as shown in Appendix 2.1) shows that two characters out of the 27 characters played a role in clustering. On the ED phenogram specimens in cluster IV all had **Dumbbell-shaped silica bodies** present and the specimens in cluster V all had DSSBs absent. One cluster differed from the rest (cluster I of ED) which had the specimens with **stomata** on the abaxial side only.

(a)

(b)

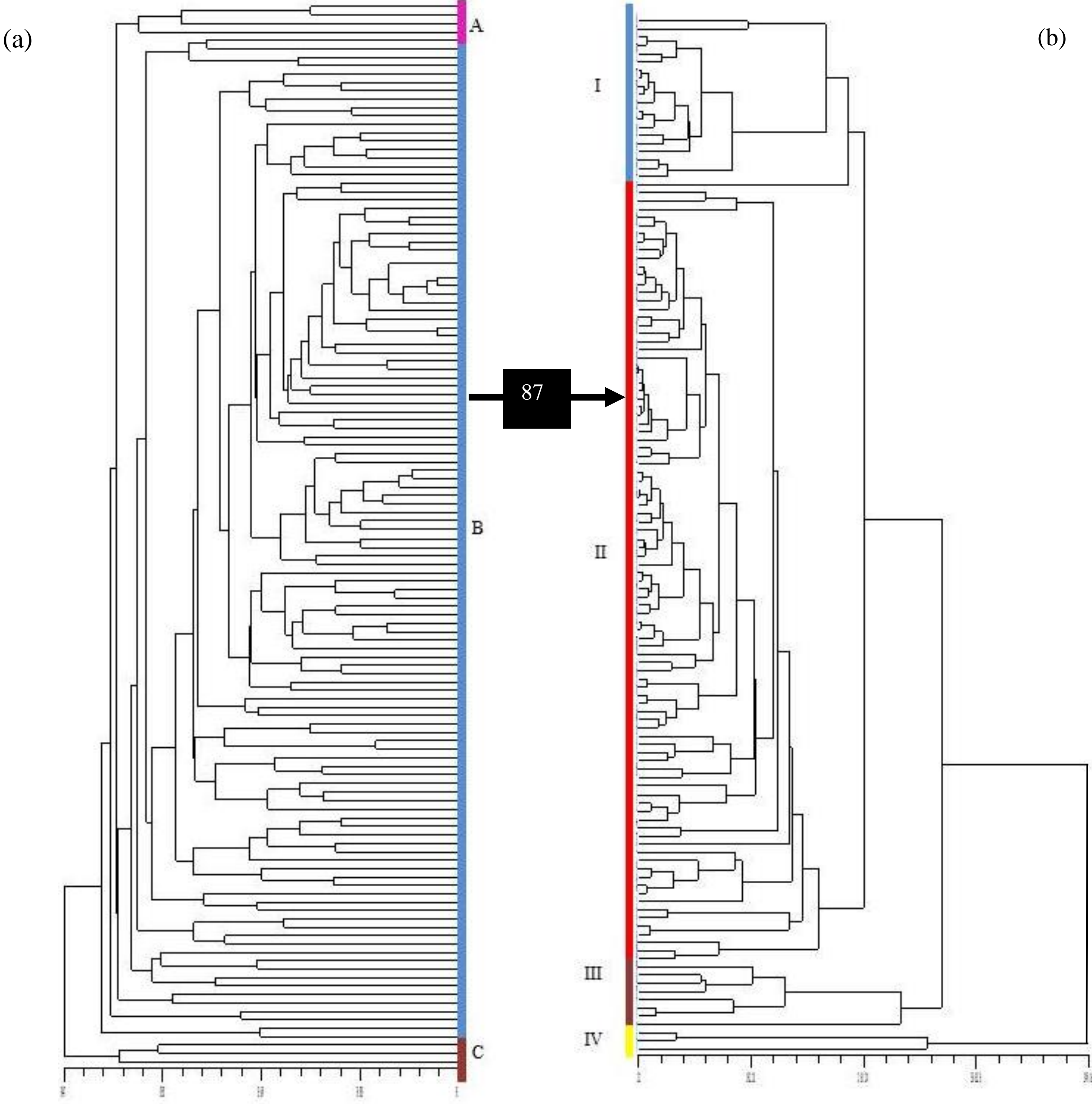
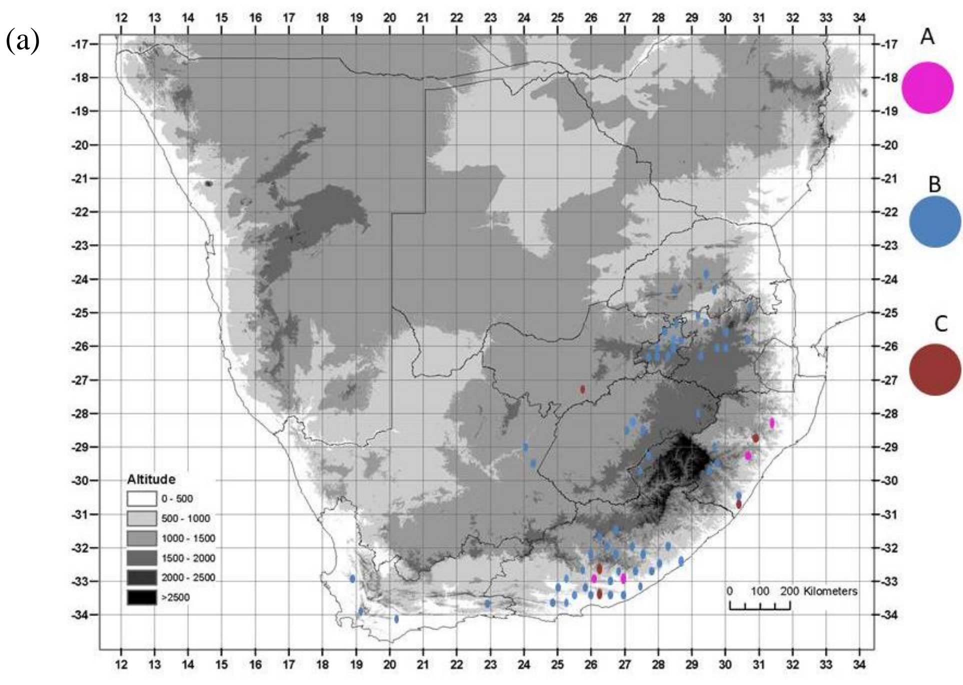


Figure 2.9: UPGMA phenograms from (a) SM coefficient (CCV = 0.75) and (b) ED coefficient (CCV = 0.31) applied to data obtained from SEM survey of herbarium specimens with the number of shared specimens between the clusters.

(a)



(b)

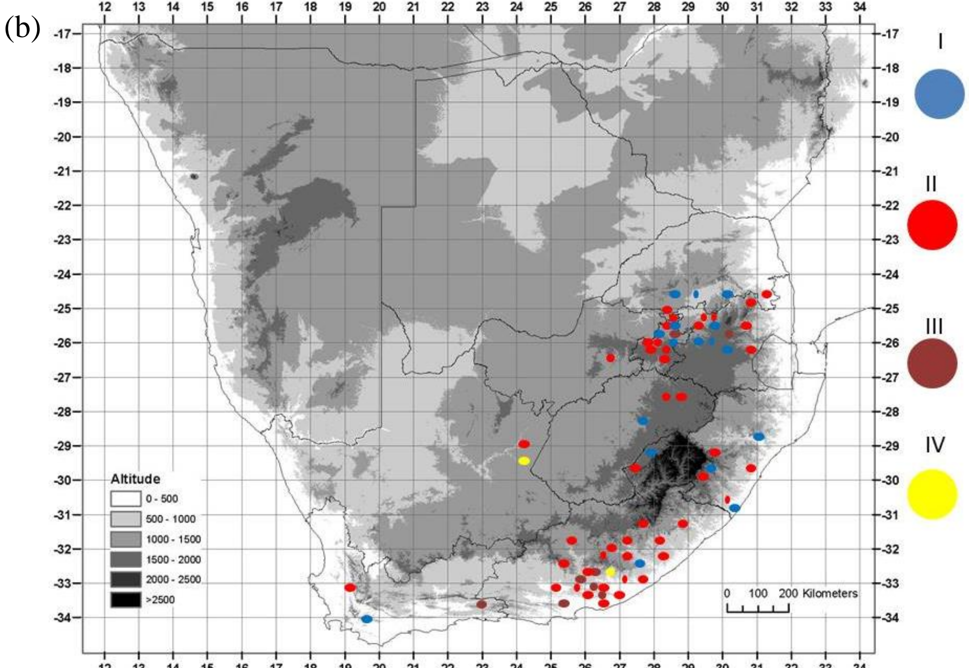


Figure 2.10: Maps showing the distribution of specimens in the clusters obtained from (a) the UPGMA-SM and (b) the UPGMA-ED SEM analyses of herbarium specimens.

Table 2.5: The CCVs obtained from tree to matrix comparison, tree-tree comparison and matrix to matrix comparison for both SM and ED analyses of SEM – herbarium specimens.

	Simple Matching	Euclidean Distance
Tree – Matrix	0.75	0.31
SM Tree – ED Tree	-0.05	
SM Matrix – ED Matrix	-0.076	

SM and ED phenograms are shown in Figure 2.9. The SM phenogram has three clusters (labelled A to C) with one big cluster (B), and ED has four clusters (labelled I to IV) with one big cluster (II). Table 2.5 shows the CCVs obtained from the data set derived from SEM of herbarium materials. The value obtained from the comparison of SM matrix to tree (0.75) shows that the phenogram is a good representation of the similarity matrix. The value obtained from ED matrix to tree comparison is low (0.31) indicating that the tree does not represent the matrix of similarity well. The value obtained from comparing the CCVs of the tree matrices (-0.05) show that there is no correspondence between the trees, and the value obtained from comparing the CCVs of the two matrices (-0.076) also shows no correspondence between the SM and ED matrices.

In order to determine which characters and states were specific to each cluster, the data sets were examined to check the character state distribution per cluster observed. The analysis of the clusters (as shown in Appendix 2.2) shows that out of the 27 chosen characters not a single one may have shaped clustering of the specimens on both SM and ED phenograms.

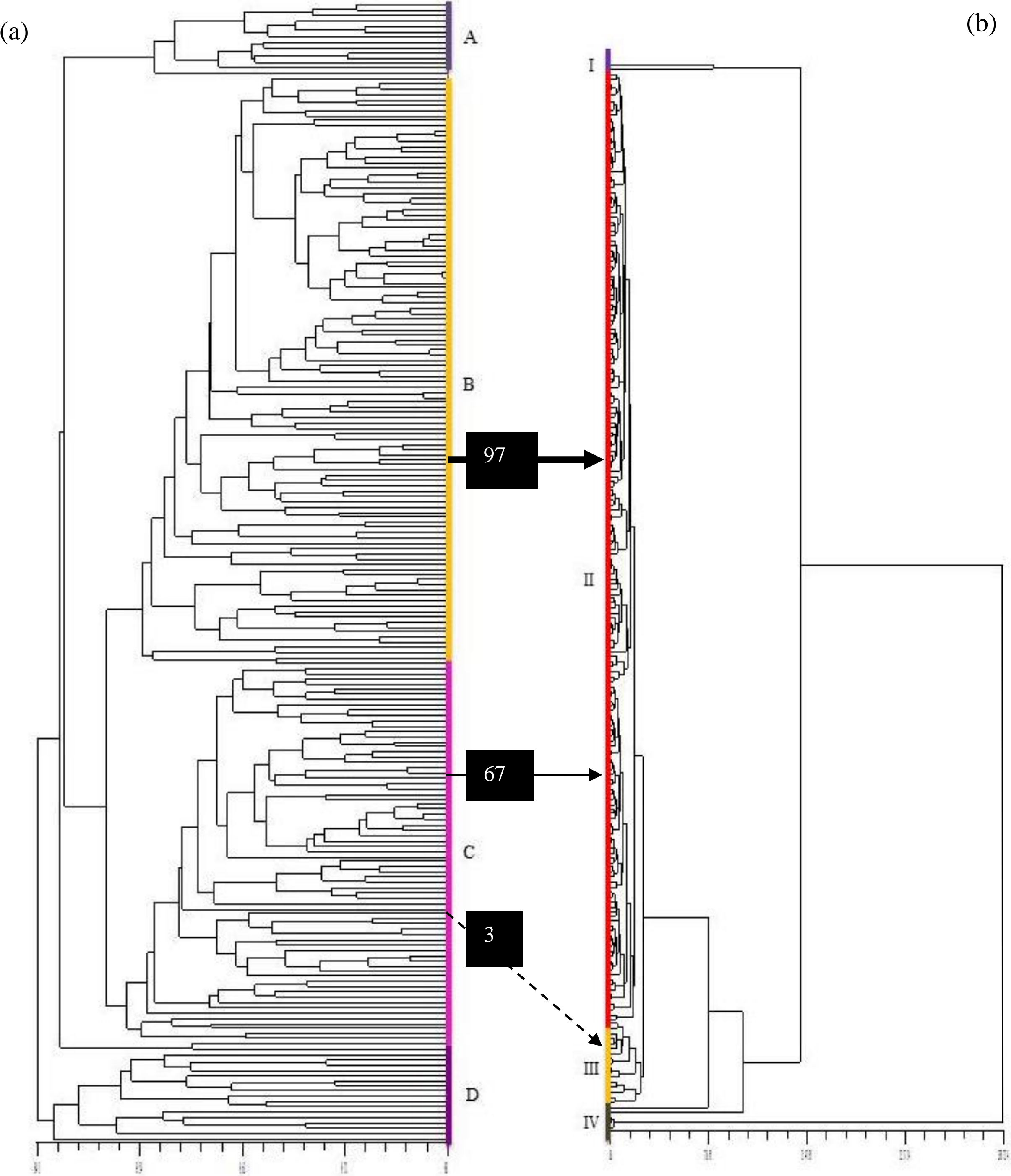
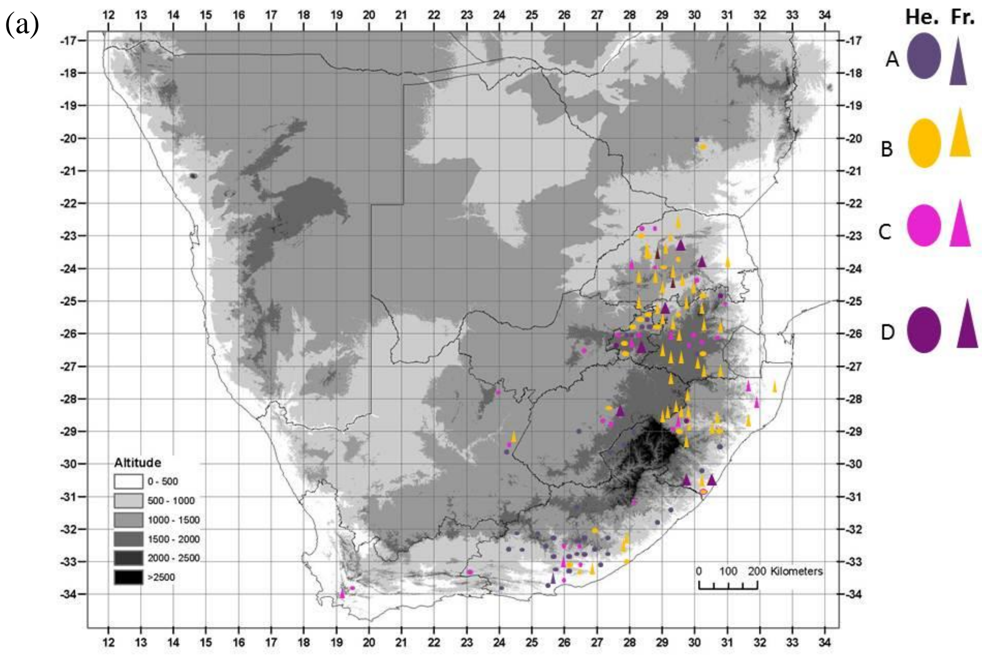


Figure 2.11: UPGMA phenograms from (a) SM coefficient (CCV =0.65) and (b) ED coefficient (CCV = 0.054) applied to data obtained from SEM survey of fresh and herbarium specimens with the number of shared specimens between the clusters.

(a)



(b)

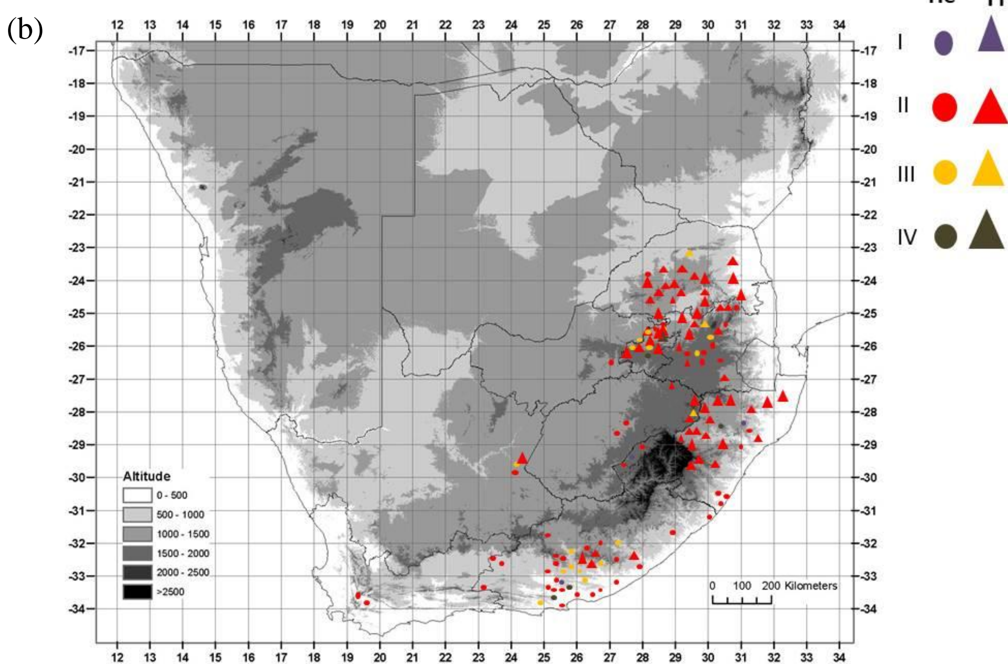


Figure 2.12: Maps showing the distribution of specimens in the clusters obtained from (a) the UPGMA-SM and (b) the UPGMA-ED SEM analyses of fresh (Fr) and herbarium (He) specimens.

Table 2.6: The CCVs obtained from tree to matrix comparison, tree-tree comparison and matrix to matrix comparison for both SM and ED analyses of SEM – data set from fresh and herbarium specimens.

	Simple Matching	Euclidean Distance
Tree – Matrix	0.65	0.54
SM Tree – ED Tree	0.033	
SM Matrix – ED Matrix	0.07	

SM and ED phenograms are shown in Figure 2.11. SM has four clusters (labelled A to D) with two big clusters (B and C), and ED has four clusters (labelled I to IV) with one big cluster (II). Table 2.6 shows the CCVs obtained from the data analysed from SEM of fresh and herbarium materials. The value obtained from the comparison of SM matrix to tree (0.65) shows that the phenogram is a fair representation of the data matrix. The value obtained from ED matrix to tree (0.54), indicates that the tree fairly represents the matrix of similarity well. The value obtained from comparing the CCVs of the tree matrices (0.033) show that there is no correspondence between the trees. Lastly, the value obtained from comparing the CCVs of the two matrices (0.07) shows no correspondence between the SM and ED matrices.

In order to determine which characters and states were specific to each cluster, the data sets were examined to check the character state distribution per cluster observed. The analysis of the clusters (as shown in Appendix 2.3) shows that out of the 27 chosen characters the presence/absence of DSSBs may have shaped clustering of the specimens on both SM and ED phenograms, especially cluster I of the ED phenogram.

2.3.2 Light Microscopy

UPGMA phenograms were created for Light Microscopy using two different distance measures, i.e. Simple Matching (clusters labelled with letters) and Euclidean Distance (clusters labelled with roman numerals). Figure 2.13 presents the results obtained from the analysis of LM of fresh specimens data set and Figure 2.14 shows the distribution maps from where the specimens were collected.

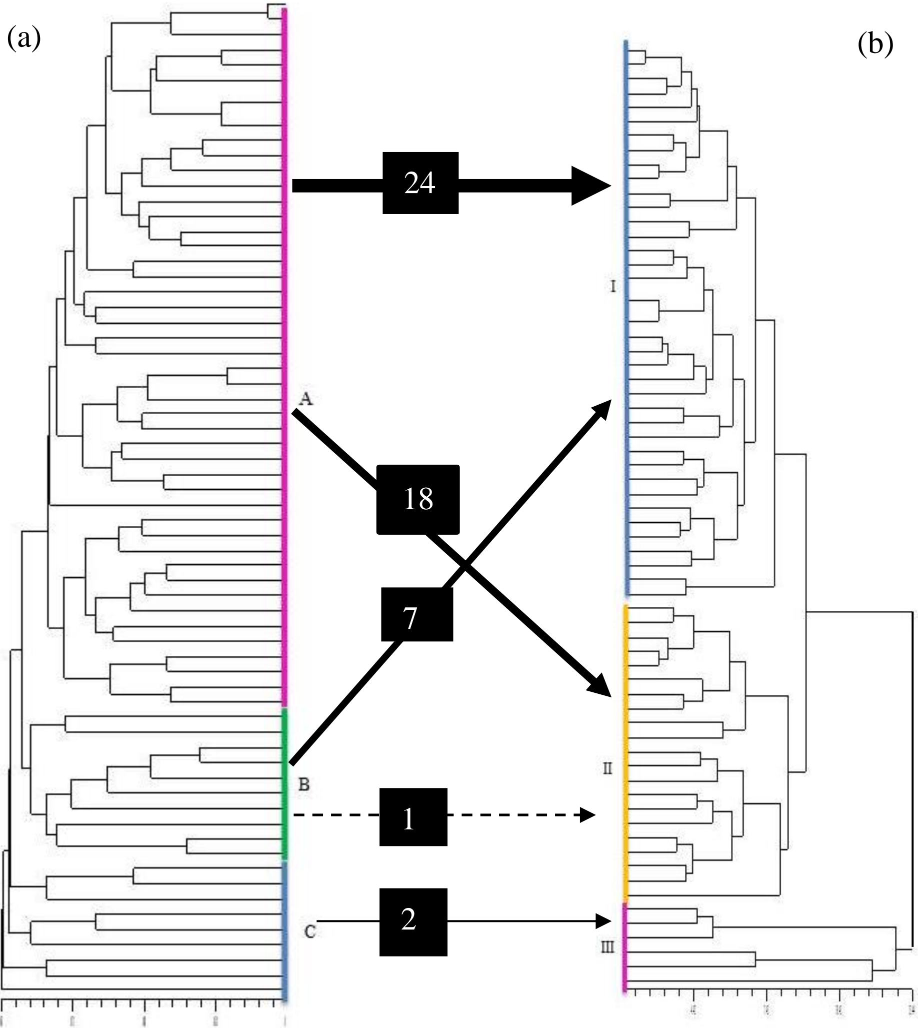


Figure 2.13: UPGMA phenograms from (a) SM coefficient (CCV = 58%) and (b) ED coefficient (CCV = 75%) applied to data obtained from LM survey of fresh specimens with the number of shared specimens between the clusters.

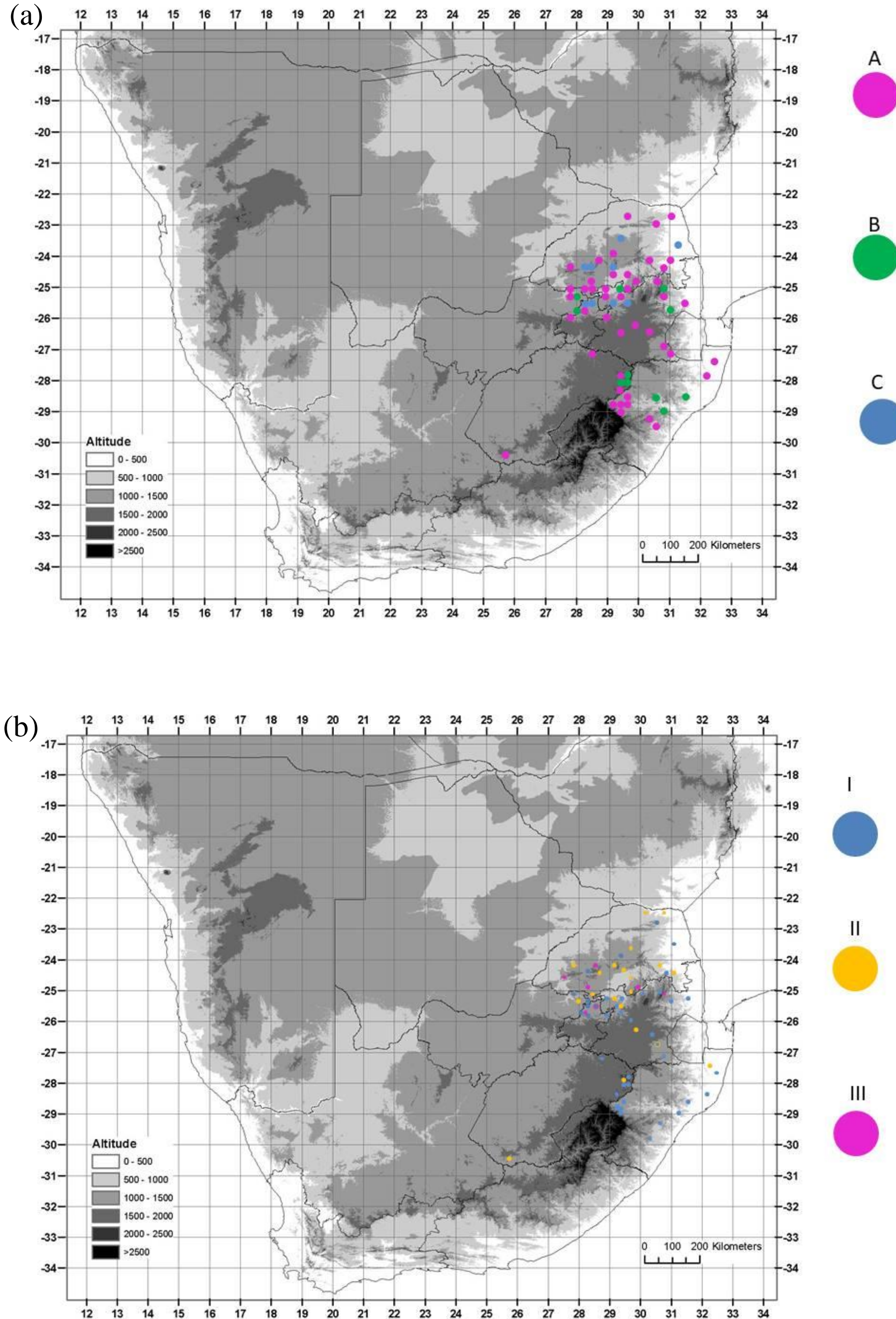


Figure 2.14: Maps showing the distribution of specimens in the clusters obtained from (a) the UPGMA-SM and (b) the UPGMA-ED LM analyses of fresh specimens

Table 2.7: The CCVs obtained between tree to matrix comparison, tree-tree comparison and matrix to matrix comparison for both SM and ED analyses of LM - fresh specimens

	Simple Matching	Euclidean Distance
Tree – Matrix	0.58	0.75
SM Tree – ED Tree	-0.20	
SM Matrix – ED Matrix	-0.33	

SM and ED phenograms from LM analysis are shown in Figure 2.13. SM has three clusters (labelled A to C) with one big cluster (A), and ED also has three clusters (labelled I to III) with one big cluster (I). Table 2.7 shows the CCVs obtained from the data analysed from LM of fresh materials. The value obtained from the comparison of SM matrix to tree (0.58) shows that the phenogram is a fair representation of the data matrix. With regards to the value obtained from ED matrix to tree (0.75), it is clearly shown that the tree is a good representation of the similarity matrix. The value obtained from comparing the CCVs of the tree matrices (-0.20) show that there is no correspondence between the trees. Lastly, the value obtained from comparing the CCVs of the two matrices (-0.33) shows no correspondence between the SM and ED matrices.

In order to determine which characters and states were specific to each cluster, the data sets were examined to check states distribution per cluster observed. There were 47 characters measured from the light microscopy micrographs. None of these characters appeared to have a role in clustering. The table with all the characters and character states is found in Appendix 2.4.

2.3.3 Scanning Electron Microscopy and Light Microscopy

The following UPGMA phenograms (Figure 2.15) were created for Scanning Electron Microscopy and Light Microscopy combined using two different distance measures, i.e. Simple Matching (clusters labelled with letters) and Euclidean Distance (clusters labelled with roman numerals), Figure 2.16 shows the distribution maps from where the specimens were collected.

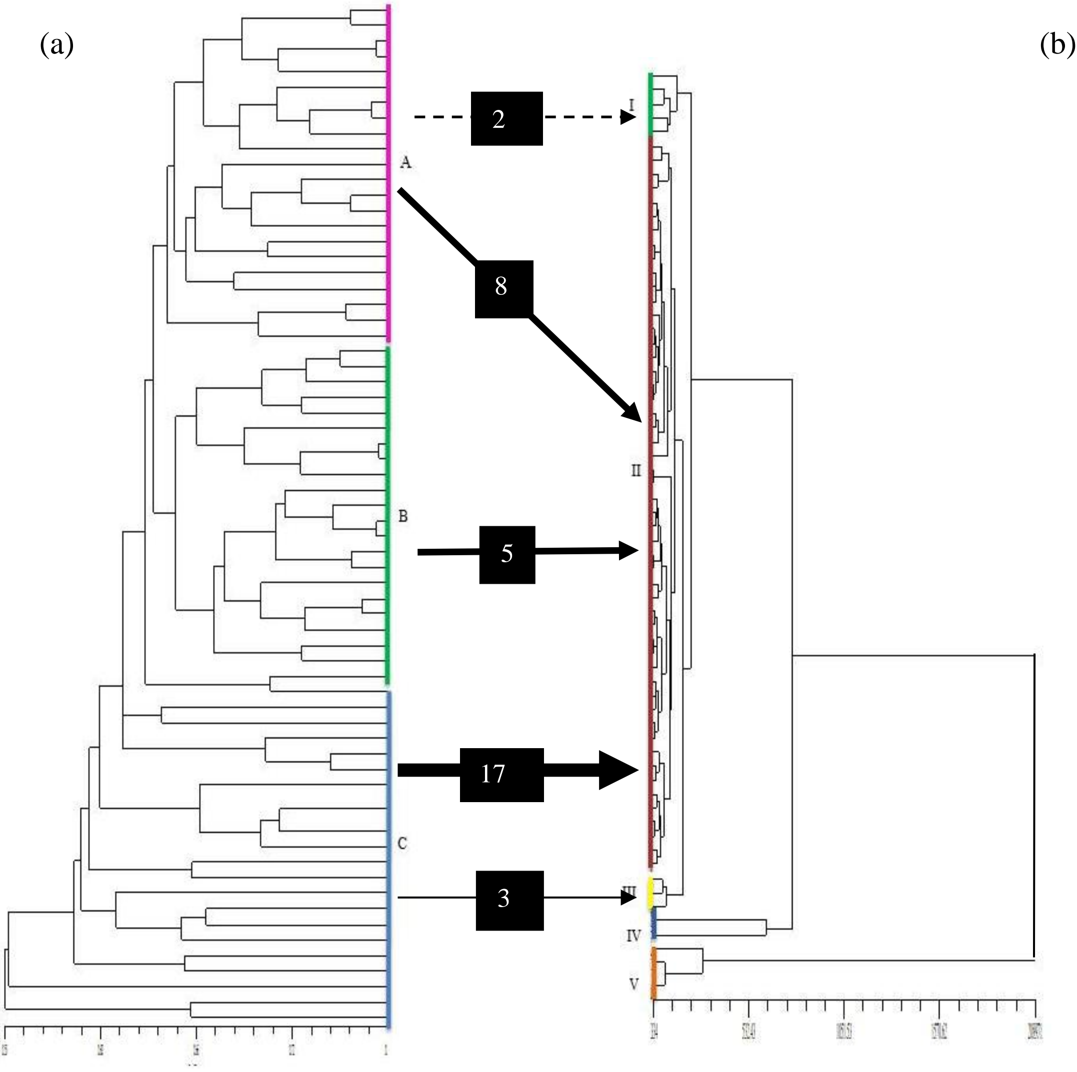
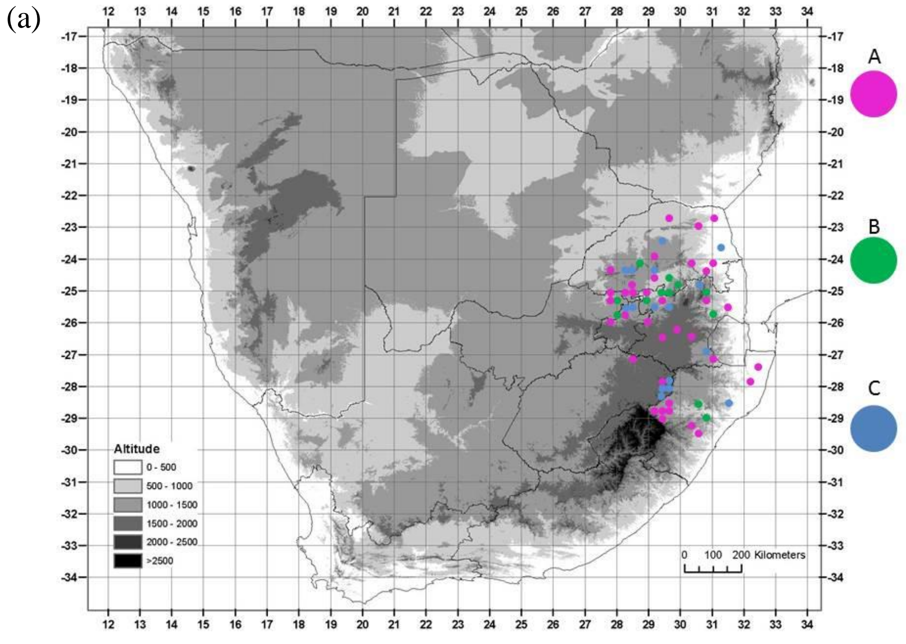


Figure 2.15: UPGMA phenograms from (a) SM coefficient (CCV = 65%) and (b) ED coefficient (CCV = 43%) applied to data obtained from LM and SEM survey of fresh specimens with the number of shared specimens between the clusters.

(a)



(b)

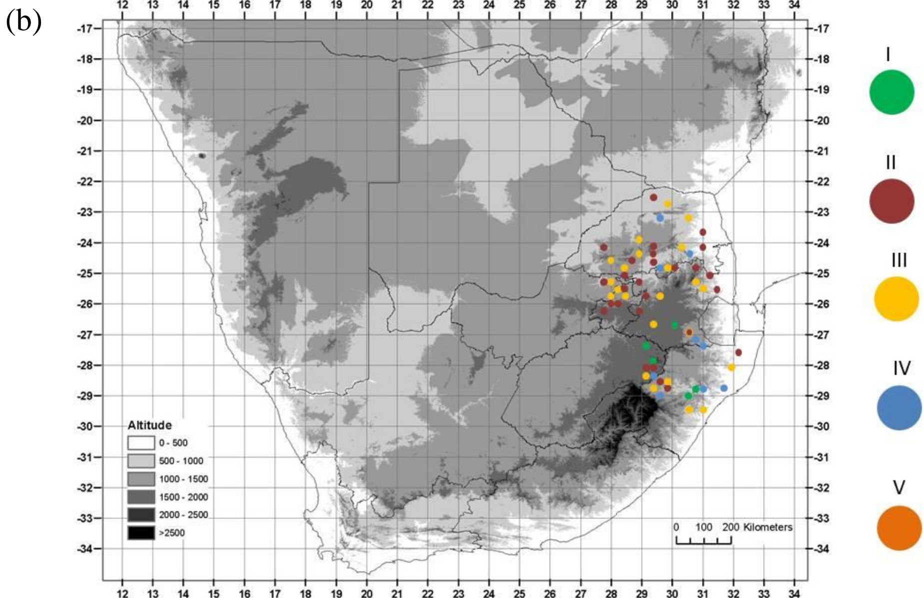


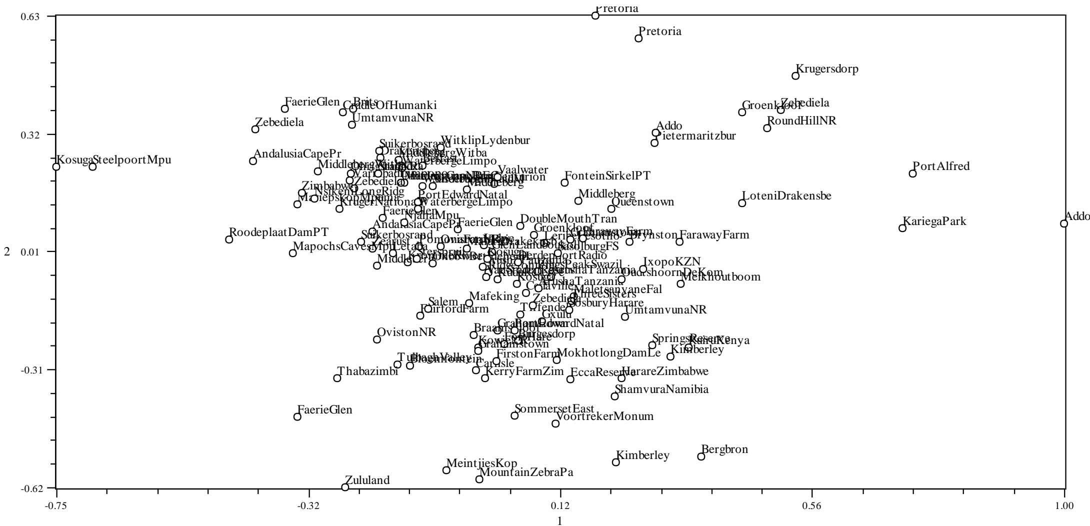
Figure 2.16: Maps showing the distribution of specimens in the clusters obtained from (a) the UPGMA-SM and (b) the UPGMA-ED LM and SEM analyses of fresh specimens.

Table 2.8: The CCVs obtained from tree to matrix comparison, tree-tree comparison and matrix to matrix comparison for both SM and ED analyses of SEM and LM combined - fresh specimens.

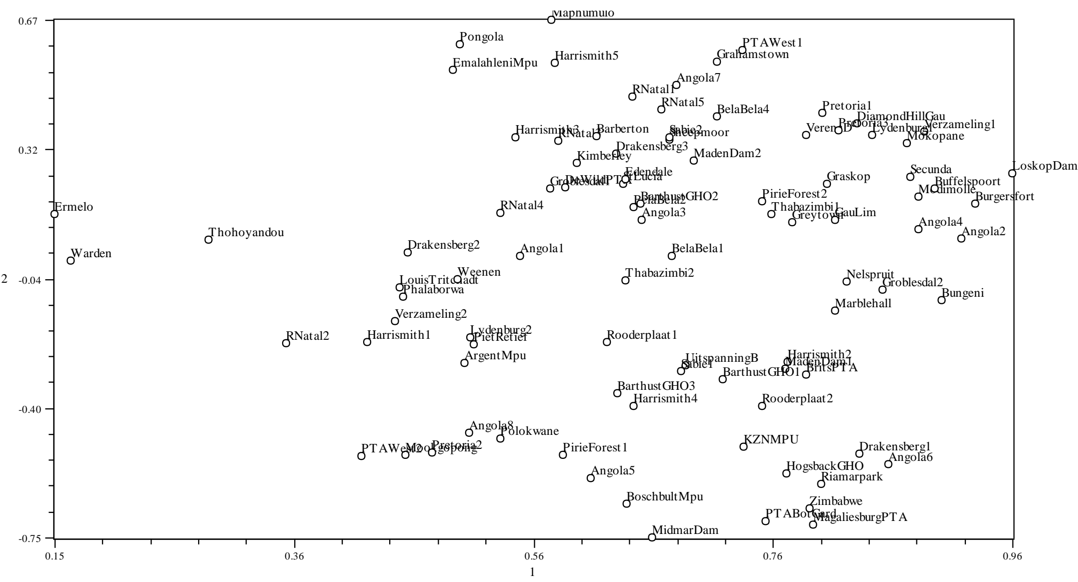
	Simple Matching	Euclidean Distance
Tree – Matrix	0.65	0.43
SM Tree – ED Tree	0.13	
SM Matrix – ED Matrix	0.038	

SM and ED phenograms from a combined SEM and LM analysis are shown in Figure 2.15. SM has three clusters (labelled A to C) of equal sizes, and ED also has five clusters (labelled I to V) with one big cluster (II). Table 2.8 shows the CCVs obtained from the data analysed from combined SEM and LM of fresh materials. The value obtained from the comparison of SM matrix to tree (0.65) shows that the phenogram is a fair representation of the data matrix. The value obtained from ED matrix to tree (0.43), shows a poor correspondence between the tree and the similarity matrix. The value obtained from comparing the CCVs of the tree matrices (0.13) show that there is no correspondence between the trees. Lastly, the value obtained from comparing the CCVs of the two matrices (0.038) shows no correspondence between the SM and ED matrices. The combined SEM and LM data did not show any characters influencing the clustering among the trees.

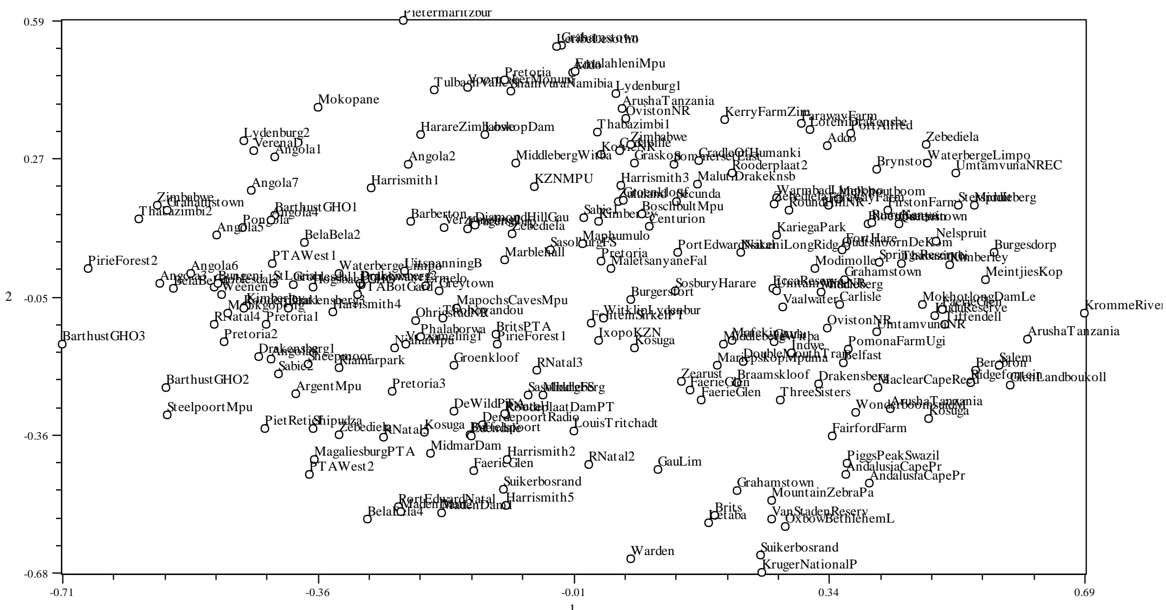
2.3.4 Principal Component Analysis



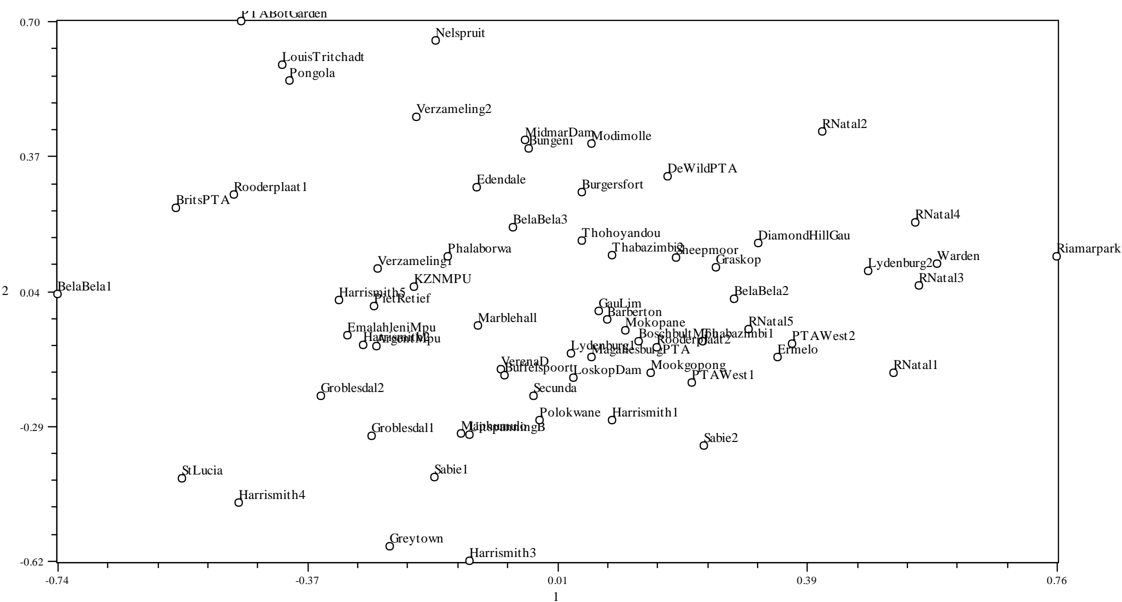
2.17 PCA of Herbarium specimens SEM data set. The first two axes shown here accounts for 78.7% of the variation in the data.



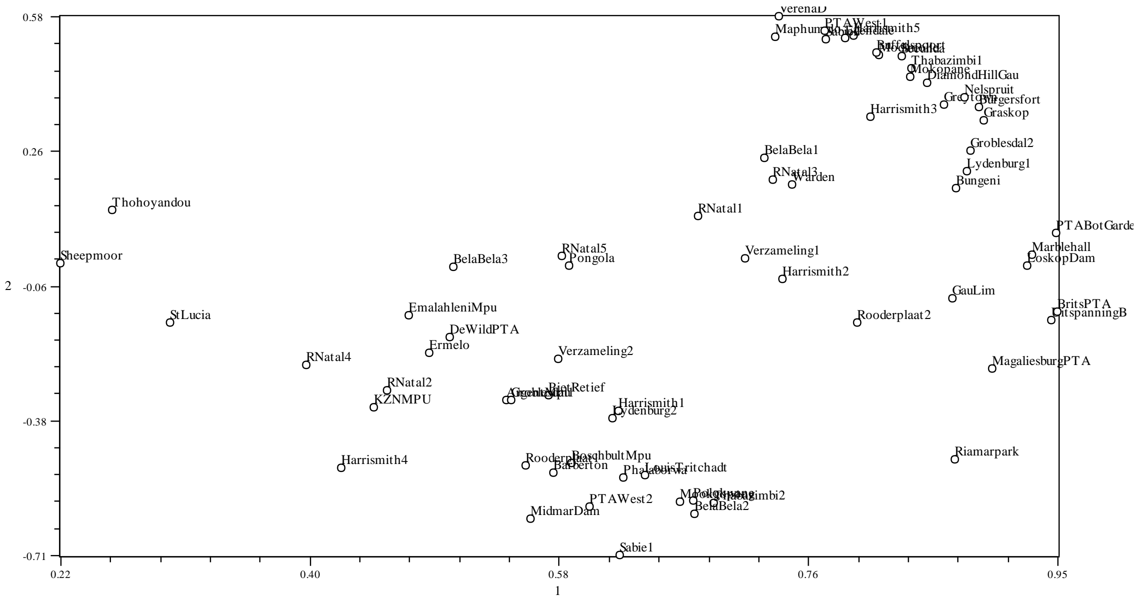
2.18 PCA of Fresh specimens SEM data set. The first two axes shown here accounts for 59.9% of the variation in the data.



2.19 PCA of Fresh and Herbarium specimens SEM data set. The first two axes shown here accounts for 18.4% of the variation in the data.



2.20 PCA of Fresh specimens LM data set. The first two axes shown here accounts for 17.5% of the variation in the data.



2.21 PCA of Fresh specimens SEM and LM data sets. The first two axes shown here accounts for 78.7% of the variation in the data.

Table 2.4: A summary of the PCA results from SEM and LM data sets

Specimens	**Eigen Values**	**Percentage (%)**	**Cumulative percentage (%)**
Herbarium SEM	85.43938057 13.66386362 10.09903855	67.8090 10.8443% 8.0151%	67.8090%, 78.6534% and 86.6685%
Fresh SEM	of 42.50149248, 12.61348267, and 10.37461988	46.1973%, 13.7103%, 11.2768%	46.1973%, 59.9076% and 71.1843%
Herbarium and Fresh SEM	23.95107388, 16.19900541 and 13.50171877	10.9867%, 7.4307% and 6.1934%;	10.9867%, 18.4175% and 24.6109%

Fresh LM	6.26819433, 5.30203452, and 4.77473360;	9.4973%, 8.0334%, 7.2344%	9.4973%, 17.5306% and 24.7651%
Fresh LM and SEM	33.74147327, 9.55823055 and 6.97738247;	51.1234%, 14.4822%, 10.5718%	51.1234%, 65.6056% and 76.1774%

The above results from PCA show that there is no signal in the data. The three values shown above (for each analysis) indicate very poor variation and therefore support the findings of the phenograms and cophenetic correlations.

2.4 <u>Conclusion</u>

The above results show very little variation within the sampled specimens to provide a hierarchical structure to the phenogram. These characters are the presence or absence of stomata on the abaxial or adaxial side; stomata length and width; presence or absence of the dumbbell-shaped silica bodies (DSSBs); length of DSSBs and the presence or absence of micro-hairs. According to Prychid *et al.* 2004, the shape and size of silica bodies can be diagnostic of a species. They can be used to distinguish between Poaceae subfamilies and below (Piperno and Pearsall, 1984). The results of the current chapter show that there was anatomical diversity among the specimens, although some of the characters may have been affected by the handling of specimens in herbaria over the years. Micro-hairs are one of the characters that are easily affected by the handling of specimens in herbaria. Fresh specimens were sampled from the mature leaves about 7cm from the collar. However, the specimens were collected at different season of the year, which may have an effect on the age of the specimen and its anatomy.

Irrespective of the data set, the specimens cannot be grouped according to anatomical differences and similarities. The results indicated that no consistent groups within *Themeda triandra* were retrieved. This means that there was no anatomical variability to explain the morphological variation observed within the species. This shows that the morphological variation is random or possibly due to adaptation to the environmental conditions where the species is found within South Africa. A similar observation was found in a study by Faria *et al.* (2012) where the differences in stomata structure of the genus *Aechmea* Ruiz & Pav. (Bromeliaceae) were due to adaptations to water availability within a region. Another study on the grass genus *Festuca* showed that the differences in appearance are a result of ecological factors and phenotypic plasticity (Martínez-Sagarra *et al.* 2017).

Even though the data shows no hierarchical taxonomic or geographic pattern based on the species leaf anatomy (SEM and LM), it has contributed to a better understanding of the anatomy of *T. triandra* species in South Africa. Further studies may be required with different anatomical and/or morphological characters that could potentially result in a clearer pattern or further confirm the above results.

3 Chapter 3: An analysis of the genetic variation in the nuclear Internal Transcribed Spacer (ITS) and External Transcribed Spacer (ETS) sequences from southern African samples of *Themeda triandra*

3.1 Introduction

Plant genetics plays an important role in plant classification (Hörandl 1998, Van Belkum *et al*. 2001). Molecular phylogenetic studies have become one of the essential tools in describing the phylogeny and evolution of plant species (Hörandl 1998, Raja *et al*. 2017) and advances in molecular techniques have provided a better understanding of species relationships (Ainouche and Bayer 1997). However, very few of these studies have resulted in new/revised classifications. Van Belkum *et al*. (2001) showed that studying the genetics of species also aids in understanding the different phenotypes observed.

As explained in the introduction, *Themeda triandra* is a species with a complex nature with varying morphologies and it is thus important to study the genetic diversity and determine whether it correlates with anatomical, morphological and cytological diversity. *T. triandra* has been hypothesised to have a number of varieties, due to its polymorphism (Gluckmann 1951). However, it may also be a case of phenotypic plasticity, which may be caused by adaptation to the different environmental conditions. Lock and Milburn (1971) and Groves *et al.* (1973) showed that the Australian and African *T. triandra* grow and flower in similar ways, therefore rejecting the hypothesis that *T. australis* is a different species.

The aim of the work described in this chapter is to use DNA sequence data from nuclear ribosomal Internal Transcribed Spacer (ITS) and External Transcribed Spacer (ETS) regions to determine if there is any genetic diversity within *T. triandra* and if this relates to the geographic distribution, morphology or what is known about the species' cytology. The study also aims to determine if the African and Australian *T. triandra* are the same species or not.

The nuclear ribosomal ITS and ETS has been evaluated and found to be the most suitable region for plant phylogenetics at species level and below (Charmet *et al*. 1997, Barker *et al.* 2005,

Gillespie *et al.* 2009, Wang *et al.* 2017). This is because these regions are faster evolving and easily amplified by polymerase chain reaction (PCR) (Ainouche and Bayer 1997, Baker *et al*. 2000). The use of ITS has become popular for plant phylogenetic studies (including grasses) at lower taxonomic levels and is widely accepted by grass systematists and recommended as a core plant DNA barcode (Doyle *et al*. 1996, Schaal *et al*. 1998, Hodkinson *et al*. 2002, Zhi-Ming *et al*. 2006, Cheng *et al*. 2016, Xu *et al.* 2017, Xuan *et al.* 2019, Peterson *et al.* 2020) due to the near-universality of the primers designed to anneal in highly conserved regions (Sang *et al*. 1995, Buckler and Holtsford 1996, Razafimandimbison *et al*. 2004, Cheng *et al*. 2016). The ITS regions show an unusual combination of highly conserved primer sites that are adjacent to highly variable sequences making it amenable for PCR amplification using universal primers (White *et al*. 1990). Buckler and Holtsford (1996) found that ITS regions have rates of nucleotide substitution that prove valuable in evaluating plant relationships at the generic and species levels. Such rates are much more accelerated in the grass family (Poaceae) mainly due to short generation times (Gaut *et al.* 1992).

The ITS nrDNA genes also evolve more rapidly and resolve lower level relationships better than other genes (Baker *et al*. 2000, Xu *et al.* 2017). As a result of these advantages, there are thousands of grass ITS sequences published on the GenBank (Hodkinson *et al*. 2002). However, there is low taxonomic resolution for some species delimitations based on ITS (Porras-Alfaro *et al*. 2014).

The use of ITS has been debatable at intraspecific level because it can be limited by biological factors such as reticulation, hybridisation and polyploidy (Sang *et al*. 1995, Bermingham and Moritz 1998, Soltis and Soltis 2000, Barker *et al*. 2005, Zheng *et al*. 2008). Reticulation occurs when gene flow results in heterozygosity of ITS types within an individual, recombination or partial gene conversion disrupts the phylogenetic signal in the ITS genealogy (Sang *et al*. 1995). Hybridisation can be viewed as beneficial in the formation of new species or varieties through polyploidy (Grant 1953, Schaal *et al*. 1998, Xu *et al.* 2017, Xuan *et al.* 2019). Hybridisation may also provide stimulus of invasiveness (Ellstrand and Schierenbeck 2000, Linder *et al.* 2014, Lambertini 2019, Naciri and Linder 2020). Polyploid plants that arise through hybridisation and have been examined with ITS markers show a polyphyletic nature (Álvarez and Wendel 2003,

Barker *et al*. 2005). Polyploidisation has negative implications on plant taxonomy and genetic diversity (Soltis and Soltis 2000). However, according to Sang *et al*. (1995), more investigations need to be done in order to further understand the evolutions of the ITS region after hybridization and polyploidization.

Other disadvantages of using ITS include its multi-copy nature, the possibility of the presence of pseudogenes, its problematic secondary structure and the possibility of contaminants, such as fungi, being amplified (Álvarez and Wendel 2003). The presence of multiple copies of a gene presents problems in determining whether the sequences being compared are paralogous (homologous genes that have diverged within one species) or orthologous (homologous genes that diverged during evolution to form different species - Baldwin 1992, Schaal *et al*. 1998). Pseudogenes are a section of chromosomes that are an imperfect copy of the gene that are often characterised by cytosine mutations at methylation sites (Buckler *et al*. 1997). ITS pseudogenes have been detected in all angiosperms (Buckler and Holtsford 1996, Buckler *et al*. 1997, Álvarez and Wendel 2003, Razafimandimbison *et al*. 2004, Zheng *et al*. 2008). Despite their disadvantage, ITS pseudogenes can be used for phylogenetic analysis where functional paralogs provide too low variation (Xiao *et al.* 2010). Active ITS regions are said to have functional constraints and the characteristics of functional ITS regions can help distinguish between functional and pseudogene ITS sequences (Buckler *et al*. 1997, Xiao *et al.* 2010, Xu *et al.* 2017). Contamination in ITS PCR amplification may occur if there are fungi in the samples being amplified. This is because ITS primers were originally made to amplify fungi and were later used for plants as well (Wang *et al*. 2017). The ITS region is said to have the highest probability of successful identification of a broad range of fungi (Raja *et al*. 2017). As a result of this the use of the universal ITS regions is discouraged and plant-specific primers have been designed (Cheng *et al.* 2016, Wang *et al*. 2017).

The use of ITS has also been criticized due to complications caused by concerted evolution taking place among members of multigene plant families (Doyle *et al*. 1996, Wang *et al.* 2016, Xu *et al.* 2017). Concerted evolution silences the contribution of one parent and possible recombination events, which then leads to incongruence between organismal and gene phylogenies (Ainouche and Bayer 1997, Soreng *et al*. 2015, Fijridiyanto and Murakami 2019,

Xuan *et al.* 2019). Concerted evolution may also explain the occurrence of paralogous genes observed within a species (Liao 1999, Xiao *et al.* 2010).

In contrast, the ETS locus has not been used extensively, as ITS has been, in phylogenetic studies of grasses (Duvall *et al*. 2003, Gillespie *et al.* 2009, Cabi *et al.* 2017, Krawczyk *et al.* 2017). However, the ETS region has been found useful in studying phylogenies of closely related plant taxa (Sallares and Brown 2004, Gillespie *et al.* 2009, Alonso *et al.* 2014, Barrett 2019, Fijridiyanto and Murakami 2019, Tkach *et al.* 2019, Nauheimer *et al.* 2019). It has also been found to represent an even more valuable instrument for phylogenetic analysis when compared to ITS (Poczai and Hyvönen 2010), having more than twice the number of variable sites than ITS (Duvall *et al*. 2003). According to Zhi-Ming *et al*. (2006), Alonso *et al*. (2014) and Fijridiyanto and Murakami (2019), ETS can be used in cases where ITS does not produce clear results, or to augment ITS data, because ETS evolves as much as 1.4 times faster. In a study where six different DNA loci were examined, ITS and ETS combined provided the best results in *Nassella neesiana* (Wang *et al*. 2017). In another study by Cabi *et al.* (2017) the use of both ITS and ETS provided a clearer view and placement of the new species *Alopecurous goekyigitiana.* Wang *et al*. (2017) also suggests that ETS promises to be the best marker for grass DNA barcoding if more robust primers are designed in order to increase its PCR success rate. However, according to Baldwin and Markos (1998) ETS lacks a highly conserved region suitable for primer design, therefore limiting its use in plant comparative studies. Although the use of ITS and ETS separately has produced variable results, the combined analysis of both datasets has resulted in better resolved and more robust trees (Gillespie *et al.* 2009, Poczai and Hyvönen 2010). The combination of these two regions has also resulted in improved resolution and increased bootstrap support for clades (Bena *et al*. 1998, Fijridiyanto and Murakami 2019), even though they show incompatibility between their DNA datasets/ alignments (Zhi-Ming *et al*. 2006). This is because these regions occur within a single transcriptional unit and evolve under similar functional constraints at comparable rates (Baldwin and Markos 1998, Zhi-Ming *et al*. 2006), hence the use of ETS and combining the two during the current study. Despite all the disadvantages associated with nuclear ribosomal DNA regions (ITS and ETS), they still represent suitable regions for plant genetic studies at and below species level (Schaal *et al*. 1998). It is for this reason that the current study utilised these two nrDNA regions.

3.2 Materials and methods

3.2.1 Sampling and DNA extraction

Themeda triandra samples were collected from various regions in southern Africa, including one sample from Zimbabwe and eight from Angola. DNA was extracted from 1-2cm of leaf or inflorescence material from the collected samples using standard CTAB genomic DNA isolation technique (Doyle and Doyle 1987).

Table 3.1: Specimen localities, collectors and whether ITS and ETS sequences were obtained KwaZulu-Natal = KZN, Mpumalanga = Mpu, Gauteng = GP, Free-State = FS, Limpopo = Lim, Eastern Cape = EC, Northern Cape = NC.

Locality	Collector	GPS	ITS	ETS
Pretoria, GP	N.P. Barker (2009)	N/A	√	√
Zimbabwe	Unknown	N/A	√	
Kalkhueve West, GP	A.E. Van Wyk	S25.850056°; E27.883139°	√	√
Weenen, KZN	Unknown	N/A	√	√
Drakensberg1, KZN	S. Ntshangase (54)	S29.47137°; E28.9938°	√	
Drakensberg2, KZN	S. Ntshangase (55)	S29.42951°; E28.9472°	√	√
GHT Barthust2, EC	S. Ntshangase (58)	N/A	√	√
Grahamstown, EC	S. Ntshangase (59)	N/A	√	√
Hogsback, EC	N.P. Barker	N/A	√	√
Grahamstown to PE, EC	N.P. Barker	N/A	√	√
Kimberley, NC	S. Ntshangase (60)	N/A	√	√
Warden, FS	S. Ntshangase (1)	S27.85188°; E 28.9592°	√	√
Harrismith 4, FS	S. Ntshangase (11)	S28.26276°; E29.12316°		√
Midmar Dam, KZN	S. Ntshangase (12)	S29.49566°; E30.16220°	√	√
Maphumulo, KZN	S. Ntshangase (13)	S29.11358°; E31.02684°		√

Greytown, KZN	S. Ntshangase (14)	S28.98470°; E30.81659°	√	√
St Lucia, KZN	S. Ntshangase (15)	S28.35575°; E32.41962°	√	√
Pongola, KZN	S. Ntshangase (17)	S27.75515°; E32.11157°	√	√
Piet Retief, Mpu	S. Ntshangase (18)	S27.20660°; E31.09293°	√	√
Verzameling1, Mpu	S. Ntshangase (19)	S26.90885°; E30.72853°	√	
Verzameling2, Mpu	S. Ntshangase (20)	S26.90885°; E30.72853°	√	√
Sheepmoor, Mpu	S. Ntshangase (21)	S26.75027°; E30.36234°	√	√
Ermelo, Mpu	S. Ntshangase (22)	S26.51356°; E29.90657°	√	√
Secunda, Mpu	S. Ntshangase (23)	S26.46438°; E29.18931°	√	√
Harrismith2, FS	S. Ntshangase (9)	S28.26690°; E29.14110°		√
Harrismith3, FS	S. Ntshangase (10)	S28.26309°; E29.14328°	√	√
Pirie Forest 1, EC	S. Ntshangase (61)	N/A	√	√
Brits, GP	S. Ntshangase (66)	S25.583333°; E27.772222°	√	√
Brits, GP	S. Ntshangase (66)	S25.583333°; E27.772222°	√	√
De Wild, GP	S. Ntshangase (67)	S25.65°; E27.822222°	√	√
De Wild, GP	S. Ntshangase (67)	S25.65°; E27.822222°	√	√
De Wild, GP	S. Ntshangase (67)	S25.65°; E27.822222°	√	√
Magaliesburg, GP	S. Ntshangase (68)	S25.706944°; E27.9°	√	√
Magaliesburg, GP	S. Ntshangase (68)	S25.706944°; E27.9°	√	√
PTAWest1, GP	S. Ntshangase (69)	S25.794444°; E27.993056°	√	√
PTAWest1, GP	S. Ntshangase (69)	S25.794444°; E27.993056°	√	√
PTAWest1, GP	S. Ntshangase (69)	S25.794444°; E27.993056°	√	√
PTAWest2, GP	S. Ntshangase (70)	S25.861111°; E28.011111°	√	√
PTAWest2, GP	S. Ntshangase (70)	S25.861111°; E28.011111°		√
PTAWest2, GP	S. Ntshangase (70)	S25.861111°; E28.011111°	√	
Brits, GP	S. Ntshangase (66)	S25.583333°; E27.772222°	√	√
Magaliesburg, GP	S. Ntshangase (68)	S25.706944°; E27.9°		√
R510, Lim	S. Ntshangase (24)	S 25.7117°; E 27.64917°	√	√
Verena D, Mpu	S. Ntshangase (33)	S 25.47208°; E 29.03927°	√	√
Riamarpark, Mpu	S. Ntshangase (34)	S 25.80934°; E 28.70253°	√	√

Thabazimbi1, Lim	S. Ntshangase (25)	S 24.91234°; E 27.28350°	√	√
Thabazimbi2, Lim	S. Ntshangase (26)	S 24.57597°; E 27.41544°	√	√
Thabazimbi2, Lim	S. Ntshangase (26)	S 24.57597°; E 27.41544°	√	
Bela Bela1, Lim	S. Ntshangase (27)	S 24.86960°; E 28.10041°	√	
Bela Bela 2, Lim	S. Ntshangase (28)	S 24.84868°; E 28.34738°	√	√
Modimolle, Lim	S. Ntshangase (29)	S 24.70976°; E 28.41278°	√	√
Marblehall1, Lim	S. Ntshangase (30)	S 24.84401°; E 28.88696°	√	√
Marblehall2, Lim	S. Ntshangase (30)	S 24.84401°; E 28.88696°		√
Groblesdal, Lim	S. Ntshangase (31)	S 25.16815°; E 29.37104°	√	√
UitspanningB, Mpu	S. Ntshangase (32)	S 25.24319°; E 29.21340°	√	√
Pirie Forest 2, EC	S. Ntshangase (62)	N/A	√	√
Angola5	N.P. Barker	N/A	√	
Angola6	N.P. Barker	N/A	√	√
Angola1	N.P. Barker	N/A	√	√
Angola7	N.P. Barker	N/A	√	√
Angola8	N.P. Barker	N/A	√	√
Angola2	N.P. Barker	N/A	√	√
Argent, Mpu	M. Cunningham	S26.01115°; E28.79572°	√	√
Diamond Hill Off-Ramp, GP	M. Cunningham	S25.79913°; E28.51610°	√	
Boschbult, Mpu	M. Cunningham	S25.56765°; E28.64466°	√	√
Gariep Dam, FS	M. Cunningham	S30.62924°; E25.49885°	√	√
Emalahleni, Mpu	S. Ntshangase (35)	S 25.88668°; E 29.27050°	√	√
Graskop, Mpu	S. Ntshangase (44)	S 24.92512°; E 30.81283°	√	√
Burgersfort, Lim	S. Ntshangase (45)	S 24.72472°; E 30.55678°	√	√
Phalaborwa, Lim	S. Ntshangase (46)	S 24.10687°; E 30.86461°	√	√
Thohoyandou, Lim	S. Ntshangase (47)	S 22.94123°; E 30.48588°	√	
Louis Tritchadt, Lim	S. Ntshangase (48)	S 23.03162°; E 29.91874°	√	√
Bungeni, Lim	S. Ntshangase (49)	S 23.20885°; E 30.17513°	√	
Polokwane, Lim	S. Ntshangase (50)	S 23.93109°; E 29.46977°	√	√

Mokopane, Lim	S. Ntshangase (51)	S 24.21767°; E 29.00510°		√
Bela Bela 3, Lim	S. Ntshangase (53)	S 25.08982°; E 28.30714°		√
Bela Bela 4, Lim	S. Ntshangase (53)	S 25.08982°; E 28.30714°		√
Loskop Dam, Mpu	S. Ntshangase (36)	S 25.41040°; E 29.36386°	√	√
Groblesdal2, Lim	S. Ntshangase (37)	S 25.17921°; E 29.54404°	√	√
Barberton, Mpu	S. Ntshangase (38)	S 25.78289°; E 31.02855°		√
Nelspruit, Mpu	S. Ntshangase (39)	S 25.64779°; E 30.97743°		√
Sabie1, Mpu	S. Ntshangase (40)	S 25.41015°; E 30.91567°	√	√
Lydenburg, Mpu	S. Ntshangase (42)	S 25.10905°; E 30.49458°	√	√
Ohrigstad, Lim	S. Ntshangase (43)	S 24.84682°; E 30.57333°	√	
Maden Dam1, EC	S. Ntshangase (63)	N/A	√	√
Maden Dam 2, EC	S. Ntshangase (64)	N/A	√	√
Skurweberg, GP	S. Ntshangase (65)	S25.791667°; E27.983333°	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	√
Australia	R. Jobson	N/A	√	
Australia	R. Jobson	N/A	√	
GenBank *T.australis*	Unknown	N/A	√	
Tswaing, GP	S. Ntshangase (73)	S25.404996°; E28.083079°	√	√

3.2.2 DNA amplification

The ITS region was amplified and sequenced using ITS_Them_ (TCGTGACCCTTAAACAAAACAG) (designed by R. Jobson, Royal Botanical Garden, Sydney) and ITS 4 (TCCTCCGCTTATTGATATGC – White *et al*. 1990). The ETS region was

amplified and sequenced using ETS Rets4-F (5'-TTGGCTACGCGAGCGCATGAG) and 18S-R (5'-AGACAAGCATATGACTACTGGCAGG) primers (designed by Dr R. Jobson, Royal Botanical Garden, Sydney).

PCR amplification consisted of a 5 minute denaturing stage at 94˚C; which was followed by 35 cycles of 94˚C for 50 seconds, 55˚C for 30 seconds and 72˚C for 2 minutes annealing and a final extension of 7 minutes at 72˚C. The PCR products were run on 1% agarose gel to view success of the PCR. The successful PCR products were then cleaned using the QIAGEN PCR purification kit according to manufacturer's instructions. The cleaned PCR products were then sequenced with BigDye terminator v.3.1. After cycle sequencing, the reactions were precipitated with 2.5mM EDTA, 100% ethanol and 70% ethanol. The products of precipitation were then sent to the Forestry and Agricultural Biotechnology Institute (FABI) (University of Pretoria) for sequencing using the ABI PRISM 3130xl and ABI PRISM 3500xl Genetic Analyzers.

3.2.3 Analysis

The raw sequence trace files were edited using Sequencher v. 5.4.6 (http://www.genecodes.com). DNA sequences were then aligned using MEGA6 with CLUSTAL W and subsequent manual corrections were made to the automatic alignment. In addition to the South African specimens analysed, Dr R. Jobson (Royal Botanic Gardens, Sydney) provided sequence data for nine Australian samples, seven *T. triandra*, one *T. arguens* and one *T. quadrivalvis*. The matrices obtained from MEGA6 were saved as NEXUS files.

Both tree building and network analyses were conducted. Networks were obtained using TCS and Median Joining (Clement *et al*. 2000) as implemented in PopArt v. 1.7 (Leigh and Bryant 2015). Phylogenetic trees were obtained using Bayesian analyses as implemented in Mr Bayes v. 3.1.2 (Huelsenbeck and Ronquist 2005).

According to Clement *et al*. (2000), the TCS method has been used extensively with nucleotide data at population levels. With this method, the distance between every pair of taxa in the source

and destination clusters is examined to determine possible connections within the species (Clement *et al*. 2000).

Prior to conducting the Bayesian analysis, JModelTest (Posada 2008) was run on the alignments to find the best-fit model of nucleotide substitution and this was used for Bayesian analysis (Posada 2008). For the Bayesian analysis (200000000 generations with a 2000 proportion of trees excluded as burnin) the phylogenetic trees were rooted on *T. arguens* as an out-group because Jobson's unpublished analyses show it to be a close relative to *T. triandra*. Phylogenetic trees were produced from Mr Bayes and TCS and Median Joining Networks for ITS, ETS and combined ITS and ETS datasets. Listed below are the characters (ITS, ETS and combined ITS and ETS respectively) for running the Bayesian analysis as obtained from the J-Model test.

```
log start;
set autoclose=yes;
Prset statefreqpr=dirichlet(1,1,1,1);
Lset nst=6 rates=invgamma;
mcmc ngen=20000000 samplefreq=200 nchains=4 savebrlens=yes startingtree=random;
sump burnin=2000 printtofile=yes;
sumt burnin=2000 contype=allcompat;
log stop;

log start;
set autoclose=yes;
Prset statefreqpr=dirichlet(1,1,1,1);
Lset nst=6 rates=invgamma;
mcmc ngen=20000000 samplefreq=200 nchains=4 savebrlens=yes startingtree=random;
sump burnin=2000 printtofile=yes;
sumt burnin=2000 contype=allcompat;
log stop;

log start;
```

```
set autoclose=yes;
Prset statefreqpr=dirichlet(1,1,1,1);
Lset nst=6 rates=invgamma;
mcmc ngen=20000000 samplefreq=200 nchains=4 savebrlens=yes startingtree=random;
sump burnin=2000 printtofile=yes;
sumt burnin=2000 contype=allcompat;
log stop;
```

3.3 Results

ITS data was obtained from 85 specimens, ETS data from 84 specimens and 67 specimens were used to produce a combined ITS and ETS data set. ITS alignments were 586bp long, ETS alignments were 448bp long and the combined ITS and ETS alignments were 1013bp long. The results are presented in the form of phylogenetic trees obtained from Bayesian analysis and Networks from Median Joining analysis.

These specimens are from 69 South African localities, 6 Angolan, 8 Australian, 1 Zimbabwean and one sample obtained from the GenBank (for ITS) and 72 South African, 5 Angolan and 7 Australian specimens (for ETS).

3.3.1 Network Analyses

Due to the similarities from the TCS network and Median Joining Network (MJN), only the results obtained from running the MJN are shown below. These show little genetic diversity between the South African specimens. However, the Australian specimens show some differences from the South African specimens and some are not grouped with these.

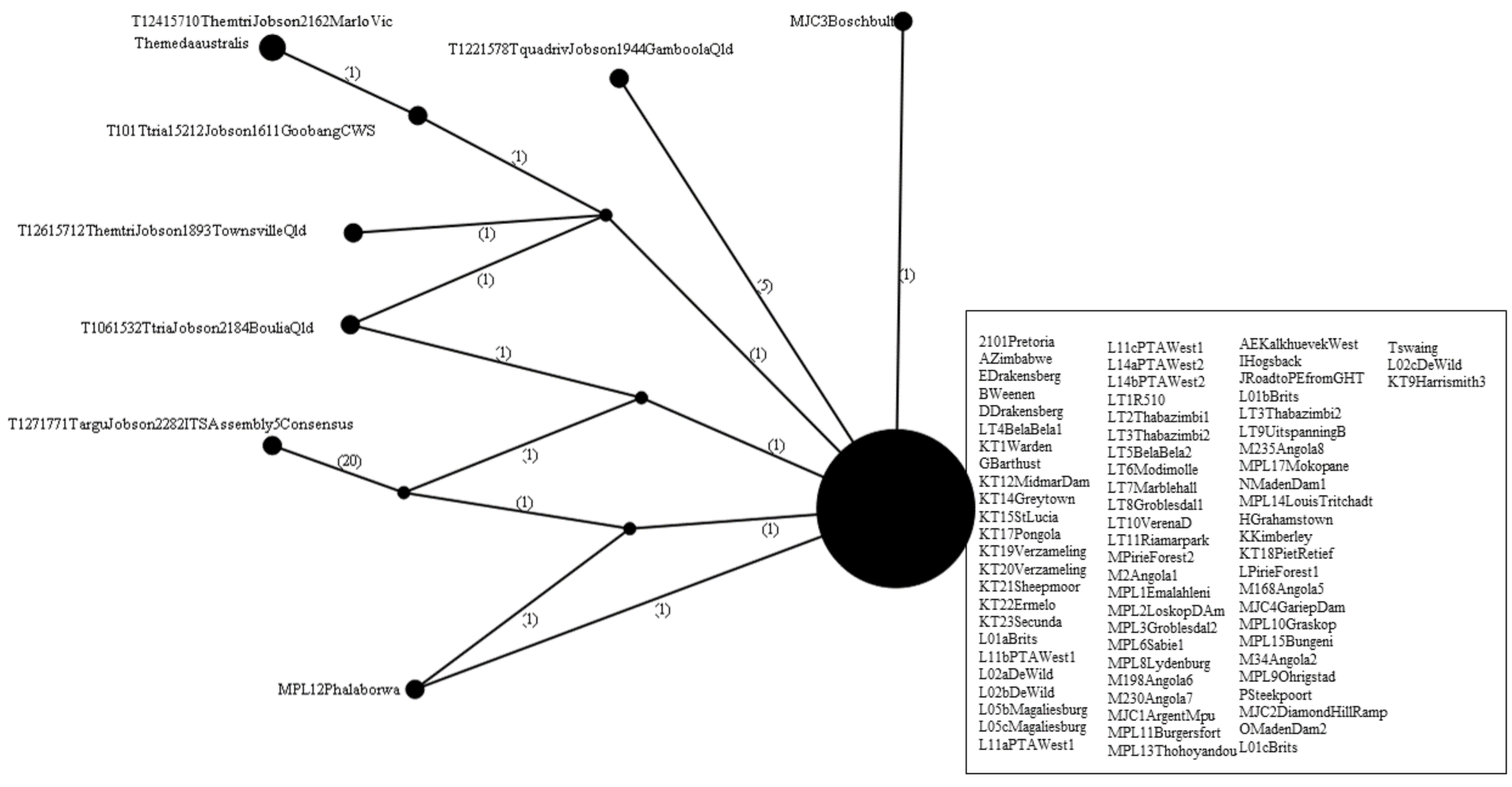


Figure 3.1: Results from Median Joining Network analysis of ITS data set. This network was obtained using an algorithm from Clement *et al* (2002). The nodes correspond to specimens collected from southern Africa and Australia. The values on the nodes are the number of mutations.

The above results show that in the MJN analysis of ITS data (Figure 3.1), the Australian specimens are not grouped with the South African specimens (source cluster) with distances of 2, 3, 5 (*T. quadrivalvis*) and 23 (*T. arguens*) mutations from the source cluster. The source (central) cluster consists of 75 South African and Angolan specimens and there are two other South African specimens that have a distance of one mutations each from the source cluster. These clusters show no geographic pattern.

Similarly, the results from the MJN analysis of ETS data (Figure 3.2) show that the Australian specimens are not grouped with the South African specimens (source cluster), with distances of 1, 2, 3, 8 (*T. quadrivalvis*) and 24 (*T.arguens*) mutations from the source cluster. The source (central) cluster consists of 70 South African and Angolan specimens. There are six other South African and Angolan specimens that have distances of 1, 3 and 5 mutations from the source cluster.

When the ITS and ETS datasets were combined, (Figure 3.3) the results were similar to those from individual data sets, with the Australian specimens showing slight difference to the South African specimens, as they did not cluster with any of them.

The Australian specimens show distances of 4, 15 (*T. quadrivalvis*) and 46 (*T.arguens*) mutations from the source cluster. The source (central) cluster consists of 57 South African and Angolan specimens. There are nine other South African and Angolan specimens that have distances of 1, 3 and 5 mutations from the source cluster.

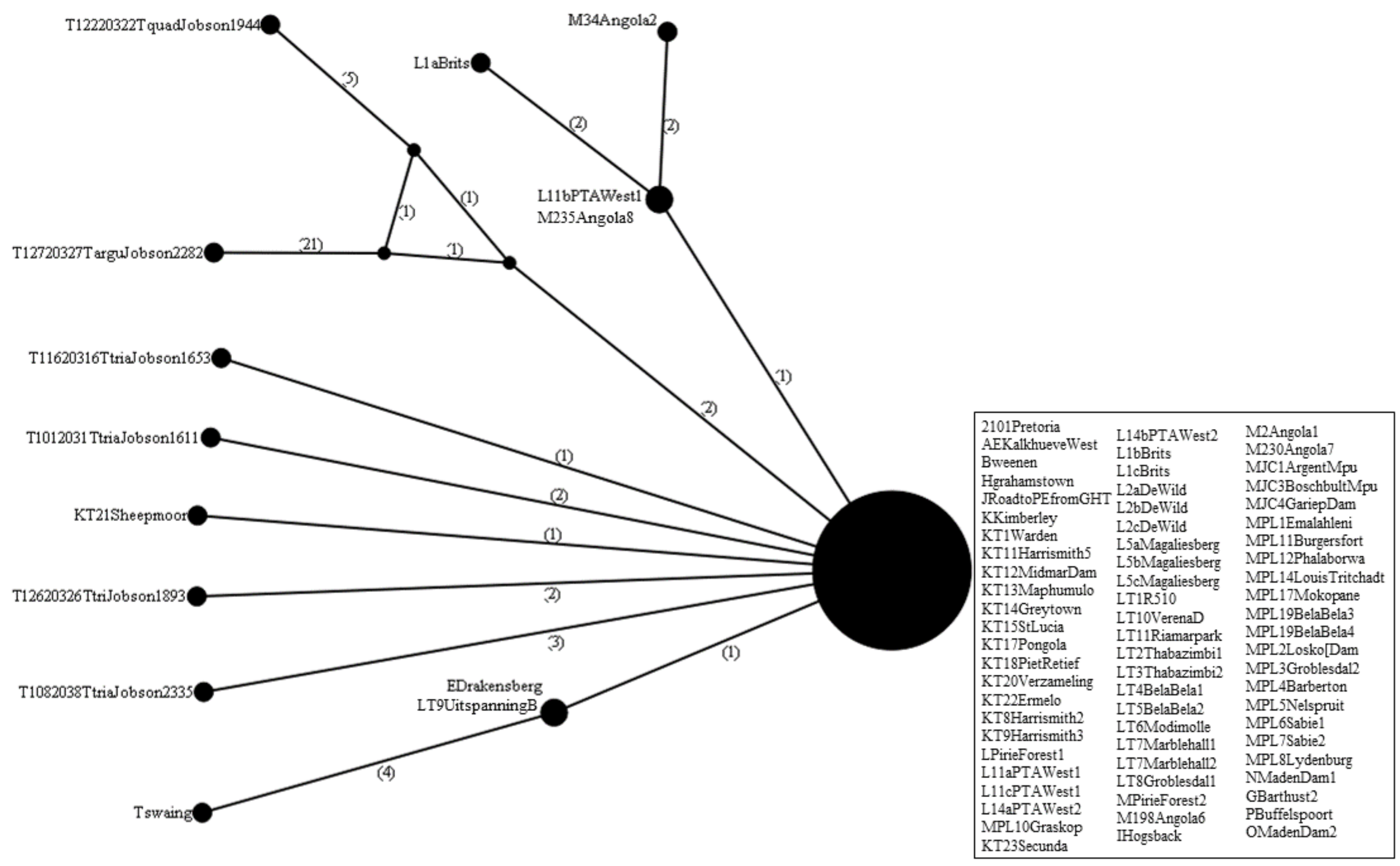


Figure 3.2: Results from Median Joining Network analysis of ETS data set. This network was obtained using an algorithm from Clement *et al* (2002). The nodes correspond to specimens collected from southern Africa and Australia. The values on the nodes are the number of mutations.

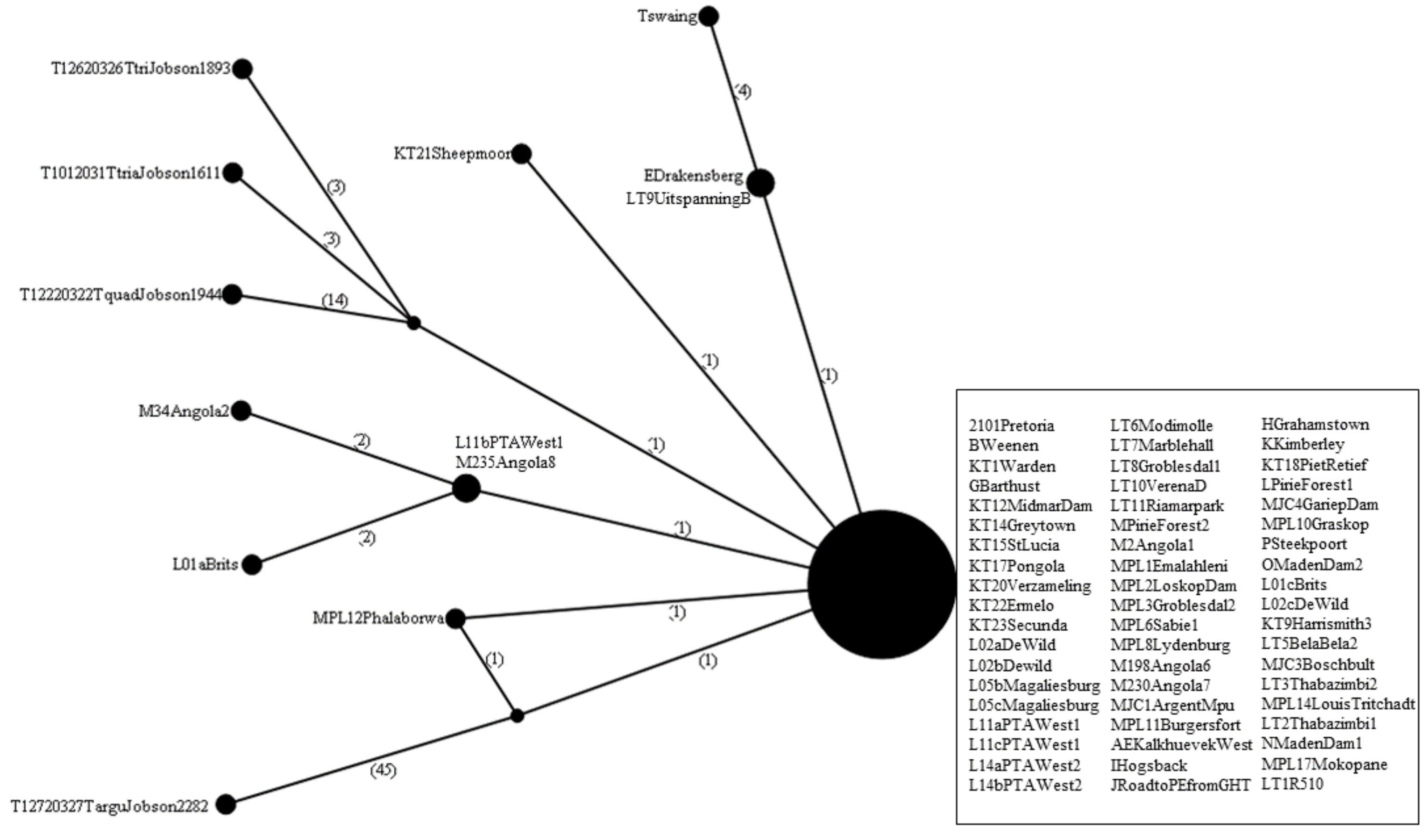


Figure 3.3: Results from Median Joining Network analysis of combined ITS and ETS data sets. This network was obtained using an algorithm from Clement *et al* (2002). The nodes correspond to specimens collected from southern Africa and Australia. The values on the nodes are the number of mutations.

3.3.2 Bayesian analyses

The following phylogenetic trees (Figures 3.4 – 3.6) were obtained from the Bayesian analyses of ITS, ETS and IITS combined with ETS data sets. According to Hall (2013) a posterior probability value less than 70% is not considered reliable. It is for this reason that only values above 70% appear on the phylogenetic trees below. A total of 586 characters were analysed for Figure 3.4 (ITS), where 550 were constant with a proportion of 0.94, 29 variable characters were parsimony-uninformative and seven were parsimony-informative. For Figure 3.5 (ETS), a total of 448 characters were analysed, where 389 characters were constant with a proportion of 0.87, 55 variable characters were parsimony-uninformative and four characters were parsimony-informative. Lastly, for Figure 3.6 (combined ITS and ETS) a total of 1013 characters were analysed, where 921 were constant with a proportion of 0.91, 85 variable characters were parsimony-uninformative and seven characters were parsimony informative.

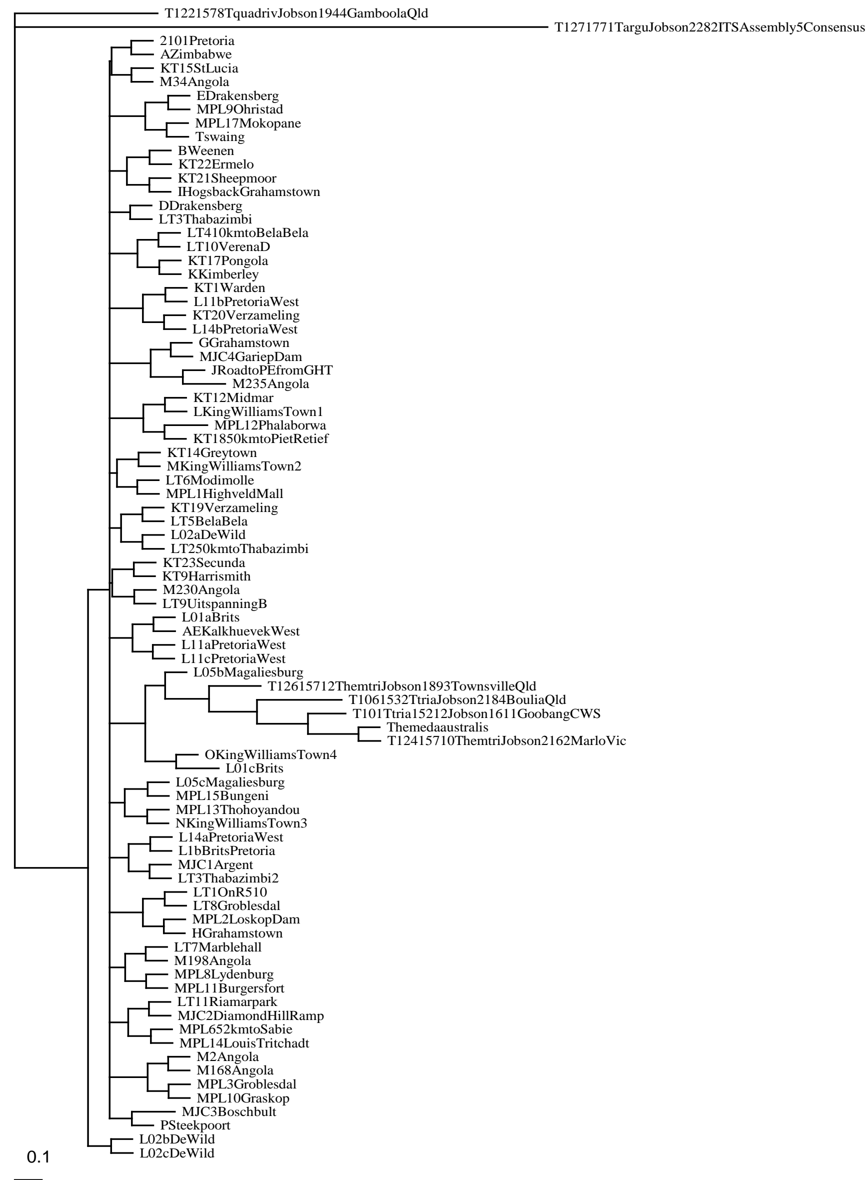


Figure 3.4: Results from the Bayesian analysis of the ITS data rooted on *T. arguens*. The posterior probabilities are all less than 70%, indicating no support for the indicated relationship.

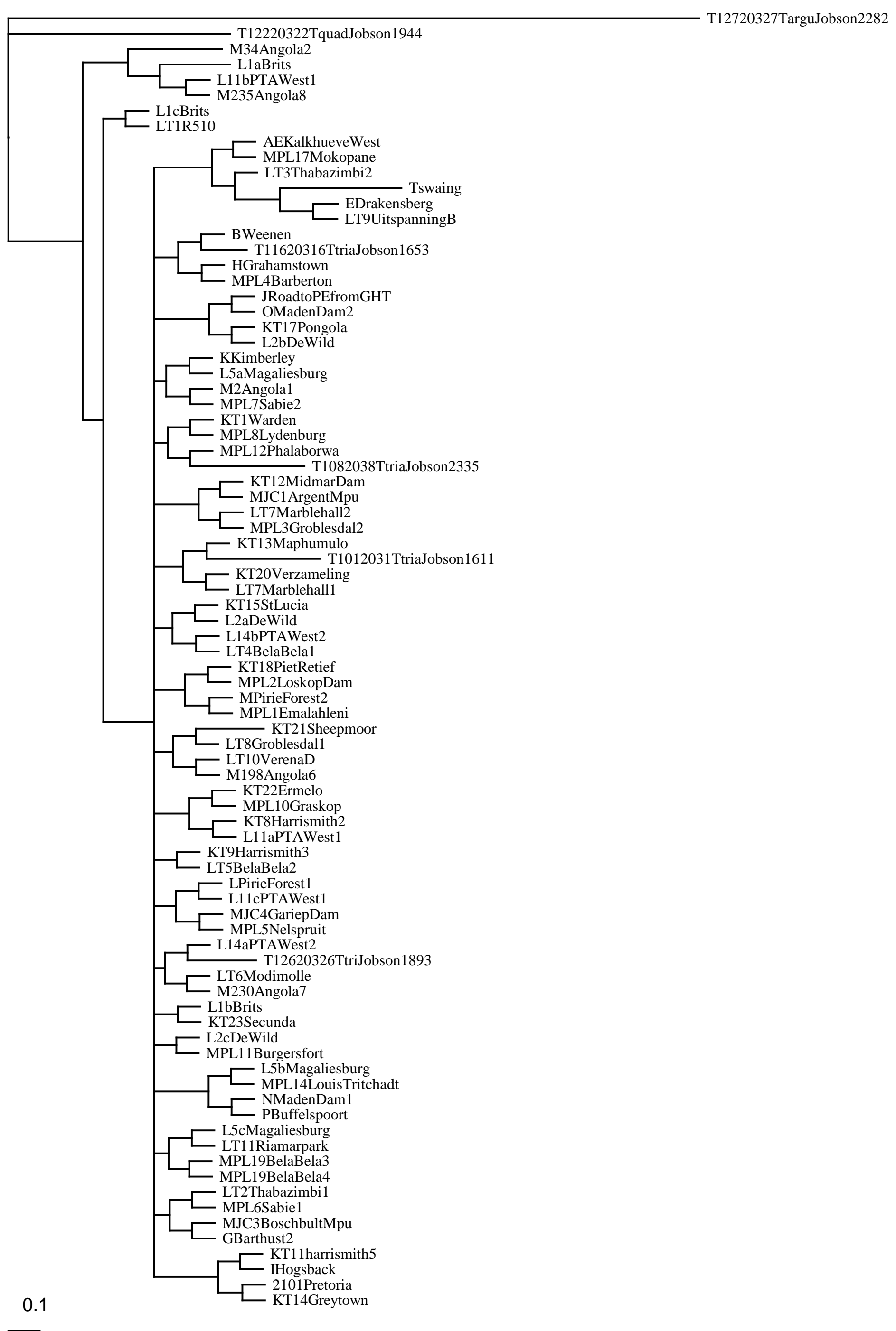


Figure 3.5: Results from the Bayesian analysis of the ETS data rooted on *T. arguens*. The posterior probabilities are all less than 70%.

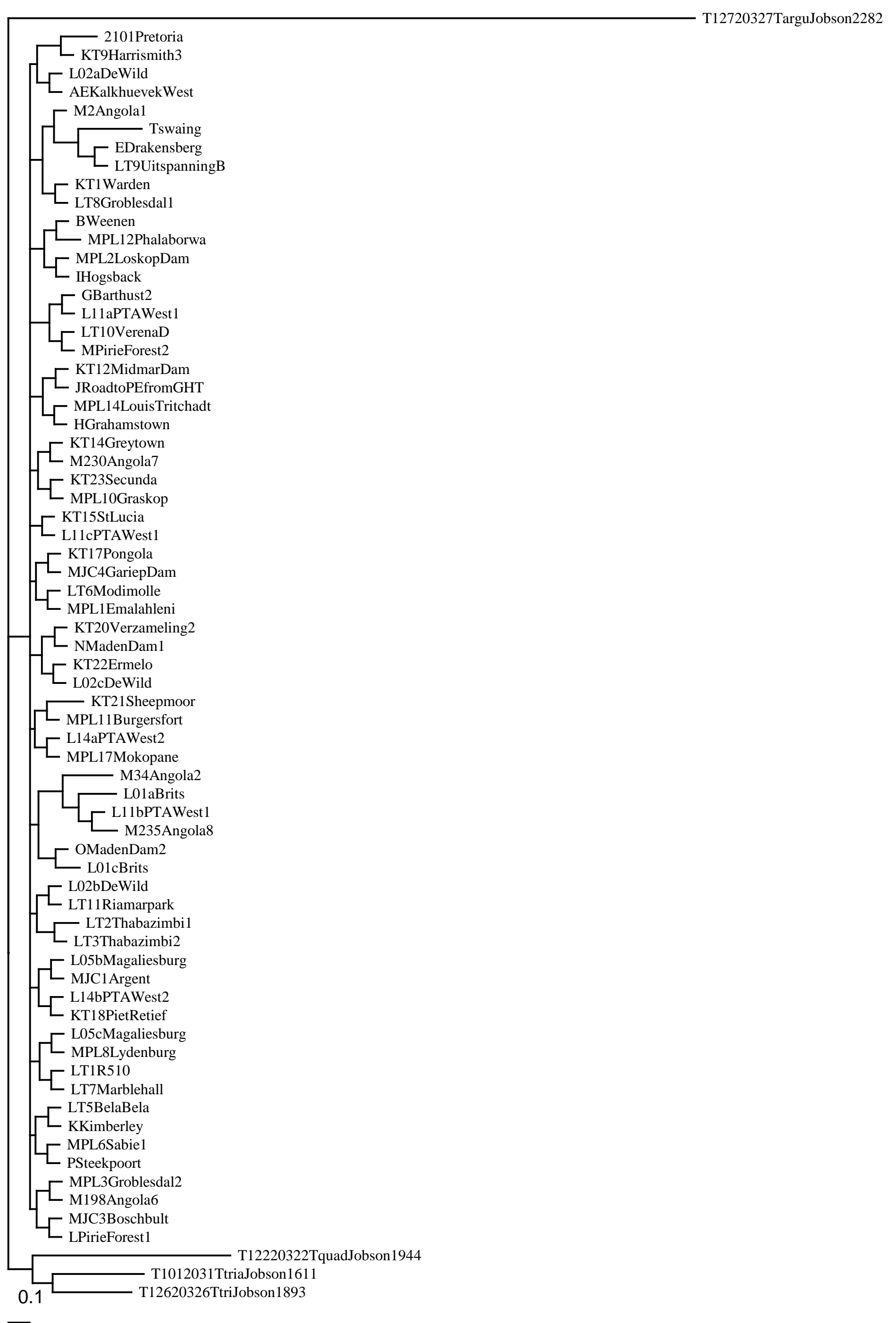


Figure 3.6: Results from the Bayesian analysis of the combined ITS and ETS data set rooted on *T. arguens*. The posterior probabilities are all less than 70%.

The phylogenetic trees obtained from Bayesian analysis have all clades where Posterior Probability values are less than 70%. These trees also show no supported groups and therefore there is no phylogenetic pattern within the samples of *T. triandra*. The Australian specimens in the analysis of ITS data (Figure 3.4) cluster together, including the *T. australis* specimen obtained from the GenBank. On Figure 3.5 (ETS analysis), the Australian specimens cluster with South African specimens, apart from each other. Lastly, where ITS and ETS were combined (Figure 3.6), the Australian *T. triandra* specimens all cluster together including *T. quadrivalvis.* This suggests that the samples from Australia and South Africa are distinct and thus there is some evidence that the recognition of *T. australis* has some merit.

3.4 Discussion

According to the above results there is limited variability, and no genetic evidence of any infra-specific groupings, and there is thus no geographic pattern observed within the *T. triandra* samples from South Africa. Australian specimens appear to show some genetic differences to the southern African specimens. *T. arguens* (L.) Hack. differs from the South African *T triandra,* whereas *T. quadrivalvis* (L.) Kuntze appears to be closely related. *T. quadrivalvis* was considered a synonym to *T. triandra* (Veldkamp 2016). This was because *T. triandra* and *T. quadrivalvis* look similar and they are both said to dominate savannas and grasslands. The one major difference between the two species is that *T. quadrivalvis* is an annual species, whilst *T. triandra* is a perennial species (Dunning *et al*. 2017). A study by Dunning *et al.* (2017) where cpDNA and nrDNA (ITS) were sequenced on 17 *Themeda* species also showed that the Australian and African *T. triandra* samples were separated. However, this is in contrast to the study by Dell'Acqua *et al.* (2014) who found no genetic differentiation between the Australian and African specimens from Kenya. This latter study used a variety of Amplified Fragment Length Polymorphism (AFLP) markers. These are said to represent a fast and reliable tool for the production of a number of genome-wide genetic markers (Dell'Acqua *et al.* 2014). Their study differed from the current study in that ITS and ETS analyse a very small region of the nuclear genome and the AFLP markers study the whole genome. However, the results are comparable to the current study.

3.5 Conclusion

The aims of this chapter were to determine whether there is any genetic structure in *T. triandra* found in southern Africa, and to determine if there are any genetic differences between the southern African and Australian species. Unfortunately, the results obtained here indicate that (like the anatomical data) there is no genetic structure within the species.

One possible reason for this results is that ITS and ETS regions are not sensitive enough to reflect recent evolution and population history. Also these regions are multi-copy in nature, and there is the possibility of pseudogenes, problematic secondary structure and possible amplification of contaminants (Álvarez and Wendel 2003). During the current study, only the forward ITS primer was species-specific. Although plant-specific primers can eliminate contamination, the above issues still remain, therefore more studies need to be conducted with recent technologies to minimise them.

The results also show that there are genetic differences between the southern African and Australian *T. triandra*, suggesting that the name *T. australis* could be applied to the Australian specimens. However, much wider sampling is required from across the full distribution range of the species.

4 Chapter 4: Synthesis

Themeda triandra is a polymorphic species with ploidy levels ranging from 2n= 20 to 100. It has been a species of interest to taxonomists and other scientists due to its variability. This variation is the main reason for the current study which aimed to answer the following questions:

1. Is there anatomical diversity within *T. triandra* specimens collected in southern Africa?

2. Is there genetic diversity within specimens collected in southern Africa and Australia?

3. To determine whether there are any species limits between South African and Australian specimens.

Results from leaf anatomy show no clear and consistent clustering of specimens and no geographic pattern among the specimens. There is, however, a little variation within the characters measured, although they did not present any taxonomic significance.

Genetic data was analysed and presented in networks and phylogenetic trees. These also showed no infra-specific groups and limited genetic diversity and no geographic pattern. The limited genetic diversity within the southern African specimens shows that the range of anatomical and morphological polymorphisms observed within the species is possibly a result of phenotypic plasticity. The Australian specimens were found to separate from those from southern Africa in both networks and phylogenetic trees. This shows that some differences do exist between the Australian and southern African specimens.

With the results showing little genetic diversity within the species within the subcontinent, it may then be suggested that the morphological differences observed in *T. triandra* may be due to environmental conditions and local adaptation. According to Bachle and Nippert (2018), climate variability has a great and common effect on physiological and phenetic responses in grass

species. These differences are used to explain species-specific responses of grasses in different conditions (Bachle and Nippert 2018, Bachle *et al.* 2018). C_4 grasses, such as *T. triandra,* are known to possess physiological traits that allow them to adapt to any unfavourable climatic conditions (Nippert *et al.* 2007, Bachle *et al.* 2018). Other such plants include *Clarkia unguiculata* Lindl. which was found to adapt to different elevations by self-fertilization and flowering earlier, before the onset of drought, especially at lower elevations (Jonas and Geber 1999).

4.1 Interpretation of these findings:

The fact that some species are variable and taxonomically difficult is not uncommon (McDade 1995). Approaches on how to accommodate these species taxonomically differ, and some workers will retain a broad and variable species, while others will divide it into smaller entities (“lumpers” and “splitters”) (Böcher 1967, McDade 1995, Spooner *et al.* 2003, Weakely 2005, Endersby 2009, Silva *et al.* 2020). A study by Böcher (1967) showed two species of the genus *Cerastium* with varying ploidy levels can either be lumped or split. Similarly, *Hildaea* is a morphologically varied genus with unclear species boundaries studied by Silva *et al.* (2020).

The concepts of splitters and lumpers, as reviewed by Endersby (2009) has been of great debate since the times of Darwin and the botanist Hooker. Hooker was said to be a lumper who reduced the number of plant species names by redefining many of the species as merely varieties. The terms “lumpers” and “splitters” are still in use this day, with splitters gaining more influence in speciation and biodiversity (Spooner *et al.* 2003, Weakely 2005, Endersby 2009). This has led to an increase in plant species names changing more than before (Weakely 2005).

Linked to this is the mix of aspects such as species concepts, ecotypes, natural variability and population genetics. In dealing with a particularly variable species of *Diospyros* from Africa, White (1955) introduced the ochlospecies concept to accommodate instances of rampant phenotypic plasticity. A change in environmental conditions may result in the phenotype

(appearance) of the species being altered to adapt to the environmental conditions (ecology and geography - White *et al*. 1990, Bayonne Mboumba and Ward 2008). Phenotypic plasticity is said to affect the ways in which species interact with each other, how they compete and can promote or hinder coexistence within an environment (Turcotte and Levine 2016). Although the ochlospecies is not a taxonomic category, but a species concept, it can be very useful in showing that a species complex cannot be accommodated in a formal taxonomic treatment (Barbosa *et al.* 2012).

According to Brown and Emery (1957) and Liebenberg (1986) *T. triandra* may be an actively evolving agamic (apomictic) species. This means that the observed morphological variation is a result of asexual reproduction where gametophytes are formed directly from the diploid cells. Apomictic lineages are sometimes referred to as micro- or intermediate species (McDade 1995). According to Stace (1998) apomictic taxa do not require a taxonomic category. Soreng (1991), studying *Poa* L. lineages, indicated that it is better to treat an apomictic lineage not as separate species, but as sub-sets of a sexual species. This reduces the number of species that may result from splitting. A study by Hörandl (1998) on an agamic species *Ranunculus auricomus* L. showed that using phenetic species concepts (based on morphology) on an agamic species may lead to splitting, resulting in a number of species instead of one. It is for this reason that the current study incorporated genetics and leaf anatomy to assess the species morphological variation. However, the results of this study show *T. traindra* to be an ochlospecies more than an agamic species.

According to (Cronk 1998) there are ten characteristics of an ochlospecies, and if a species meets six or more of these it can then be considered an ochlospecies. These characteristics are:

1. A species which shows a non-hierarchical polymorphic variation.
2. Character-state distribution shows only partial correlation with the geography and ecology.
3. Characters vary independently, not in a correlated fashion.
4. Complexity is not due to hybridisation or to a special breeding system between currently recognisable species.

5. Geographically and ecologically widespread, occurring in a variety of climates and vegetation zones.
6. At a particular locality two distinct and non-intergrading forms may be found and other forms may be found at other localities, but taken together the forms intergrade and the classification breaks down.
7. They sometimes have a closely related, but morphologically monotypic species.
8. Similar variants may occur in widely separated localities and appear to be polytopic in origin.
9. They occur in medium to large genera, with more than 50 species.
10. There is often an untenable proliferation of synonyms.

According to Barbosa *et al.* (2012) the complexity seen in ochlospecies may be a result of "ancient hybridization or introgression events and to events related to expansion and retraction of populations during the Pleistocene Epoch".

Classifying species with a wide range of morphologies has been a great challenge for taxonomists (Harrington and Gadek 2009). A number of studies done on plant species and genera, without a clear geographic pattern have led to taxonomists assigning the term ochlospecies to taxonomically problematic species and species complexes (Pipoly 1983, Huxley and Jebb 1993, Neuba *et al.* 2006, Bennett *et al.* 2008, Harrington and Gadek 2009, Barbosa *et al.* 2012, Applequist 2015, Henderson 2020). It is worth briefly discussing these studies in which the ochlospecies concept is applied.

Barbosa *et al.* (2012) showed *Vellozia hirsuta* Goethart & Henrard to be an ochlospecies due to its variation in morphology and leaf anatomy. As in *Themeda triandra*, the variation seen in *V. hirsuta* is quite complex and does not follow any geographic pattern and therefore became very challenging to classify. In the end, the authors concluded that *V. hirsuta* is a single species with a wide morphological range (Barbosa *et al.* 2012). Huxley and Jebb (1993) described *Myrmecodia tuberosa* Jack as an ochlospecies due to its morphological variation that could not be related to

its geography. There had been variants with informal names described, but further studying of the many character combinations showed it to be an ochlospecies. The previously named variants were therefore eliminated and *M. tuberosa* remained one species with varying morphologies (Huxley and Jebb 1993).

A similar situation is seen in *Strobilanthes echinata* Nees (Bennett *et al.* 2008). *S. echinata* is polymorphic without a clear geographic pattern. The species has been described under many different names, but with further morphological studies and reference to White (1962), it was concluded that it is indeed an ochlospecies. The genus *Allophylus* L. is another example of a species complex (Stace 1998, Harrington and Gadek 2009). Initially, 255 species were described in the genus, but further studies by Leenhouts (1967) concluded that *Allophylus cobbe* (L.) Räusch is a single species with a wide range of morphologies and 255 synonyms, therefore making it an ochlospecies.

Another genus with a widespread morphology is *Sambucus.* According to Applequist (2015) there has not been any formal taxonomic treatment that has been satisfactory for the genus. Applequist (2015) reduced the number of species in the genus from thirty to nine where eight of the initially described species were found to be an ochlospecies complex. The species in *Sambucus* are so complex that neither lumping nor splitting them seemed appropriate (Applequist 2015). It was for this reason that the ochlospecies concept was considered and *Sambucus nigra* was then accepted as the formal name for the species (Applequist 2015).

Henderson (2020) found species in the palm genus *Calamus* to be similar to each other and taxonomically difficult. The previously described species, subspecies and varieties were grouped together as one morphologically variable species. Lima *et al*. (2015) showed that *M. tomentosa* is an ochlospecies because there was no genetic diversity to support the morphological differences within the species,)a case similar to *Themeda triandra*.

Other species that have been considered as ochlospecies include, *Dodonaea viscosa* Jacq. which shows a wide morphologiacal variation which does not correlate to its geography (Harrington and Gadek 2009). Neuba *et al.* (2006) studied *Leptactina benguelensis* (Welw. ex Benth. & Hook.f.) and found that it meets six of the characteristics of an ochlospecies described by Cronk (1998) resulting in the suppression of the subspecies and leaving *L. benguelensis* as a single species with multiple morphological forms. Similarly *Cybianthus spicatus* (H.B.K.) was found to be an ochlospecies due to its polymorphic nature which did not relate to its geography (Pipoly 1983).

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Apart from White's original study on *Diospyros*, other African taxa considered to meet the requirements of the ochlospecies concept include *Vachellia karoo* (Hayne) Banfi & Galasso, where a study using ISSR DNA Barcoding techniques found this taxon to be an ochlospecies (Taylor and Barker 2012). Howis (2007) also used DNA data to show that some species of *Gazania* that were previously considered separate species because of their morphology were placed in a morphologically and genetically overlapping continuum that comprised an ochlospecies.

The ochlospecies concept has thus provided a more satisfactory way of classifying species and genera that do not conform to formal taxonomic treatments. *Themeda triandra,* with its polymorphic nature is another example of the many species that can be considered under the ochlospecies concept. As noted above, there are 10 characteristics of an ochlospecies, and a taxon must meet at least six of these to be considered an ochlospecies.

According to the current study, *T. triandra* meets characteristics 1, 2, 3, 4, 5 and 8, which qualifies it to be an ochlospecies.

While this is perhaps not a taxonomically convenient finding, the range of variability of this species means that (in the absence of data that can provide a finer resolution) this is the best lens through which *Themeda triandra* can be viewed. Variability is a biological reality that cannot always be conveniently partitioned and labelled.

4.2 Future perspectives

Further studies need to be conducted using more sophisticated DNA markers, such as Amplified Fragment Length Polymorphism (AFLP) markers. It has been shown that AFLP markers are the most appropriate and efficient method for studying systematics in plant groups where variation cannot be detected through nuclear and chloroplast sequence analyses (Hodkinson et al. 2000; Ward 2011).

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6 Appendix

Table 2.1: Details of specimens used in anatomical survey, including locality, collector and whether they are fresh or herbarium specimens, and the nature of the anatomical method used (Scanning Electron Microscopy, light microscopy or both). KwaZulu-Natal = KZN, Mpumalanga = Mpu, Gauteng = GP, Free-State = FS, Limpopo = Lim, Eastern Cape = EC, Northern Cape = NC

Collector	Fresh	Herbarium	Locality	GPS Co-ordinates	SEM	LM
S. Ntshangase (1)	√		Warden, FS	S27.85188°; E 28.9592°	√	√
S. Ntshangase (2)	√		Royal Natal 1, KZN	S28.71217°; E28.93358°	√	√
S. Ntshangase (3)	√		Royal Natal 2, KZN	S28.71073°; E28.92605°	√	√
S. Ntshangase (4)	√		Royal Natal 3, KZN	S28.71012°; E28.91412°	√	√
S. Ntshangase (5)	√		Royal Natal 4, KZN	S28.72620°; E28.92976°	√	√
S. Ntshangase (6)	√		Royal Natal 5, KZN	S28.68802°; E28.94857°	√	√
S. Ntshangase (7)	√		Road from Royal natal to Harrismith, KZN	S28.51175°; E29.07344°	√	√
S. Ntshangase (8)	√		Harrismith 1, FS	S28.26690°; E29.14110°	√	√
S. Ntshangase (9)	√		Harrismith 2, FS	S28.26309°; E29.14328°	√	√
S. Ntshangase (10)	√		Harrismith 3, FS	S28.26507°; E29.14053°	√	√
S. Ntshangase (11)	√		Harrismith 4, FS	S28.26276°; E29.12316°	√	√

S. Ntshangase (12)	√		Midmar Dam, KZN	S29.49566°; E30.16220°	√	√
S. Ntshangase (13)	√		Maphumulo, KZN	S29.11358°; E31.02684°	√	√
S. Ntshangase (14)	√		Greytown, KZN	S28.98470°; E30.81659°	√	√
S. Ntshangase (15)	√		St. Lucia, KZN	S28.35575°; E32.41962°	√	√
S. Ntshangase (16)	√		N2 towards Pongola, KZN	S27.75515°; E32.11157°	√	√
S. Ntshangase (17)	√		Pongola, KZN	S27.42693°; E31.86554°	√	√
S. Ntshangase (18)	√		Piet Retief, Mpu	S27.20660°; E31.09293°	√	√
S. Ntshangase (19)	√		Verzameling, Mpu	S26.90885°; E30.72853°	√	√
S. Ntshangase (20)	√		Verzameling, Mpu	S26.90885°; E30.72853°	√	√
S. Ntshangase (21)	√		Sheepmoor, Mpu	S26.75027°; E30.36234°	√	√
S. Ntshangase (22)	√		Ermelo, Mpu	S26.51356°; E29.90657°	√	√

S. Ntshangase (23)	√		Secunda, Mpu	S26.46438°; E29.18931°	√	√
S. Ntshangase (24)	√		R510 - Lim	S 25.7117°; E 27.64917°	√	√
S. Ntshangase (25)	√		Thabazimbi 1, Lim	S 24.91234°; E 27.28350°	√	√
S. Ntshangase (26)	√		Thabazimbi 2, Lim	S 24.57597°; E 27.41544°	√	√
S. Ntshangase (27)	√		Bela Bela 1, Lim	S 24.86960°; E 28.10041°	√	√
S. Ntshangase (28)	√		Bela Bela 2, Lim	S 24.84868°; E 28.34738°	√	√
S. Ntshangase (29)	√		Modimolle, Lim	S 24.70976°; E 28.41278°	√	√
S. Ntshangase (30)	√		Marblehall, Lim	S 24.84401°; E 28.88696°	√	√
S. Ntshangase (31)	√		Groblesdal, Lim	S 25.16815°; E 29.37104°	√	√
S. Ntshangase (32)	√		Uitspanning B, Mpu	S 25.24319°; E 29.21340°	√	√
S. Ntshangase (33)	√		Verena-D, Mpu	S 25.47208°; E 29.03927°	√	√

S. Ntshangase (34)	√		Riamarpark, Mpu	S 25.80934°; E 28.70253°	√	√
S. Ntshangase (35)	√		Emalahleni, Mpu	S 25.88668°; E 29.27050°	√	√
S. Ntshangase (36)	√		Loskop Dam, Mpu	S 25.41040°; E 29.36386°	√	√
S. Ntshangase (37)	√		Groblesdal, Lim	S 25.17921°; E 29.54404°	√	√
S. Ntshangase (38)	√		Barberton, Mpu	S 25.78289°; E 31.02855°	√	√
S. Ntshangase (39)	√		Nelspruit, Mpu	S 25.64779°; E 30.97743°	√	√
S. Ntshangase (40)	√		Sabie 1, Mpu	S 25.41015°; E 30.91567°	√	√
S. Ntshangase (41)	√		Sabie 2, Mpu	S 25.14502°; E 30.75759°	√	√
S. Ntshangase (42)	√		Lydenburg, Mpu	S 25.10905°; E 30.49458°	√	√
S. Ntshangase (43)	√		Ohrigstad, Lim	S 24.84682°; E 30.57333°	√	√
S. Ntshangase (44)	√		Graskop, Mpu	S 24.92512°; E 30.81283°	√	√

S. Ntshangase (45)	√		Burgersfort, Lim	S 24.72472°; E 30.55678°	√	√
S. Ntshangase (46)	√		Phalaborwa, Lim	S 24.10687°; E 30.86461°	√	√
S. Ntshangase (47)	√		Thohoyandou, Lim	S 22.94123°; E 30.48588°	√	√
S. Ntshangase (48)	√		Louis Tritchadt, Lim	S 23.03162°; E 29.91874°	√	√
S. Ntshangase (49)	√		Bungeni, Lim	S 23.20885°; E 30.17513°	√	√
S. Ntshangase (50)	√		Polokwane, Lim	S 23.93109°; E 29.46977°	√	√
S. Ntshangase (51)	√		Mokopane, Lim	S 24.21767°; E 29.00510°	√	√
S. Ntshangase (52)	√		Mookgopong, Lim	S 24.62459°; E 28.59945°	√	√
S. Ntshangase (53)	√		Bela Bela, Lim	S 25.08982°; E 28.30714°	√	√
M. Cunningham	√		Argent, Mpu	S26.01115°; E28.79572°	√	√
M. Cunningham	√		Diamond Hill-Off-ramp, GP	S25.79913°; E28.51610°	√	√
M. Cunningham	√		Boschbult, Mpu	S25.56765°; E28.64466°	√	√

M. Cunningham	√		Gariep Dam, FS	S30.62924°; E25.49885°		√
Unknown	√		Zimbabwe	N/A	√	
Unknown	√		Weenen, KZN	N/A	√	
S. Ntshangase (54)	√		Drakensberg 1, KZN	S29.47137°; E28.9938°	√	
S. Ntshangase (55)	√		Drakensberg 2, KZN	S29.42951°; E28.9472°	√	
S. Ntshangase (56)	√		Drakensberg 3, KZN	S29.44847°; E28.9384°	√	
S. Ntshangase (57)	√		Barthust 1, EC	N/A	√	
S. Ntshangase (58)	√		Barthust 2, EC	N/A	√	
S. Ntshangase (59)	√		Barthust 3, EC	N/A	√	
Prof Nigel Barker	√		Hogsback, EC	N/A	√	
Prof Nigel Barker	√		Port Elizabeth, EC	N/A	√	
S. Ntshangase (60)	√		Kimberley, NC	N/A	√	
S. Ntshangase (61)	√		Pirie Forest 1, EC	N/A	√	
S. Ntshangase	√		Pirie Forest 2, EC	N/A	√	

(62)						
S. Ntshangase (63)	√		Maden Dam 1, EC	N/A	√	
S. Ntshangase (64)	√		Maden Dam 2, EC	N/A	√	
S. Ntshangase (65)	√		Skurweberg, GP	S25.791667°; E27.983333°	√	√
N.P. Barker	√		Angola 1	N/A	√	
N.P. Barker	√		Angola 2	N/A	√	
N.P. Barker	√		Angola 3	N/A	√	
N.P. Barker	√		Angola 4	N/A	√	
N.P. Barker	√		Angola 5	N/A	√	
N.P. Barker	√		Angola 6	N/A	√	
N.P. Barker	√		Angola 7	N/A	√	
N.P. Barker	√		Angola 8	N/A	√	
N.P. Barker (2099)	√		Pretoria 1, GP	N/A	√	
N.P. Barker (2100)	√		Pretoria 2, GP	N/A	√	
N.P. Barker (2101)	√		Pretoria 3, GP	N/A	√	
S. Ntshangase	√		Brits; PTA, GP	S25.583333°; E27.772222°	√	√

(66)						
S. Ntshangase (67)	√		De Wild; PTA, GP	S25.65°; E27.822222°	√	√
S. Ntshangase (68)	√		Magaliesburg Range; PTA, GP	S25.706944°; E27.9°	√	√
S. Ntshangase (69)	√		Pretoria West-1, GP	S25.794444°; E27.993056°	√	√
S. Ntshangase (70)	√		Pretoria West-2, GP	S25.861111°; E28.011111°	√	√
S. Ntshangase (71)	√		PTA Botanical garden, GP	S25.739369°; E28.273635°	√	√
S. Ntshangase (72)	√		Edendale, KZN	S29.615850°; E30.370443°	√	√
N.P. Barker and D. Masia	√		Roodeplaat 1, GP	GP S34.665°; E21.694°	√	√
N.P. Barker and D. Masia	√		Roodeplaat 2, GP	GP S34.665°; E21.694°	√	√
S. Ntshangase (73)	√		Tswaing crater, GP	S25.404996°; E28.083079°		√
C.M. Breen (31) - GRA		√	Zululand	N/A	√	
A.B. Low (12094)		√	Tulbagh Valley	N/A	√	

- GRA						
J. Allsop (7) - GRA		√	Pietermaritzburg	N/A	√	
J. Anderson (ORFS199) - GRA		√	Oviston_NR	N/A	√	
L. Smook (3972) - GRA		√	Sommerset_East	N/A	√	
E. Bebster (5) - GRA		√	Grahamstown	N/A	√	
Abraham (s.n.) - GRA		√	Kowie_NR	N/A	√	
B.P. Botha (5668) - GRA		√	Addo	N/A	√	
M.R. Court (5) - GRA		√	Voortreker_Monument	N/A	√	
E.A. Gotzee (214) - GRA		√	Shamvura_Namibia	N/A	√	
H. Burrows (s.n.) - GRA		√	Harare_Zimbabwe	N/A	√	
Phillipson (4492) - GRA		√	Leribe_Lesotho	N/A	√	

Phillipson (5088) - GRA		√	Arusha_Tanzania	N/A	√	
De Bie (s.n.) - GRA		√	Bloemfontein	N/A	√	
P. Christonsen (132) - GRA		√	Ruiru_Kenya	N/A	√	
M. hurter (s.n.) - GRA		√	Cedaville	N/A	√	
D.F. Moult (29) - GRA		√	Kimberley	N/A	√	
S. McCartn (16) - GRA		√	Bergbron	N/A	√	
E. Brink (s.n.) - GRA		√	Ecca_Reserve	N/A	√	
K. Bowker (s.n.) - GRA		√	Carlisle	N/A	√	
H. Burrows (4418) - GRA		√	Kariega_Park	N/A	√	
G. Siom (893) - GRA		√	Arusha_Tanzania	N/A	√	
A. Mogg (34292) - GRA		√	Kerry_Farm_Zimbabwe	N/A	√	

R. Lupke (6132) - GRA		√	Mokhotlong_Dam_Lesotho	N/A	√	
C. Chielchinskey (50) - GRA		√	Tiffendell	N/A	√	
P.N. Sebothoma (48) - GRA		√	Queenstown	N/A	√	
J. Victor (698) - GRA		√	Burgesdorp	N/A	√	
G. Youthead (733) - GRA		√	Kromme_River	N/A	√	
J. Jessop (1014) - GRA		√	Melkhoutboom	N/A	√	
S. Dickinson (55) - GRA		√	Brynston	N/A	√	
S. Gower (17) - GRA		√	Meintjies_Kop	N/A	√	
G. Buchanan (24565) - GRA		√	Ridgefontein	N/A	√	
P. Burdett (103) - GRA		√	Kudu_Reserve	N/A	√	
E. Archibald (3351) - GRA		√	Middleberg	N/A	√	

E. Archibald (3874) - GRA		√	Addo	N/A	√	
E. Warren (115) - GRA		√	Port_Alfred	N/A	√	
Gibbs-Russell (3068) - GRA		√	Fort_Hare	N/A	√	
A. Jacot-Gillarmod (8856) - GRA		√	Faraway_Farm	N/A	√	
M. Olivier (2462) - GRA		√	Springs_Reserve	N/A	√	
E. Archibald (530) - GRA		√	Maletsanyane_Falls_Lesotho	N/A	√	
D. Verwoed (4) - GRA		√	Double_Mouth_Transkei	N/A	√	
L. Smook (4004) – GRA		√	Braamskloof	N/A	√	
K. Dahlstrand (2861) - GRA		√	Kosuga	N/A	√	
G. Swart (s.n.) – GRA		√	Firston_Farm	N/A	√	
Gibbs-Russell		√	Gxulu	N/A	√	

(3452) - GRA						
A. Jacot-Gillarmod (9213) - GRA		√	Faraway_Farm	N/A	√	
J. Wirminghaus (132) – GRA		√	Round_Hill_NR	N/A	√	
L. Britten (716) – GRA		√	Three_Sisters	N/A	√	
I. Hepburn (180) - GRA		√	Sterspruit	N/A	√	
D. Cotterrell (s.n.) – GRA		√	Fairford_Farm	N/A	√	
L. Britten (4631) – GRA		√	Pomona_Farm_Ugi	N/A	√	
Forward (s.n.) – GRA		√	Piggs_Peak_Swaziland	N/A	√	
N. Fanshaw (214) – GRA		√	Zearust	N/A	√	
J. Anderson (ORFS180) – GRA		√	Oviston_NR	N/A	√	
W. Berrington –		√	Van_Staden_Reserve	N/A	√	

GRA						
Bonny and Uschi (ub190) – GRA		√	Mountain_Zebra_Park_Craddock	N/A	√	
T. Dold (1900) – GRA		√	Grahamstown	N/A	√	
A.M. Makwarela (75) – GRA		√	Indwe	N/A	√	
R. Lubke (3418) – GRA		√	Oudtshoorn_De_Kombuis	N/A	√	
K. Simpson and B. Ripley (24) – GRA		√	Grahamstown	N/A	√	
R.E. Geroeau (6535) – GRA		√	Arusha_Tanzania	N/A	√	
T. Hill (12) – GRA		√	Loteni_Drakensberg	N/A	√	
J.M. Todd (96) – GRA		√	Sosbury_Harare	N/A	√	
K. Easton (73) – GRA		√	Kimberley	N/A	√	
C.D. Hobson (16) – GRA		√	Salem	N/A	√	

J. Hebblethwaite (s.n.) -PRU		√	Oxbow_Bethlehem_Lesotho	N/A	√	
M.P. Robertson (69) –PRU		√	Sani Pass, Maluti-Drakensberg	N/A	√	
S. Malan (688) –PRU		√	Faerie Glen	N/A	√	
Group 1/8 –PRU		√	Faerie Glen	N/A	√	
A. Abbott (5881) –PRU		√	Woodcliffs trails NW of Reed park	N/A	√	
B.J. Coetzee (2) –PRU		√	Wonderboomsuid, Magaliesberg	N/A	√	
P. van Staden –PRU		√	Kransberg National Park Thabazimbi	N/A	√	
P.C. Zietsman (3713) -PRU		√	Glen Landboukollege Bloemfontein	N/A	√	
N.P. Barker (619) -PRU		√	Brits, Beestekral Game reserve	N/A	√	
N. van Rooyen (1627) -PRU		√	Waterberge, Ellisras District	N/A	√	
A. Oosterhuis (1) –PRU		√	about 2km from Fonteine-Sirkel	N/A	√	
H. Herne (22) -		√	Cape province: Anda	N/A	√	

PRU			Lusia			
K.J. Bloem (5) – PRU		√	Steenkampsberg Natuur Reservaat	N/A	√	
C. Watson (4) – PRU		√	Kruger National Park	N/A	√	
M. Mathee (355) –PRU		√	Potlale Nature Reserve, Zebediela	N/A	√	
A.A. Gubb (2868) –PRU		√	Farm Eureka, South of Rooigrond	N/A	√	
S.P. Bester (243) –PRU		√	Drakensberg. 2km to Lily Vale	N/A	√	
A. Abbott (8116) –PRU		√	Umtamvuna Nature Reserve	N/A	√	
L.A. Coetzer (868) –PRU		√	Farm Lekkerbreek, Wambad District.	N/A	√	
R. Ayres (007) - PRU		√	Gauteng, 30km from Krugersdorp	N/A	√	
A.E. van Wyk (13657) –PRU		√	Centurion, Gauteng	N/A	√	
C.J. du Plessis (664) –PRU		√	Middelburg (Doornkop)	N/A	√	
G.J. Bredenkamp		√	Suiker bosrand-Tussen	N/A	√	

(248) –PRU			Fees			
H.P.V.D. Schyef (5989) –PRU		√	Marieskop, Eastern Transvaal.	N/A	√	
H.G. (1700) – PRU		√	Pretoria, about 18 miles SE of Pretoria	N/A	√	
H. Herne (7) – PRU		√	Cape province, Anda Lusia	N/A	√	
W.M. O'Donovan (s.n.) –PRU		√	Charleswood District. Zimbabwe	N/A	√	
A. Krige (130) – PRU		√	Cradle of Human kind	N/A	√	
R. Smit (2801) – PRU		√	Mapoch's caves, Roossenekal	N/A	√	
G.K. Theron (550) –PRU		√	Sasolburg. OUS, Hoeveldtuin	N/A	√	
J.C. Scheepers (527) –PRU		√	Duiwelskloof, Letaba District	N/A	√	
P.J. Robbertse (422) –PRU		√	Zebediela, Mogotokloof, Blare harig	N/A	√	
P.J. Robbertse (591) –PRU		√	Zebediela, Mogotokloof	N/A	√	

P.J. Robbertse (600) –PRU		√	Zebediela	N/A	√	
C.J. du Plessis (217) –PRU		√	Middelburg (Doornkop)	N/A	√	
A. Abbott (328) – PRU		√	Umtamvuna Nature Reserve	N/A	√	
G.K. Theron (696) –PRU		√	Middelburg/Witbank, Loskop dam	N/A	√	
G.K. Theron (2052) –PRU		√	Middelburg/Witbank, Loskop	N/A	√	
G.K. Theron (71) –PRU		√	Groenkloof. Langs, Totius straad	N/A	√	
G.F. Bredenkamp (787) –PRU		√	Suikerboorand	N/A	√	
Hon’s Wildlife Management (3) – PRU		√	Derdepoort Radio station. Transvaal	N/A	√	
M. Potgieter (312) –PRU		√	Maitland Nature Reserve. Kosuga	N/A	√	
M. Potgieter (312) –PRU		√	Maitland Nature Reserve. Kosuga	N/A	√	
A. Abbott (6168)		√	Nsikeni, Long Ridge	N/A	√	

–PRU						
A. Abbott (3976) –PRU		√	Umtamvuna Nature Reserve	N/A	√	
R.H. Archer (75) –PRU		√	Vaalwater, Klatenberge	N/A	√	
Group 2/89 –PRU		√	Coregon Str., Faerie Glen	N/A	√	
G.G. Catto (12) –PRU		√	Groenkloof National Park, Pretoria	N/A	√	
J.P. Kluge (368) –PRU		√	Witklip, Lydenburg, Transvaal	N/A	√	
G.K. Theron (3419) –PRU		√	Ohrigstad Natuur Reservaat	N/A	√	
Group 4/32 –PRU		√	Faerie Glen	N/A	√	
G.K. Theron (550) –PRU		√	Sasolburg. OVS	N/A	√	
A. Abbott (7966) –PRU		√	Port Edward, Natal	N/A	√	
A. Abbott (7965) –PRU		√	Port Edward, Natal	N/A	√	
S.J. Siebert (678) –PRU		√	Steelpoort, Mpumalanga	N/A	√	

N. van Rooyen (1627) –PRU		√	Waterberge, 45km N of Vaalwater	N/A	√	
N. van Rooyen (1058) –PRU		√	NKW, Punda Milia, Shipudza	N/A	√	
N. van Rooyen (2807) –PRU		√	Roodeplaat dam, Pretoria	N/A	√	
H.G. (1700) – PRU		√	Pretoria	N/A	√	
J.P.J. van Vuuren (10) –PRU		√	Mpumalanga, Njaha, Graskop	N/A	√	
J.P.J. van Vuuren (33) -PRU		√	KZN, Ixopo, 6.4km from town	N/A	√	

Table 6.1: Characters and character states as obtained from the SEM observations of freshly collected specimens from Simple Matching and Euclidean Distance phenogram analyses.

Character	**Fresh Euclidean**	**Fresh SM**
Macro-Hairs presence/absence	**Cluster I:** none of the specimens have macro-hairs. **Cluster II:** 9/28 specimens have macro-	**Cluster A:** 6/37 specimens have macro-hairs. **Cluster B:** One of the two

	hairs. **Cluster III:** 7/54 specimens have macro-hairs. **Cluster IV:** None of the specimens have macro-hairs **Cluster V:** None of the specimens have macro-hairs	specimens has macro-hairs. **Cluster C:** 10/48 specimens have macro-hairs. **Cluster D:** None of the two have macro-hairs. **Cluster E:** None of the two have macro-hairs
Macro-hair frequency	**Cluster II:** 4/9 specimens have an occasional frequency. **Cluster III:** 3/7 specimens have an occasional frequency.	**Cluster A:** 1/6 specimens has a dense frequency. **Cluster B:** The one specimen has a dense frequency. **Cluster C:** 7/10 specimens have a dense frequency.
Macro-hair length	Specimens have varying macro-hair lengths.	**Cluster A:** Lengths vary, however, ±1400µm is common. **Cluster B:** The one specimen has a macro-hair length of 908.2µm. **Cluster C:** Specimens have varying macro-hair lengths.
Micro-Hairs presence/absence	**Cluster I:** One of the two specimens has micro-hairs. **Cluster II:** 26/28 specimens have micro-hairs. **Cluster III**: 49/54	**Cluster A:** 35/37 specimens have micro-hairs. **Cluster B:** None of the two

	specimens have micro-hairs. **Cluster IV**: All two specimens have micro-hairs. **Cluster V:** All three specimens have micro-hairs.	specimens have micro-hairs. **Cluster C:** 44/48 specimens have micro-hairs. **Cluster D:** Both specimens have micro-hairs. **Cluster E:** Both specimens have micro-hairs
Micro-hair frequency	**Cluster I:** the one specimen has an occasional frequency. **Cluster II:** 3/26 specimens have a dense micro-hair frequency. **Cluster III:** 10/49 specimens have a dense micro-hair frequency. **Cluster IV:** All two specimens have an occasional micro-hair frequency. **Cluster V:** All three specimens have an occasional micro-hair frequency.	**Cluster A:** 6/35 specimens have a dense micro-hair frequency. **Cluster C:** 7/44 specimens have a dense micro-hair frequency. **Cluster D:** Both specimens have an occasional micro-hair frequency. **Cluster E:** One of the two specimens has a dense micro-hair frequency.
Micro-hair length	The specimens have micro-hairs that vary from 25µm to 70µm in length.	The specimens have varying lengths.
Large-Prickle Hairs presence/absence	**Cluster I:** Both specimens have large-prickle hairs. **Cluster II:** All the specimens have large-	**Cluster A:** All 37 specimens have large-prickle hairs. **Cluster B:** Both specimens have

	prickle hairs. **Cluster III:** 51/54 specimens have large-prickle hairs. **Cluster IV:** All two specimens have large-prickle hairs. **Cluster V:** All three specimens have large-prickle hairs.	large-prickle hairs. **Cluster C:** 45/48 specimens have large prickle hairs. **Cluster D:** Both specimens have large-prickle hairs. **Cluster E:** Both specimens have large-prickle hairs.
Large-prickles density	**Cluster I:** One of the two specimens has a dense large-prickle hair frequency. **Cluster II:** 6/28 specimens have an occasional frequency. **Cluster III:** 6/51 specimens have an occasional frequency. **Cluster IV:** One of the two specimens has an occasional frequency. **Cluster V:** All three specimens have a dense large-prickle hair frequency.	**Cluster A:** 5/37 specimens have an occasional frequency. **Cluster B:** Both specimens have a dense large-prickle hair frequency. **Cluster C:** 7/45 specimens have an occasional large prickle-hair frequency. **Cluster D:** Both specimens have an occasional large prickle-hair frequency. **Cluster E:** Both specimens have a dense large prickle-hair frequency.
Large prickles base	**Cluster I:** One of the two specimens has a	**Cluster A:** 6/37 specimens have

structure	base structure with a collar. **Cluster II:** 4/28 specimens have a base structure without a collar. **Cluster III:** 5/51 specimens have a base structure without a collar. **Cluster IV:** One of the two specimens has a base structure without a collar. **Cluster V:** All three specimens have a base structure with a collar.	no collar at their base. **Cluster B:** Both specimens have a collar at their large-prickle hair base. **Cluster C:** 4/45 specimens have no collar at their base. **Cluster D:** Both specimens have a collar at their large-prickle hair base. **Cluster E:** One of the two specimens does not have a collar at the base of the large-prickle hairs.
Large-prickles length	**Cluster I:** The lengths are 60µm and 30µm. **Cluster II:** The lengths vary from 30µm to 90µm. **Cluster III:** The lengths vary from 23µm to 104µm. **Cluster IV:** The lengths vary from 47µm to 48µm. **Cluster V:** The lengths vary from 54µm	**Cluster A:** The large-prickle lengths range from 40µm to 88µm. **Cluster B:** The large-prickle lengths range from 35µm to 60µm. **Cluster C:** The large-prickle lengths range from 30µm to 104µm.

	to 67µm.	**Cluster D:** The large-prickle lengths range from 48µm to 54µm. **Cluster E:** The large-prickle lengths range from 52µm to 70µm.
Large prickles size uniformity	**Cluster I:** Both specimens have uniform large-prickle hair lengths. **Cluster II:** 3/28 specimens have non-uniform large-prickle hairs. **Cluster III:** 5/51 specimens have non-uniform large-prickle hairs. **Cluster IV:** All two specimens have uniform large-prickle hairs. **Cluster V:** All three specimens have uniform large-prickle hairs.	**Cluster A:** 6/37 specimens have non-uniform large prickle hairs. **Cluster B:** One of the two specimens has non-uniform large prickle hairs. **Cluster C:** 2/45 specimens have non-uniform large prickle hairs. **Cluster D:** Both specimens have uniform large prickle hairs. **Cluster E:** Both specimens have uniform large prickle hairs
Large-prickles orientation	**Cluster I:** Both specimens have large-prickle hairs that are not erect. **Cluster II:** 3/28 specimens have erect large-prickle hairs. **Cluster III:** 7/51 specimens have erect	**Cluster A:** 5/37 specimens have erect large-prickle hairs. **Cluster B:** One of the two specimens has erect large-prickle hairs.

	large-prickle hairs. **Cluster IV:** One of the two specimens ha erect large-prickle hairs. **Cluster V:** one of the three specimens has erect large-prickle hairs.	**Cluster C:** 6/45 specimens have erected large prickle hairs. **Cluster D:** Both specimens have non-erect large prickle hairs. **Cluster E:** Both specimens have non-erect large prickle hairs.
Large prickles distribution	**Cluster I:** Only one of the two specimens has large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster II:** 6/28 specimens have large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster III:** 5/51 specimens have large-prickle hairs distributed on all vascular bundles including the midrib **Cluster IV:** Large-prickle hairs are distributed on only the 1˚ and 2˚ vascular bundles on all two specimens. **Cluster V:** Large-prickle hairs are distributed on only the 1˚ and 2˚ vascular bundles on all three specimens.	**Cluster A:** 6/37 specimens have large prickle hairs distributed on all vascular bundles including the midrib. **Cluster B:** Both specimens have large prickle hairs distributed on the 1˚ and 2˚ vascular bundles only **Cluster C:** 7/47 specimens have large prickle hairs distributed on all vascular bundles including the midrib. **Cluster D:** Both specimens have large prickle hairs distributed on the 1˚ and 2˚ vascular bundles

		only. **Cluster E:** Both specimens have large prickle hairs distributed on the 1˚ and 2˚ vascular bundles only.
Long-Barb Prickles presence/absence	**Cluster I:** Both specimens have long-barb prickles. **Cluster II:** 7/28 specimens do not have long-barb prickles. **Cluster III:** 13/54 specimens do not have long-barb prickles. **Cluster IV:** One of the two specimens does not have long-barb prickles. **Cluster IV:** One of the three specimens does not have long-barb prickles	**Cluster A:** 7/37 specimens do not have long-barb prickles. **Cluster B:** Both specimens have long-barb prickles. **Cluster C:** 11/48 specimens do not have long-barb prickles. **Cluster D:** None of the two specimens has long-barb prickles. **Cluster E:** One of the two specimens has long-barb prickles.
Long-barb prickles length	The lengths of all the long-barb prickle on the specimens on all clusters (I to V) vary from 15µm to 35µm.	The lengths of all the specimens from all clusters (A to E) vary from 7.5µm to 35µm.
Long-barb prickles density	**Cluster I:** One of the two specimens has a dense frequency. **Cluster II:** 3/21 specimens have a dense	**Cluster A:** 10/30 specimens have a dense frequency of long barb prickles.

	frequency. **Cluster III:** 12/41 specimens have a dense frequency. **Cluster IV:** The one specimen has a dense frequency. **Cluster V**: Both specimens have an occasional long-barb prickle density.	**Cluster B:** One of the specimens has a dense frequency of long-barb prickles. **Cluster C:** 8/39 specimens have a dense frequency of long barb prickles. **Cluster E:** The one specimen with long barb prickles has an occasional frequency.
Leaf-Margin Prickles	**Cluster I:** All specimens have leaf-margin prickles. **Cluster II:** 1/28 specimens does not have leaf-margin prickles. **Cluster III:** 4/54 specimens do not have leaf-margin prickles. **Cluster IV:** One of the two specimens does not have leaf-margin prickles. **Cluster V:** All three specimens have leaf-margin prickles.	**Cluster A:** Only one of the 37 specimens does not leaf-margin prickles. **Cluster B:** Both specimens have leaf-margin prickles. **Cluster C:** 4/48 specimens do not have leaf-margin prickles. **Cluster D:** One of the two specimens does not have leaf-margin prickles. **Cluster E:** Both specimens have leaf-margin prickles.
Leaf margin prickles	Leaf-margin prickles have varying lengths	Leaf-margin prickles have

length	on all the clusters (I to V)	varying lengths on all the clusters (A to E)
Silica bodies	**Cluster I:** Only one of the specimens has DSSBs **Cluster II:** 20/28 specimens have DSSBs. **Cluster III:** 36/54 specimens have DSSBs. **Cluster IV:** Both specimens have DSSBs. **Cluster V:** None of the three specimens has DSSBs.	**Cluster A:** 8/37 specimens do not show DSSBs. **Cluster B:** None of the two specimens show DSSBs**.** **Cluster C:** 20/48 specimens do not show DSSBs. **Cluster D:** None of the two specimens show DSSBs**.** **Cluster E:** Only one of the two specimens does not have DSSBs.
Papillae	**Cluster I:** One of the two specimens has papillae. **Cluster II:** All 28 specimens have papillae (on both the abaxial and adaxial sides). **Cluster III:** 5/54 specimens do not have papillae. **Cluster IV:** All two specimens have papillae. **Cluster V:** All three specimens have	**Cluster A:** All the 37 specimens have papillae. **Cluster B:** Both specimens have papillae. **Cluster C:** 6/48 specimens do not have papillae. **Cluster D:** Both specimens have papillae. **Cluster E:** Both specimens have papillae.

	papillae.	
Papillae diameter	Papillae on all the specimens, on all the clusters have varying diameters, with 7.7µm being the most commonly occurring length.	Papillae on all the specimens, on all the clusters have varying diameters.
Stomata	**Cluster I:** Both specimens have stomata on the abaxial side only. **Cluster II:** All 28 specimens have stomata, with 9/28 specimens having stomata on both abaxial and adaxial sides. **Cluster III:** All 54 specimens have stomata, with 14/54 specimens having stomata on both abaxial and adaxial sides. **Cluster IV:** Both specimens have stomata, with one specimen having stomata on both abaxial and adaxial sides. **Cluster V:** All three specimens have stomata, with one specimen having stomata on both abaxial and adaxial sides.	**Clusters A to E:** All specimens have stomata.
Stomata length	Stomata on all the clusters have varying lengths.	Stomata on all the clusters have varying lengths.
Stomata width	Stomata on all the clusters have varying	Stomata on all the clusters have

	widths.	varying widths.
Epidermal Cells	**Cluster I:** None of the two specimens have visible epidermal cells. **Cluster II:** 17/28 specimens do not have their epidermal cells visible. **Cluster III:** 37/54 specimens do not have their epidermal cells visible. **Cluster IV:** None of the two specimens have their epidermal cells visible. **Cluster V:** None of the three specimens have their epidermal cells visible	**Cluster A:** 12/37 specimens have visible epidermal cells. **Cluster B:** None of the two specimens have visible epidermal cells **Cluster C:** 16/48 specimens have visible epidermal cells. **Cluster D:** None of the two specimens have visible epidermal cells. **Cluster E:** None of the two specimens have visible epidermal cells.
Cell length	Cells are of varying lengths.	Cells are of varying lengths.
Cell width	Cells are of varying widths.	Cells are of varying widths.
Cell shape	**Cluster II:** 1/17 specimens has varying cell shapes. **Cluster III:** 1/37 specimens has varying cell shapes.	**Cluster A:** All 12 specimens have constant epidermal cell shapes. **Cluster C:** 2/16 specimens have varying epidermal cell shapes.

Table 6.2: Characters and character states as obtained from the SEM observations of herbarium specimens from Simple Matching and Euclidean Distance phenogram analyses.

Character	Herbarium (Euclidean Distance)	Herbarium (Simple Matching)
Macro-Hairs presence/absence	**Cluster I:** 4/21 specimens have macro-hairs. **Cluster II:** 17/94 specimens have macro-hairs. **Cluster III:** None of the 8 specimens has macro-hairs. **Cluster IV:** One of the three specimens has macro-hairs.	**Cluster A:** One of four specimens has macro-hairs. **Cluster B:** 16/116 specimens have macro-hairs. **Cluster C:** 3/5 specimens have macro-hairs.
Macro-hair frequency	**Cluster I:** One of the four specimens has a dense macro-hair frequency. **Cluster II:** 9/17 specimens have a dense macro-hair frequency. **Cluster IV:** The one specimen has an occasional macro-hair frequency.	**Cluster A:** The one specimen has an occasional macro-hair frequency. **Cluster B:** 8/16 specimens have an occasional macro-hair frequency. **Cluster C:** 1/3 specimens has an occasional macro-hair frequency.
Macro-hair length	The lengths of all the macro-hairs on all clusters vary, with the shortest being	Specimens have varying macro-hair lengths.

	257µm and the longest being about 2000µm.	
Micro-Hairs presence/absence	**Cluster I:** 13/21 specimens do not have micro-hairs. **Cluster II:** 68/94 specimens do not have micro-hairs. **Cluster III:** Only two of the 8 specimens have micro-hairs. **Cluster IV:** None of the three specimens has micro-hairs.	**Cluster A:** 2/4 specimens have micro-hairs. **Cluster B:** 28/116 specimens have micro-hairs. **Cluster C:** 2/5 specimens have micro-hairs.
Micro-hair frequency	**Cluster I:** 3/8 specimens have a dense micro-hair frequency. **Cluster II:** Only one of the 26 specimens has a dense micro-hair frequency. **Cluster III:** Both specimens have an occasional micro-hair frequency.	**Cluster A:** Both specimens have an occasional frequency. **Cluster B:** 3/28 specimens have an occasional micro-hair frequency. **Cluster C:** Both specimens have an occasional micro-hair frequency.
Micro-hair length	The specimens have varying lengths.	**Cluster A:** The lengths are 22µm and 89µm. **Cluster B:** The specimens have varying lengths.

		Cluster C: Both lengths are above 50μm and below 60μm.
Large-Prickle Hairs presence/absence	**Cluster I:** All 21 specimens have large-prickle hairs. **Cluster II:** All 94 specimens have large-prickle hairs. **Cluster III:** All 8 specimens have large-prickle hairs. **Cluster IV:** All 3 specimens have large-prickle hairs. • This makes this character taxonomically insignificant.	**Cluster A:** All four specimens have large prickle hairs. **Cluster B:** All 116 specimens have large prickle hairs. **Cluster C:** 1/5 specimens does not have large prickle hairs.
Large-prickles density	**Cluster I:** 2/21 specimens have an occasional large-prickle hair frequency. **Cluster II:** 28/94 specimens have an occasional large-prickle frequency. **Cluster III:** One of the 8 specimens has an occasional large-prickle hair frequency. **Cluster IV:** One of the three specimens has an occasional large-prickle hair frequency.	**Cluster A:** Three of the four specimens have a dense large prickle hair frequency. **Cluster B:** 30/116 specimens have an occasional large prickle hair frequency. **Cluster C:** 2/4 specimens have a dense large prickle hair frequency.

Large-prickles base structure	**Cluster I:** All the 21 specimens have a base structure that has a collar. **Cluster II:** All the 94 specimens have a base structure that has a collar. **Cluster III:** All the 8 specimens have a base structure that has a collar. **Cluster IV:** All the three specimens have a base structure that has a collar. • The base structure character also shows no taxonomic importance for the current study.	**Cluster A:** All 4 specimens have base structures with a collar. **Cluster B:** All 116 specimens have base structures with a collar. **Cluster C:** All 4 specimens have base structures with a collar.
Large-prickles length	The lengths vary greatly on all the clusters, from as little as 20µm to over 100µm.	**Cluster A:** The large prickle hair lengths range from 59µm to 60µm. **Cluster B:** The lengths vary greatly. **Cluster C:** The lengths vary from 32µm to 117µm.
Large-prickles size uniformity	**Cluster I:** 4/21 specimens have non-uniform large-prickle hair lengths. **Cluster II:** 15/94 specimens have non-uniform large-prickle hair lengths.	**Cluster A:** All the specimens have large prickle hairs of uniform sizes. **Cluster B:** 19/116 have large

	Cluster III: 2/8 specimens have non-uniform large prickle hair lengths. **Cluster IV:** All three specimens have uniform large-prickle lengths.	prickle hairs of non-uniform sizes. **Cluster C:** Three of the four specimens have large prickle hairs of uniform sizes.
Large-prickles orientation	**Cluster I:** 2/21 specimens have erect large-prickle hairs. **Cluster II:** 11/94 specimens have erect large-prickle hairs. **Cluster III:** None of the 8 specimens have erect large-prickle hairs. **Cluster IV:** None of the three specimens have erect large-prickle hairs.	**Cluster A:** All specimens have un-erect large prickle hairs. **Cluster B:** 12/116 specimens have un-erect large prickle hairs. **Cluster C:** All four specimens have un-erect large prickle hairs.
Large-prickles distribution	**Cluster I:** 2/21 specimens have large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster II:** 12/94 specimens have large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster III:** 1/8 specimens have large-prickle hairs distributed on the 1° and 2	**Cluster A:** All four specimens have large prickle hairs distributed on all vascular bundles excluding the midrib. **Cluster B:** 13/116 specimens have large prickle hairs distributed on all vascular bundles including the midrib. **Cluster C:** 2/4 specimens have

	˚ vascular bundles only. **Cluster IV:** All three specimens have large-prickle hairs distributed on all vascular bundles including the midrib.	large prickle hairs distributed on all vascular bundles including the midrib.
Long-Barb Prickles presence/absence	**Cluster I:** 11/21 specimens have long-barb prickles. **Cluster II:** 39/94 specimens have long-barb prickles. **Cluster III:** 3/8 specimens have long-barb prickles. **Cluster IV:** All three specimens have long-barb prickles.	**Cluster A:** 3/4 specimens have long-barb prickles. **Cluster B:** 48/116 specimens have long-barb prickles. **Cluster C:** 1/5 specimens has long-barb prickles.
Long barb prickles length	**Cluster I:** The lengths range from 15µm to 36µm, with one extreme 127µm from Zululand. **Cluster II:** The lengths range from 18µm to 160µm **Cluster III:** The lengths range from 16µm to 55µm **Cluster IV:** The lengths range from 21µm to 53µm.	**Cluster A:** The lengths range from 28µm to 127µm. **Cluster B:** The lengths range from 15µm to 160µm. **Cluster C:** The only specimen in this cluster has a 42µm long barb prickle.

Long barb prickles density	**Cluster I:** All 11 specimens have an occasional density of long-barb prickles. **Cluster II:** 4/39 specimens have a dense frequency of long-barb prickles. **Cluster III:** All 3 specimens have an occasional density of long-barb prickles. **Cluster IV:** All 3 specimens have an occasional density of long-barb prickles.	**Cluster A:** All three specimens have an occasional long barb frequency. **Cluster B:** 5/48 specimens have a dense long barb prickle frequency. **Cluster C:** The one specimen has a dense long barb frequency.
Leaf-Margin Prickles	**Cluster I:** 3/21 specimens do not have leaf-margin prickles. **Cluster II:** 28/94 specimens do not have leaf-margin prickles. **Cluster III:** One of the eight specimens does not have leaf-margin prickles. **Cluster IV:** One of the three specimens does not have leaf-margin prickles.	**Cluster A:** 2/4 specimens do not have leaf-margin prickles. **Cluster B:** 35/116 specimens do not have leaf-margin prickles. **Cluster C:** All five specimens have leaf-margin prickles.
Leaf margin prickles length	Leaf-margin prickles have varying lengths on all the clusters (I to IV)	Leaf-margin prickles have varying lengths on all the clusters (A to C)
Silica bodies	**Cluster I:** 9/21 specimens have DSSBs. **Cluster II:** 34/94 specimens have DSSBs.	**Cluster A:** All four specimens have DSSBs. **Cluster B:** 43/116 specimens

	Cluster III: 6/8 specimens have DSSBs. **Cluster IV:** All three specimens have DSSBs.	have DSSBs. **Cluster C:** 3/5 specimens have DSSBs.
Papillae	**Cluster I:** 2/21 specimens do not have papillae. **Cluster II:** 5/94 specimens do not have papillae. **Cluster III:** All 8 specimens have papillae. **Cluster IV:** All three specimens have papillae.	**Cluster A:** All the specimens have papillae. **Cluster B:** 8/116 specimens do not have papillae. **Cluster C:** All the specimens have papillae.
Papillae diameter	Papillae have varying diameters.	Papillae have varying diameters.
Stomata	**Cluster I to IV:** All the specimens have stomata.	**Cluster A:** All four specimens have stomata. **Cluster B:** All 116 specimens have stomata. **Cluster C:** All five specimens have stomata.
Stomata length	Stomata on all the clusters have varying lengths.	Stomata on all the clusters have varying lengths.
Stomata width	Stomata on all the clusters have varying widths.	Stomata on all the clusters have varying widths.

Epidermal Cells	**Cluster I:** 6/21 specimens have visible epidermal cells. **Cluster II:** 45/94 specimens have visible epidermal cells. **Cluster III:** 4/8 specimens have visible epidermal cells. **Cluster IV:** All three specimens have visible epidermal cells.	**Cluster A:** All four specimens have visible epidermal cells. **Cluster B:** 44/116 specimens have visible epidermal cells. **Cluster C:** All specimens have visible epidermal cells.
Cell length	Cells are of varying lengths.	Cells are of varying lengths.
Cell width	Cells are of varying widths.	Cells are of varying widths.
Cell shape	**Cluster A:** 2/6 specimens have cells of varying shapes. **Cluster B:** 20/45 specimens have cells of varying shapes. **Cluster C:** 2/7 specimens have cells of varying shapes.	**Cluster A:** One of the four specimens has varying epidermal cell shapes. **Cluster B:** 20/44 specimens have varying epidermal cell shapes. **Cluster C:** 1/5 specimens has varying epidermal cell shapes.

Table 6.3: Characters and character states as obtained from the SEM observations of fresh and herbarium specimens from Simple Matching and Euclidean Distance phenogram analyses.

Character	Fresh and Herbarium (ED)	Fresh and Herbarium (SM)
Macro-Hairs presence/absence	**Cluster I:** None of the two specimens have macro-hairs. **Cluster II:** 37/194 specimens have macro-hairs. **Cluster III:** 4/17 specimens have macro-hairs. **Cluster IV:** None of the five specimens have macro-hairs.	**Cluster A:** 1/14 specimens has macro-hairs. **Cluster B:** 12/112 specimens have macro-hairs. **Cluster C:** 9/73 specimens have macro-hairs. **Cluster D:** 16/19 specimens have macro-hairs.
Macro-hair frequency	**Cluster II:** 18/37 specimens have a dense macro-hair frequency. **Cluster III:** Three of the four specimens have a dense macro-hair frequency.	**Cluster A:** The one specimen has an occasional frequency of macro-hairs. **Cluster B:** 9/12 specimens have an occasional macro-hair frequency. **Cluster C:** 5/9 specimens have an occasional macro-hair frequency. **Cluster D:** 4/16 specimens have an occasional macro-hair frequency.
Macro-hair length	Specimens have varying macro-hair lengths.	Macro-hair lengths vary greatly from less than 1000 µm to over 4000µm.
Micro-Hairs	**Cluster I:** Both specimens have	**Cluster A:** 4/14 specimens have

presence/absence	micro-hairs. **Cluster II:** 84/194 specimens do not have micro-hairs. **Cluster III:** 8/17 specimens have micro-hairs. **Cluster IV:** 3/5 specimens have micro-hairs.	micro-hairs. **Cluster B:** 43/112 specimens have micro-hairs. **Cluster C:** 64/73 specimens have micro-hairs. **Cluster D:** 11/19 specimens have micro-hairs.
Micro-hair frequency	**Cluster I:** Both specimens have an occasional micro-hair frequency. **Cluster II:** 17/84 specimens have a dense micro-hair frequency. **Cluster III:** All 8 specimens have an occasional micro-hair frequency. **Cluster IV:** 1/3 specimens has a dense micro-hair frequency.	**Cluster A:** All four specimens have occasional micro-hair frequency. **Cluster B:** 5/43 specimens have a dense micro-hair frequency. **Cluster C:** 11/64 specimens have a dense micro-hair frequency. **Cluster D:** 1/11 specimens has a dense micro-hair frequency.
Micro-hair length	The specimens have varying lengths.	Specimens have micro-hair lengths that vary greatly.
Large-Prickle Hairs presence/absence	**Cluster I:** Both specimens have large-prickle hairs. **Cluster II:** 4/194 specimens do not have large-prickle hairs. **Cluster III:** All 17 specimens have	**Cluster A:** All the 14 specimens have large-prickle hairs. **Cluster B:** Only one of the 112 specimens does not have large-prickle hairs.

	large-prickle hairs. **Cluster IV:** All five specimens have large-prickle hairs.	**Cluster C:** Three of 73 specimens do not have large-prickle hairs. **Cluster D:** All the 19 specimens have large-prickle hairs.
Large-prickles density	**Cluster I:** Both specimens have a dense large-prickle hair frequency. **Cluster II:** 145/190 specimens have a dense large-prickle hair frequency. **Cluster III:** 13/17 specimens have a dense large-prickle hair frequency. **Cluster IV:** All five specimens have a dense large-prickle hair frequency.	**Cluster A:** 4/14 specimens have an occasional large-prickle hair frequency. **Cluster B:** 27/111 specimens have an occasional large-prickle hair frequency. **Cluster C:** 12/70 specimens have an occasional large-prickle hair frequency. **Cluster D:** 3/19 specimens have an occasional large-prickle hair frequency.
Large-prickles base structure	**Cluster I:** Both specimens have base structures with a collar. **Cluster II:** 10/190 specimens have base structures without a collar. **Cluster III:** 1/17 has a base structure without a collar.	**Cluster A:** All 14 specimens have large-prickle hairs with a collar. **Cluster B:** 2/111 specimens have large-prickle hairs without a collar. **Cluster C:** 8/70 specimens have large-prickle hairs without a collar.

	Cluster IV: All specimens have base structures with a collar.	**Cluster D:** 1/19 specimens has large-prickle hairs without a collar.
Large-prickles length	The lengths vary greatly on all the clusters.	The lengths vary greatly on all the clusters.
Large-prickles size uniformity	**Cluster I:** One of the two specimens has uniform large-prickle hair lengths. **Cluster II:** 26/190 specimens have non-uniform large-prickle hair sizes. **Cluster III:** 2/17 specimens have non-uniform large-prickle hair sizes. **Cluster IV:** 1/5 specimens has non-uniform large-prickle hair sizes.	**Cluster A:** 3/14 specimens have non-uniform large-prickle lengths. **Cluster B:** 16/111 specimens have non-uniform large-prickle lengths. **Cluster C:** 7/70 specimens have non-uniform large-prickle lengths. **Cluster D:** 5/19 specimens have non-uniform large-prickle lengths.
Large-prickles orientation	**Cluster I:** Both specimens have un-erect large-prickle hairs. **Cluster II:** 23/190 specimens have erect large-prickle hairs. **Cluster III:** 2/17 specimens have erect large-prickle hairs. **Cluster D:** 1/5 specimens has erect large-prickle hairs.	**Cluster A:** All 14 specimens have non erect large-prickle hairs. **Cluster B:** 13/111 specimens have erect large-prickle hairs. **Cluster C:** 9/70 specimens have erect large-prickle hairs. **Cluster D:** 2/19 specimens have erect large-prickle hairs.
Large-prickles	**Cluster I:** Only one of the two	**Cluster A:** 2/14 specimens have

distribution	specimens has large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster II:** 26/190 specimens have large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster III:** All 17 specimens have large-prickle hairs distributed on primary and secondary vascular bundle excluding the midrib. **Cluster IV:** 2/5 specimens have large-prickle hairs distributed on all vascular bundles including the midrib.	large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster B:** 18/111 specimens have large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster C:** 9/70 specimens have large-prickle hairs distributed on all vascular bundles including the midrib. **Cluster D:** 2/19 specimens have large-prickle hairs distributed on all vascular bundles including the midrib.
Long-Barb Prickles presence/absence	**Cluster I:** One of the two specimens has long-barb prickles. **Cluster II:** 108/194 specimens have long-barb prickles. **Cluster III:** 11/17 specimens have long-barb prickles. **Cluster IV:** 3/5 specimens have	**Cluster A:** 5/14 specimens have long-barb prickles. **Cluster B:** 55/112 specimens have long-barb prickles. **Cluster C:** 51/73 specimens have long-barb prickles. **Cluster D:** 11/19 specimens have

	long-barb prickles.	long-barb prickles.
Long-barb prickles length	The lengths of all the specimens on all clusters (I to IV) vary greatly.	The lengths of all the specimens on all clusters (A to D) vary greatly.
Long-barb prickles density	**Cluster I:** The one specimen has an occasional long-barb prickle frequency. **Cluster II:** 18/108 specimens have a dense long-barb prickle frequency. **Cluster III:** 3/11 specimens have a dense long-barb prickle frequency. **Cluster IV:** All three specimens have an occasional long-barb prickle frequency.	**Cluster A:** All five specimens have an occasional long-barb frequency. **Cluster B:** 6/55 specimens have a dense long-barb frequency. **Cluster C:** 14/51 specimens have a dense long-barb frequency. **Cluster D:** 2/11 specimens have a dense long-barb frequency.
Leaf-Margin Prickles	**Cluster I:** One of the two specimens does not have leaf-margin prickles. **Cluster II:** 31/194 specimens do not have leaf-margin prickles. **Cluster III:** 8/17 specimens do have leaf-margin prickles. **Cluster IV:** one of the specimens does not have leaf-margin prickles.	**Cluster A:** 3/14 specimens do not have leaf margin prickles. **Cluster B:** 21/112 specimens do not have leaf margin prickles. **Cluster C:** 5/73 specimens do not have leaf margin prickles. **Cluster D:** 6/19 specimens do not have leaf margin prickles.

Leaf margin prickles length	Leaf-margin prickle lengths vary from as little as 20µm to as big as 500µm	Leaf-margin prickles have varying lengths on all the clusters (A to D)
Silica bodies	**Cluster I:** Both specimens have DSSBs. **Cluster II:** 92/194 specimens do not have DSSBs. **Cluster III:** 9/17 specimens do not have DSSBs. **Cluster IV:** One of the five specimens does not have DSSBs.	**Cluster A:** 4/14 specimens do not have DSSBs. **Cluster B:** 62/112 specimens do not have DSSBs. **Cluster C:** 25/73 specimens do not have DSSBs. **Cluster D:** 14/19 specimens do not have DSSBs.
Papillae	**Cluster I:** Both specimens have papillae. **Cluster II:** 12/194 specimens do not have papillae. **Cluster III:** One of the 17 specimens does not have papillae. **Cluster IV:** All five specimens have papillae.	**Cluster A:** All 14 specimens have papillae. **Cluster B:** 5/112 specimens do not have papillae. **Cluster C:** 6/73 specimens do not have papillae. **Cluster D:** 2/19 specimens do not have papillae.
Papillae diameter	Papillae diameter varies greatly, most specimens have diameters less	Papillae diameter varies greatly, with a majority of the specimens

	than 10µm.	with diameters of less than 10µm.
Stomata	**Cluster I:** Both specimens have stomata visible on both sides (ab- and ad-). **Cluster II:** All 194 specimens have stomata visible, 44 specimens have stomata visible on both sides. **Cluster III:** All 17 specimens have stomata visible; four specimens have stomata visible on both sides. **Cluster IV:** All specimens have stomata visible, but none have stomata visible on both sides.	**Cluster A to D:** All the specimens have stomata.
Stomata length	Stomata on all the clusters have varying lengths.	Stomata on all the clusters have varying lengths.
Stomata width	Stomata on all the clusters have varying widths.	Stomata on all the clusters have varying widths.
Epidermal Cells	**Cluster I:** Both specimens have epidermal cells visible. **Cluster II:** 75/194 specimens have their epidermal cells visible. **Cluster III:** 5/17 specimens have	**Cluster A:** 2/14 specimens do not have epidermal cells visible. **Cluster B:** 46/112pecimens have epidermal cells visible. **Cluster C:** 18/73 specimens have

	their epidermal cells visible. **Cluster IV:** 2/5 specimens have their epidermal cells visible.	epidermal cells visible. **Cluster D:** 8/19 specimens have epidermal cells visible.
Cell length	Cells are of varying lengths	Cells are of varying lengths
Cell width	Cells are varying widths	Cells are varying widths
Cell shape	**Cluster I:** One of the two specimens has cells of varying shapes. **Cluster II:** 23/75 specimens have epidermal cells of varying shapes. **Cluster III:** 2/5 specimens have epidermal cells of varying shapes. **Cluster IV:** Both specimens have epidermal cells of constant shapes.	**Cluster A:** 5/12 specimens have varying epidermal cell shapes. **Cluster B:** 15/18 specimens have varying epidermal cell shapes. **Cluster C:** 1/18 specimens has varying epidermal cell shapes. **Cluster D:** 3/8 specimens have varying epidermal cell shapes.

Table 6.4: Characters and character states as obtained from the LM observations of freshly collected specimens from Simple Matching and Euclidean Distance phenogram analyses.

Character	**Fresh (Euclidean Distance)**	**Fresh (Simple Matching)**
Outline of open leaves	**Cluster I:** 3/38 specimens have V-shaped leaves, none of them have U-shaped leaves, 4/38 specimens have	**Cluster A:** 4/47 specimens have V-shaped leaves, none have U-shaped leaves; 2/47 specimens have wide

	wide open leaves of almost 180˚, 8/38 specimens have narrow leaves of about 45˚, 9/38 specimens have leaves with a concave shape and 14/38 specimens have convex-shaped leaves. **Cluster II:** 2/21 specimens have wide open leaves of almost 180˚, 4/21 specimens have narrow leaves of about 45 ˚, 4/21 specimens have concave shaped leaves and 11/21 specimens have convex-shaped leaves. **Cluster III:** 1/6 specimens has V-shaped leaves, 1/6 specimens has narrow leaves of about 45˚ and 4/6 specimens have convex-shaped leaves.	open leaves of almost 180˚; 12/47 specimens have narrow leaves of about 45˚; 7/47 specimens have leaves with a concave shape and 22/47 specimens have convex-shaped leaves. **Cluster B:** 4/10 specimens have wide open leaves of almost 180 ○ , 1/10 specimens has narrow leaves of about 45 ○ , 2/10 specimens have concave shaped leaves and 3/10 specimens have convex-shaped leaves. **Cluster C:** 4/10 specimens have concave shaped leaves and 6/10 specimens have convex-shaped leaves
Ribs on the adaxial side: Absent = 0;	**Cluster I:** 24/38 specimens have ribs on the adaxial side of the leaves.	**Cluster A:** 39/47 specimens have ribs on the adaxial side of the

Present = 1	**Cluster II:** 18/21 specimens have ribs on the adaxial side of the leaves. **Cluster III:** 5/6 specimens have ribs on the adaxial side of the leaves.	leaves. **Cluster B:** 4/10 specimens have ribs on the adaxial side of the leaves. **Cluster C:** 7/10 specimens has ribs on the adaxial side of the leaves.
Ribs on the abaxial side: Absent = 0; Present = 1	**Cluster I:** 29/38 specimens do not have ribs on the abaxial side of the leaves. **Cluster II:** 12/21 specimens do not have ribs on the abaxial side of the leaves. **Cluster III:** 4/6 specimens do not have ribs on the abaxial side of the leaves.	**Cluster A:** 20/47 specimens have ribs on the abaxial side of the leaves. **Cluster B:** None of the 10 specimens has ribs on the abaxial side of the leaves. **Cluster C:** 1/10 specimens has ribs on the abaxial side of the leaves.
On the adaxial side: Ribs only over the 1˚ and 2˚ bundles = 0; Over only 1˚ or 2˚ bundles = 1	**Cluster I:** 20/24 specimens have ribs over both the 1˚ and 2˚ vascular bundles. 4/24 specimens have ribs over 1˚or 2˚ vascular bundles. **Cluster II:** 17/18 specimens have ribs over both the 1˚ and 2˚ vascular	**Cluster A:** 36/39specimens have ribs over both the 1 ○ and 2 ○ vascular bundles. 3/39 specimens have ribs over 1 ○ or 2 ○ vascular bundles. **Cluster B:** ¾ specimens have ribs

	bundles. 1/18 specimen has ribs over 1° or 2° vascular bundles. **Cluster III:** 4/5 specimens have ribs over both the 1° and 2° vascular bundles. 1/5 specimen has ribs over 1° or 2° vascular bundles.	over both the 1○ and 2○ vascular bundles. 1/4 specimens has ribs over 1○ or 2○ vascular bundles. **Cluster C:** 5/7 specimens have ribs over both the 1○ and 2○ vascular bundles. 2/7 specimens have ribs over 1○ or 2○ vascular bundles.
On the abaxial side: Ribs opposite 1° bundles = 0; Opposite both 1° and 2° bundles = 1	**Cluster I:** All 9 specimens have ribs opposite the 1° bundles. **Cluster II:** 5/9 specimens have ribs opposite the 1° bundles. 4/9 specimens have ribs opposite both 1° and 2° bundles. **Cluster III:** Both specimens have ribs opposite the 1° bundles.	**Cluster A:** 16/20 specimens have ribs opposite the 1° bundles; and 4/20 specimens have ribs opposite both 1° and 2° bundles. **Cluster B:** None of the specimens have ribs on the abaxial side. **Cluster C:** The one specimen has ribs opposite the 1° bundles.
Total number of 1° vascular bundles	**Cluster I:** The numbers of 1° vascular bundles are all 5 and below	**Cluster A:** The numbers of 1○ vascular bundles are all 6 and below

	(minimum is 2). **Cluster II:** The numbers of 1° vascular bundles ranges from 3 to 6. **Cluster III:** The numbers of 1° vascular bundles ranges from 3 to 6.	(minimum is 2, and the most common numbers are 3 and 4). **Cluster B:** The numbers of 1 ○ vascular bundles are all 6 and below (minimum is 2, and the most common number is 3). **Cluster C:** The numbers of 1 ○ vascular bundles are all 5 and below (minimum is 3).
Total number of 2° vascular bundles	**Cluster I:** The numbers of 2° vascular bundles ranges from 5 to 11. **Cluster II:** The numbers of 2° vascular bundles ranges from 4 to 12. **Cluster III:** The numbers of 2° vascular bundles ranges from 3 to 14.	**Cluster A:** The numbers of 2 ○ vascular bundles ranges from 4 to 12. **Cluster B:** The numbers of 2 ○ vascular bundles ranges from 5 to 14. **Cluster C:** The numbers of 2 ○ vascular bundles ranges from 3 to 11.
Total number of 3°	**Cluster I:** The numbers of 3°	**Cluster A:** The numbers of 3 ○

vascular bundles	vascular bundles ranges from 6 to 16. **Cluster II:** The numbers of 3˚ vascular bundles ranges from 13 to 20. **Cluster III:** The numbers of 3˚ vascular bundles ranges from 19 to 26.	vascular bundles ranges from 8 to 21. **Cluster B:** The numbers of 3 ○ vascular bundles ranges from 6 to 24. **Cluster C:** The numbers of 3 ○ vascular bundles ranges from 8 to 26.
1˚ bundles fewer than 2˚ and 3˚ bundles = 0; They are an equal number = 1	**Cluster I:** All 38 specimens have 1˚ bundles fewer than 2˚ and 3˚ bundles. **Cluster II:** 19/21 specimens have 1˚ bundles fewer than 2˚ and 3 ○ bundles; 2 specimens have an equal number of 1˚, 2˚ and 3˚. **Cluster III:** 4/6 specimens have 1˚ bundles fewer than 2˚ and 3˚ bundles; 2/6 specimens have an equal number of 1˚, 2˚ and 3˚.	**Cluster A:** 45/47 specimens have less 1˚ bundles than the 2˚ and 3˚ bundles; and 2/47 specimens have equal numbers of 1˚, 2˚ and 3˚ bundles. **Cluster B:** All 10 specimens have less 1˚ bundles than the 2˚ and 3˚ bundles. **Cluster C:** 8/10 specimens have less 1˚ bundles than the 2˚ and 3˚ bundles; and 2/10 specimens have

		equal numbers of 1°, 2° and 3° bundles.
Two 3° bundles occur between the larger bundles = 0; More than two 3° bundles occur between larger bundles = 1	**Cluster I:** 7/38 specimens have two 3° bundles occurring between the larger bundles; and 31/38 with more than two 3° bundles occurring between larger bundles. **Cluster II:** 1/21 specimen has two 3 ° bundles occurring between the larger bundles; and 20/21 with more than two 3° bundles occurring between larger bundles. **Cluster III:** All six specimens have more than two 3° bundles occurring between larger bundles.	**Cluster A:** 5/47 specimens have two 3° bundles occurring between the larger bundles; and 42/47 with more than two 3° bundles occurring between larger bundles. **Cluster B:** 1/10 specimen has two 3 ° bundles occurring between the larger bundles; and 9/10 with more than two 3° bundles occurring between larger bundles. **Cluster C:** 3/10 specimen has two 3 ° bundles occurring between the larger bundles; and 7/10 with more than two 3° bundles.
Two 2° bundles occur between 1° = 0; More	**Cluster I:** 17/38 specimens have two 2° bundles occurring between 1°	**Cluster A:** 22/47 specimens have two 2° bundles occurring between 1

than two 2˚ bundles occur between 1˚ bundles = 1	bundles; and 21/38 with more than two 2˚ bundles occurring between 1˚ bundles. **Cluster II:** 12/21 specimens have two 2˚ bundles occurring between 1˚ bundles; and 9/21 with more than two 2˚ bundles occurring between 1˚ bundles. **Cluster II:** 1/5 specimen has two 2˚ bundles occurring between 1˚ bundles; and 5/6 with more than two 2˚ bundles occurring between 1˚ bundles.	˚ bundles; and 25/47 with more than two 2˚ bundles occurring between 1˚ bundles. **Cluster B:** 4/10 specimens have two 2˚ bundles occurring between 1 ˚ bundles; and 6/10 with more than two 2˚ bundles occurring between 1 ˚ bundles. **Cluster C:** 4/10 specimen has two 2 ˚ bundles occurring between 1˚ bundles; and 6/10 with more than two 2˚ bundles occurring between 1 ˚ bundles.
3˚ Vascular bundles: Positioned at the centre of the blade = 0; They are closer to either the abaxial = 1; The adaxial side of the leaf = 2	**Cluster I:** All the 38 specimens have 3˚ vascular bundles positioned closer to the abaxial side of the leaf.	**Cluster A:** All the 47 specimens have 3˚ vascular bundles positioned closer to the abaxial side of the leaf. **Cluster B:** All the 10 specimens

	Cluster II: 12/21 specimens have 3˚ vascular bundles positioned closer at the centre of the leaf blade; and 9/21 specimens have 3˚ vascular bundles positioned closer to the abaxial side of the leaf. **Cluster III:** All six specimens have 3˚ vascular bundles positioned closer to the abaxial side of the leaf.	have 3˚ vascular bundles positioned closer to the abaxial side of the leaf. **Cluster C:** All the 10 specimens have 3˚ vascular bundles positioned closer to the abaxial side of the leaf.
3˚ bundles: With a circular shape = 0; With an elliptical shape = 1	**Cluster I:** All 38 specimens have 3˚ bundles with a circular shape. **Cluster II:** 20 specimens have 3˚ bundles with a circular shape; and one with an elliptical shape. **Cluster III:** All 6 specimens have 3˚ bundles with a circular shape.	**Cluster A:** 46/47 specimens have 3 ˚bundles with a circular shape; and 1/47 with an elliptical shape. **Cluster B:** All 10 specimens have 3 ˚bundles with a circular shape. **Cluster C:** All 10 specimens have 3 ˚bundles with a circular shape.
2˚ bundles: With an elliptical shape = 0; With a round/circular	**Cluster I:** 14/38 specimens have 2˚ bundles with an elliptical shape; and	**Cluster A:** 26/47 specimens have 2 ˚bundles with an elliptical shape;

shape = 1	24 have a circular shape. **Cluster II:** 9/21 specimens have 2˚ bundles with an elliptical shape; and 12 have a circular shape. **Cluster III:** All six specimens have 2˚ bundles with a circular shape.	and 21/47 have a circular shape. **Cluster B:** 5/10 specimens have 2˚ bundles with an elliptical shape; and 5/10 have a circular shape. **Cluster C:** 6/10 specimens have 2˚ bundles with an elliptical shape; and 4/10 have a circular shape.
1˚ bundles: With an egg-shape, i.e. broad on the adaxial side = 0; With a circular shape = 1	**Cluster I:** All 38 specimens have 1˚ bundles with an egg shape. **Cluster II:** 20 specimens have 1˚ bundles with an egg shape; and 1 specimen has 1˚ bundles with a circular shape. **Cluster III:** All 6 specimens have 1˚ bundles with an egg shape.	**Cluster A:** 46/47 specimens have 1˚ bundles with an egg shape; and 1/47 specimens has 1˚ bundles with a circular shape. **Cluster B:** All 10 specimens have 1˚ bundles with an egg shape. **Cluster C:** All 10 specimens have 1˚ bundles with an egg shape.
Phloem of the 1˚ bundle: Completely surrounded by thick-walled fibres =	**Cluster I:** 37/38 specimens have the phloem of the 1˚ bundle completely	**Cluster A:** 45/47 specimens have the phloem of the 1˚ bundle

0; Divided by the intrusion of small fibres = 1	surrounded by thick-walled fibres; 1/38 has phloem divided by the intrusions of small fibres. **Cluster II:** 19/21 specimens have the phloem of the 1˚ bundle completely surrounded by thick-walled fibres; 2/21 have phloem divided by the intrusions of small fibres. **Cluster III:** All six specimens have the phloem of the 1˚ bundle completely surrounded by thick-walled fibres.	completely surrounded by thick-walled fibres; 2/47 has phloem divided by the intrusions of small fibres. **Cluster B:** All 10 specimens have the phloem of the 1˚ bundle completely surrounded by thick-walled fibres. **Cluster C:** 8/10 specimens have the phloem of the 1˚ bundle completely surrounded by thick-walled fibres; 2/10 has phloem divided by the intrusions of small fibres.
Lysigenous cavity: Absent on the 1˚ bundles = 0; Present = 1	**Cluster I:** 7/38 specimens do not have the lysigenous cavity on their 1˚ vascular bundles. **Cluster II:** 2/21 specimens do not have the lysigenous cavity on their 1˚ vascular bundles. **Cluster III:** 1/6 specimens does not	**Cluster A:** 5/47 specimens do not have the lysigenous cavity on their 1˚ vascular bundles. **Cluster B:** 4/10 specimens do not have the lysigenous cavity on their 1˚ vascular bundles. **Cluster C:** 1/9 specimens does not

	have the lysigenous cavity on its 1° vascular bundles.	have the lysigenous cavity on its 1° vascular bundles.
Vascular bundle sheath: incomplete due to slight interruptions from narrow/ wide girders of 1-3 or more fibres = 0; Sheath complete, i.e. completely surrounds the 1° bundle = 1	**Cluster I:** 36/38 specimens have an incomplete vascular bundle sheath; and 2/38 specimens have a complete vascular bundle sheath. **Cluster II:** 19/21 specimens have an incomplete vascular bundle sheath; and 2/21 specimens have a complete vascular bundle sheath. **Cluster III:** All six specimens have an incomplete vascular bundle sheath.	**Cluster A:** 46/47 specimens have an incomplete vascular bundle sheath; and 1/47 specimens have a complete vascular bundle sheath. **Cluster B:** 8/10 specimens have an incomplete vascular bundle sheath; and 2/10 specimens have a complete vascular bundle sheath. **Cluster C:** 8/10 specimens have an incomplete vascular bundle sheath; and 2/10 specimens have a complete vascular bundle sheath.
Length of extension of sheath: 1-2 cells long = 0; 2-5 cells long = 1	**Cluster I:** All 38 specimens have a sheath that is 1-2 cells long. **Cluster II:** All 21 specimens have a sheath that is 1-2 cells long. **Cluster III:** All 6 specimens have a sheath that is 1-2 cells long.	**Cluster A:** All 47 specimens have a sheath that is 1-2 cells long. **Cluster B:** All 10 specimens have a sheath that is 1-2 cells long. **Cluster C:** All 10 specimens have a sheath that is 1-2 cells long.
Number of cells	**Cluster I:** The number of cells	**Cluster A:** The number of cells

comprising the 1° bundle sheath.	comprising the 1° sheath varies from 9-19 cells. **Cluster II:** The number of cells comprising the 1° sheath varies from 12-21 cells. **Cluster III:** The number of cells comprising the 1° sheath varies from 9-20 cells.	comprising the 1° sheath varies from 9-21 cells. **Cluster B:** The number of cells comprising the 1° sheath varies from 9-20 cells. **Cluster C:** The number of cells comprising the 1° sheath varies from 10-19 cells.
Number of cells comprising the 2° bundle sheath.	**Cluster I:** The number of cells comprising the 2° sheath varies from 6-11 cells (with 7 being the most common). **Cluster II:** The number of cells comprising the 2° sheath varies from 6-12 cells. **Cluster III:** The number of cells comprising the 2° sheath varies from 7-10 cells.	**Cluster A:** The number of cells comprising the 2° sheath varies from 6-12 cells. **Cluster B:** The number of cells comprising the 2° sheath varies from 7-12 cells. **Cluster C:** The number of cells comprising the 2° sheath varies from 6-10 cells.
Number of cells	**Cluster I:** The number of cells	**Cluster A:** The number of cells

comprising the 3° bundle sheath.	comprising the 3° sheath varies from 4-7 cells. **Cluster II:** The number of cells comprising the 3° sheath varies from 5-9 cells. **Cluster III:** The number of cells comprising the 3° sheath varies from 4-6 cells (with 6 being the most common).	comprising the 3° sheath varies from 4-9 cells. **Cluster B:** The number of cells comprising the 3° sheath varies from 5-7 cells. **Cluster C:** The number of cells comprising the 3° sheath varies from 4-7 cells.
Sclerenchyma: Associated with 1° vascular bundles only = 0; Associated with either 2° or 3° bundles = 1	**Cluster I:** 12/38 specimens have sclerenchyma associated with 1° vascular bundles; and 26/38 have sclerenchyma associated with 2° or 3° vascular bundles. **Cluster II:** 4/21 specimens have sclerenchyma associated with 1° vascular bundles; and 17/21 have sclerenchyma associated with 2° or 3	**Cluster A:** 9/47 specimens have sclerenchyma associated with 1° vascular bundles; and 38/47 have sclerenchyma associated with 2° or 3° vascular bundles. **Cluster B:** 9/10 specimens have sclerenchyma associated with 1° vascular bundles; and 1/10 has sclerenchyma associated with 2° or

	° vascular bundles. **Cluster III:** 4/6 specimens have sclerenchyma associated with 1° vascular bundles; and 2/6 have sclerenchyma associated with 2° or 3° vascular bundles.	3° vascular bundles. **Cluster C:** 2/10 specimens have sclerenchyma associated with 1° vascular bundles; and 8/10 have sclerenchyma associated with 2° or 3° vascular bundles.
On the adaxial side sclerenchyma: Narrow, with only 2-4 fibres wide = 0; With more than 4 fibres = 1	**Cluster I:** All 38 specimens have narrow sclerenchyma with 2-4 fibres on the adaxial side. **Cluster II:** 19/21 specimens have narrow sclerenchyma with 2-4 fibres, and 2/21 have more than 4 fibres on the adaxial side. **Cluster III:** All six specimens have narrow sclerenchyma with 2-4 fibres on the adaxial side.	**Cluster A:** All 47 specimens have narrow sclerenchyma with 2-4 fibres on the adaxial side. **Cluster B:** All 10 specimens have narrow sclerenchyma with 2-4 fibres on the adaxial side. **Cluster C:** 8/10 specimens have narrow sclerenchyma with 2-4 fibres, and 2/10 have more than 4 fibres on the adaxial side.
1° vascular bundle girders on the adaxial side: Well developed,	**Cluster I:** 28/38 specimens have 1° vascular bundle girders that are well	**Cluster A:** 38/47 specimens have 1° vascular bundle girders that are

conspicuous and narrowing towards the 1° bundle = 0; They form a straight/horizontal band towards the 1° bundle = 1	developed, conspicuous and narrowing towards the 1° bundle on the adaxial side; and 10/38 specimens have girders that form a straight band towards the 1° bundle. **Cluster II:** 19/21 specimens have 1° vascular bundle girders that are well developed, conspicuous and narrowing towards the 1° bundle on the adaxial side; and 2/21 specimens have girders that form a straight band towards the 1° bundle. **Cluster III:** 4/6 specimens have 1° vascular bundle girders that are well developed, conspicuous and narrowing towards the 1° bundle on the adaxial side; and 2/6 specimens have girders that form a straight band	well developed, conspicuous and narrowing towards the 1° bundle on the adaxial side; and 9/47 specimens have girders that form a straight band towards the 1° bundle. **Cluster B:** 9/10 specimens have 1° vascular bundle girders that are well developed, conspicuous and narrowing towards the 1° bundle on the adaxial side; and 1/10 specimens has girders that form a straight band towards the 1° bundle. **Cluster C:** 5/10 specimens have 1° vascular bundle girders that are well developed, conspicuous and narrowing towards the 1° bundle on the adaxial side; and 5/10 specimens have girders that form a straight

	towards the 1° bundle.	band towards the 1° bundle.
2° vascular bundle girders on the adaxial side: Well developed, conspicuous and narrowing towards the 2° bundle = 0; They form a straight/horizontal band towards the 2° bundle = 1	**Cluster I:** 34/38 specimens have 2° vascular bundle girders that are well developed, conspicuous and narrowing towards the 2° bundle on the adaxial side; and 4/38 specimens have girders that form a straight band towards the 2° bundle. **Cluster II:** All 21 specimens have 2° vascular bundle girders that are well developed, conspicuous and narrowing towards the 2° bundle on the adaxial side. **Cluster III:** 5/6 specimens have 2° vascular bundle girders that are well developed, conspicuous and narrowing towards the 2° bundle on the adaxial side; and 1/6 specimens	**Cluster A:** 44/47 specimens have 2° vascular bundle girders that are well developed, conspicuous and narrowing towards the 2° bundle on the adaxial side; and 3/47 specimens have girders that form a straight band towards the 2° bundle. **Cluster B:** All 10 specimens have 2° vascular bundle girders that are well developed, conspicuous and narrowing towards the 2° bundle on the adaxial side. **Cluster C:** 8/10 specimens have 2° vascular bundle girders that are well developed, conspicuous and narrowing towards the 2° bundle on the adaxial side; and 2/10 specimens

	has girders that form a straight band towards the 2° bundle.	have girders that form a straight band towards the 2° bundle.
Girder fibres of the adaxial and the abaxial side: In contact with the cells of the single or outer 1° bundle sheath = 0; Fibres interrupt the cells of the single or outer 1° bundle sheath = 1	**Cluster I:** 37/38 specimens have girder fibres that are in contact with the cells of the single or outer 1° bundle sheath on the adaxial and abaxial sides; and 1/38 specimens has fibres that interrupt the cells of the single or outer 1° bundle sheath. **Cluster II:** All 21 specimens have girder fibres that are in contact with the cells of the single or outer 1° bundle sheath on the adaxial and abaxial sides. **Cluster III:** All 6 specimens have girder fibres that are in contact with the cells of the single or outer 1° bundle sheath on the adaxial and abaxial sides.	**Cluster A:** All 47 specimens have girder fibres that are in contact with the cells of the single or outer 1° bundle sheath on the adaxial and abaxial sides. **Cluster B:** 9/10 specimens have girder fibres that are in contact with the cells of the single or outer 1° bundle sheath on the adaxial and abaxial sides; and 1/10 specimens has fibres that interrupt the cells of the single or outer 1° bundle sheath. **Cluster C:** All 10 specimens have girder fibres that are in contact with the cells of the single or outer 1° bundle sheath on the adaxial and abaxial sides.

Girder fibres of the adaxial and the abaxial side: In contact with the cells of the single or outer 2˚ bundle sheath = 0; Fibres interrupt the cells of the single or outer 2˚ bundle sheath = 1	**Cluster I:** 34/38 specimens have girder fibres that are in contact with the cells of the single or outer 2˚ bundle sheath on the adaxial and abaxial sides; and 4/38 specimens have fibres that interrupt the cells of the single or outer 2˚ bundle sheath. **Cluster II:** 20/21 specimens have girder fibres that are in contact with the cells of the single or outer 2˚ bundle sheath on the adaxial and abaxial sides; and 1/21 specimens has fibres that interrupt the cells of the single or outer 2˚ bundle sheath. **Cluster III:** All 6 specimens have girder fibres that are in contact with the cells of the single or outer 2˚ bundle sheath on the adaxial and abaxial sides.	**Cluster A:** 45/47 specimens have girder fibres that are in contact with the cells of the single or outer 2˚ bundle sheath on the adaxial and abaxial sides; and 2/47 specimens have fibres that interrupt the cells of the single or outer 2˚ bundle sheath. **Cluster B:** All 10 specimens have girder fibres that are in contact with the cells of the single or outer 2˚ bundle sheath on the adaxial and abaxial sides. **Cluster A:** 7/10 specimens have girder fibres that are in contact with the cells of the single or outer 2˚ bundle sheath on the adaxial and abaxial sides; and 3/10 specimens have fibres that interrupt the cells of the single or outer 2˚ bundle sheath.

Sclerenchyma of the abaxial side: Deep and wide than that of the adaxial side = 0; Is the same size as that of the adaxial side = 1	**Custer I:** 31/38 specimens have sclerenchyma of the abaxial side that is deep and wider than that of the adaxial side; and 7/38 specimens have sclerenchyma of the abaxial side that is the same size as that of the adaxial side. **Cluster II:** 20/21 specimens have sclerenchyma of the abaxial side that is deep and wider than that of the adaxial side; and 1/21 specimens have sclerenchyma of the abaxial side that is the same size as that of the adaxial side. **Cluster III:** 3/6 specimens have sclerenchyma of the abaxial side that is deep and wider than that of the adaxial side; and 3/6 specimens have sclerenchyma of the abaxial side that is the same size as that of the adaxial side.	**Custer A:** 41/47 specimens have sclerenchyma of the abaxial side that is deep and wider than that of the adaxial side; and 6/47 specimens have sclerenchyma of the abaxial side that is the same size as that of the adaxial side. **Cluster B:** 8/10 specimens have sclerenchyma of the abaxial side that is deep and wider than that of the adaxial side; and 2/10 specimens have sclerenchyma of the abaxial side that is the same size as that of the adaxial side. **Cluster C:** 7/10 specimens have sclerenchyma of the abaxial side that is deep and wider than that of the adaxial side; and 3/10 specimens have sclerenchyma of the abaxial side that is the same size as that of the adaxial side.
Girder on the abaxial	**Cluster I:** 16/38 specimens have	**Cluster A:** 9/47 specimens have

side: Narrowing towards the 1° vascular bundle, i.e. triangular/trapezoidal = 0; Forms a straight/horizontal band = 1	girders on the abaxial side narrowing towards the 1° vascular bundle; and 22/38 specimens have girders on the abaxial side forming a straight band. **Cluster II:** 3/21 specimens have girders on the abaxial side narrowing towards the 1° vascular bundle; and 18/21 specimens have girders on the abaxial side forming a straight band. **Cluster III:** 2/6 specimens have girders on the abaxial side narrowing towards the 1° vascular bundle; and 4/6 specimens have girders on the abaxial side forming a straight band.	girders on the abaxial side narrowing towards the 1° vascular bundle; and 38/47 specimens have girders on the abaxial side forming a straight band. **Cluster B:** 8/10 specimens have girders on the abaxial side narrowing towards the 1° vascular bundle; and 2/10 specimens have girders on the abaxial side forming a straight band. **Cluster C:** 5/10 specimens have girders on the abaxial side narrowing towards the 1° vascular bundle; and 5/10 specimens have girders on the abaxial side forming a straight band.
Sclerenchyma in leaf margins: Absent = 0; Present, comprising a couple of fibres = 1; or	**Cluster I:** 4/38 specimens do not have sclerenchyma on the leaf margins; and 34/38 specimens have sclerenchyma in the leaf margins	**Cluster A:** 5/47 specimens do not have sclerenchyma on the leaf margins; and 42/47 specimens have sclerenchyma in the leaf margins

present, with a width less than 3˚ bundle = 2	comprising a couple of fibres. **Cluster II:** 5/21 specimens do not have sclerenchyma on the leaf margins; and 16/21 specimens have sclerenchyma in the leaf margins comprising a couple of fibres. **Cluster III:** 1/6 specimens does not have sclerenchyma on the leaf margins; and 5/6 specimens have sclerenchyma in the leaf margins comprising a couple of fibres.	comprising a couple of fibres. **Cluster B:** 3/10 specimens do not have sclerenchyma on the leaf margins; and 7/10 specimens have sclerenchyma in the leaf margins comprising a couple of fibres. **Cluster C:** 2/10 specimens do not have sclerenchyma on the leaf margins; and 8/10 specimens have sclerenchyma in the leaf margins comprising a couple of fibres.
The marginal cap: Pointed = 0; Round = 1; Crescent-shaped, i.e. sclerenchyma extends shortly along both the abaxial and adaxial sides of the leaf = 2	**Cluster I:** 17/38 specimens have pointed marginal caps; and 21/38 specimens have round marginal caps. **Cluster II:** 11/21 specimens have pointed marginal caps; and 10/21 specimens have round marginal caps. **Cluster III:** 5/6 specimens have pointed marginal caps; and 1/5 specimens has round marginal caps.	**Cluster A:** 25/47 specimens have pointed marginal caps; and 22/47 specimens have round marginal caps. **Cluster B:** 5/10 specimens have pointed marginal caps; and 5/10 specimens have round marginal caps. **Cluster C:** 5/10 specimens have pointed marginal caps; and 5/10 specimens has round marginal caps.

Chlorenchyma: Radiate = 0; Irregular = 1	**Cluster I:** All 38 specimens have radiate Chlorenchyma. **Cluster II:** All 21 specimens have radiate Chlorenchyma. **Cluster III:** All 6 specimens have radiate Chlorenchyma.	**Cluster A:** All 47 specimens have radiate Chlorenchyma. **Cluster B:** All 10 specimens have radiate Chlorenchyma. **Cluster C:** All 10 specimens have radiate Chlorenchyma.
One layer of Chlorenchyma cells around bundles = 0; More than one layers = 1	**Cluster I:** All 38 specimens have one layer of Chlorenchyma cells around bundles. **Cluster II:** All 21 specimens have one layer of Chlorenchyma cells around bundles. **Cluster III:** All 6 specimens have one layer of Chlorenchyma cells around bundles.	**Cluster A:** All 47 specimens have one layer of Chlorenchyma cells around bundles. **Cluster B:** All 10 specimens have one layer of Chlorenchyma cells around bundles. **Cluster C:** All 10 specimens have one layer of Chlorenchyma cells around bundles.
Radiating mesophyll groups: Separated by irregular Chlorenchyma and intercellular air-spaces = 0; Separated by bulliform and colourless cell groups =	**Cluster I:** 26/38 specimens have radiating mesophyll groups that are separated by irregular Chlorenchyma and intercellular air-spaces; and 12/38 specimens have radiating mesophyll groups that are separated by bulliform and colourless cells.	**Cluster A:** 34/47 specimens have radiating mesophyll groups that are separated by irregular Chlorenchyma and intercellular air-spaces; and 13/47 specimens have radiating mesophyll groups that are separated by bulliform and

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Radiating cells: Reduced to two strips of Chlorenchyma by large girders or colourless parenchyma = 0; Completely surrounding the bundles = 1	**Cluster I:** 28/38 specimens have radiating cells that are reduced to two strips of Chlorenchyma by large girders or colourless parenchyma; and 10/38 specimens have radiating cells that are completely surrounding the bundles. **Cluster II:** 18/21 specimens have	**Cluster A:** 36/47 specimens have radiating cells that are reduced to two strips of Chlorenchyma by large girders or colourless parenchyma; and 11/47 specimens have radiating cells that are completely surrounding the bundles. **Cluster B:** All 10 specimens have

	radiating cells that are reduced to two strips of Chlorenchyma by large girders or colourless parenchyma; and 3/21 specimens have radiating cells that are completely surrounding the bundles. **Cluster III:** 3/6 specimens have radiating cells that are reduced to two strips of Chlorenchyma by large girders or colourless parenchyma; and 3/6 specimens have radiating cells that are completely surrounding the bundles.	radiating cells that are reduced to two strips of Chlorenchyma by large girders or colourless parenchyma. **Cluster C:** 7/10 specimens have radiating cells that are reduced to two strips of Chlorenchyma by large girders or colourless parenchyma; and 3/10 specimens have radiating cells that are completely surrounding the bundles.
Colourless cells: Absent = 0; Present = 1	**Cluster I:** 11/38 specimens do not have colourless cells; and 27/38 specimens have colourless cells. **Custer II:** 7/21 specimens do not have colourless cells; and 14/21 specimens have colourless cells. **Cluster III:** 4/6 specimens do not have colourless cells; and 2/6 specimens have colourless cells.	**Cluster A:** 14/47 specimens do not have colourless cells; and 33/47 specimens have colourless cells. **Custer B:** 7/10 specimens do not have colourless cells; and 3/10 specimens have colourless cells. **Cluster C:** 1/10 specimens does not have colourless cells; and 9/10 specimens have colourless cells.

Colourless cells: Closely associated with bulliform cells = 0; Not closely associated with bulliform cells = 1	**Cluster I:** All 27 specimens have colourless cells that are closely associated with bulliform cells. **Cluster II:** 13/14 specimens have colourless cells that are closely associated with bulliform cells; and 1/14 specimens has colourless cells that are not closely associated with bulliform cells. **Cluster III:** All 2 specimens have colourless cells that are closely associated with bulliform cells.	**Cluster A:** All 33 specimens have colourless cells that are closely associated with bulliform cells. **Cluster B:** 2/3 specimens have colourless cells that are closely associated with bulliform cells; and 1/3 specimens has colourless cells that are not closely associated with bulliform cells. **Cluster C:** All 9 specimens have colourless cells that are closely associated with bulliform cells.
Colourless cells: Smaller than bulliform cells; are uninflated, often the same size as the Chlorenchyma cells = 0; Have similar size or shape as the bulliform cells, and are inflated = 1	**Cluster I:** All 27 specimens have colourless cells that are smaller than the bulliform cells. **Cluster II:** All 14 specimens have colourless cells that are smaller than the bulliform cells. **Cluster III:** All 2 specimens have colourless cells that are smaller than the bulliform cells.	**Cluster A:** All 33 specimens have colourless cells that are smaller than the bulliform cells. **Cluster B:** All 3 specimens have colourless cells that are smaller than the bulliform cells. **Cluster C:** All 9 specimens have colourless cells that are smaller than the bulliform cells.
Colourless cells:	**Cluster I:** All 27 specimens have	**Cluster A:** All 33 specimens have

Narrower than the bulliform cells = 0; Are the same width as the bulliform cells = 1	narrower colourless cells than the bulliform cells. **Cluster II:** All 14 specimens have narrower colourless cells than the bulliform cells. **Cluster III:** All 2 specimens have narrower colourless cells than the bulliform cells.	narrower colourless cells than the bulliform cells. **Cluster B:** All 3 specimens have narrower colourless cells than the bulliform cells. **Cluster C:** All 9 specimens have narrower colourless cells than the bulliform cells.
Only one extension of colourless cells from each bulliform cell group = 0; Two extensions, one on either side = 1	**Cluster I:** All 27 specimens have only one extension of colourless cells from each bulliform cell group. **Cluster II:** All 14 specimens have only one extension of colourless cells from each bulliform cell group. **Cluster III:** All 2 specimens have only one extension of colourless cells from each bulliform cell group.	**Cluster A:** All 33 specimens have only one extension of colourless cells from each bulliform cell group. **Cluster B:** All 3 specimens have only one extension of colourless cells from each bulliform cell group. **Cluster C:** All 9 specimens have only one extension of colourless cells from each bulliform cell group.
Bulliform cells: Absent = 0; Present in groups = 1; present, but not in groups = 2	**Cluster I:** All 38 specimens have bulliform cells occurring in groups. **Cluster II:** All 21 specimens have bulliform cells occurring in groups. **Cluster III:** All 6 specimens have	**Cluster A:** All 47 specimens have bulliform cells occurring in groups. **Cluster B:** All 10 specimens have bulliform cells occurring in groups. **Cluster C:** All 10 specimens have

	bulliform cells occurring in groups.	bulliform cells occurring in groups.
Bulliform cells occupy less than ¼ of the leaf thickness = 0; Occupy more than ¼ of the leaf thickness = 1	**Cluster I:** 4/38 specimens have bulliform cells that occupy less than ¼ of the leaf thickness; and 34/38 specimens have bulliform cells that occupy more than ¼ of the leaf thickness. **Cluster II:** 4/21 specimens have bulliform cells that occupy less than ¼ of the leaf thickness; and 17/21 specimens have bulliform cells that occupy more than ¼ of the leaf thickness. **Cluster III:** 1/6 specimens has bulliform cells that occupy less than ¼ of the leaf thickness; and 5/6 specimens have bulliform cells that occupy more than ¼ of the leaf thickness.	**Cluster A:** 5/47 specimens have bulliform cells that occupy less than ¼ of the leaf thickness; and 42/47 specimens have bulliform cells that occupy more than ¼ of the leaf thickness. **Cluster B:** All 10 specimens have bulliform cells that occupy more than ¼ of the leaf thickness. **Cluster C:** 4/10 specimens has bulliform cells that occupy less than ¼ of the leaf thickness; and 6/10 specimens have bulliform cells that occupy more than ¼ of the leaf thickness.
Outer walls of typical epidermal cells: Slightly thickened or	**Cluster I:** All 38 specimens have slightly thickened outer walls of the epidermal cells.	**Cluster A:** All 47 specimens have slightly thickened outer walls of the epidermal cells.

with a thin cuticle = 0; Not thickened with a very thin cuticle = 1	**Cluster II:** All 21 specimens have slightly thickened outer walls of the epidermal cells. **Cluster III:** All 6 specimens have slightly thickened outer walls of the epidermal cells.	**Cluster B:** All 10 specimens have slightly thickened outer walls of the epidermal cells. **Cluster C:** All 10 specimens have slightly thickened outer walls of the epidermal cells.