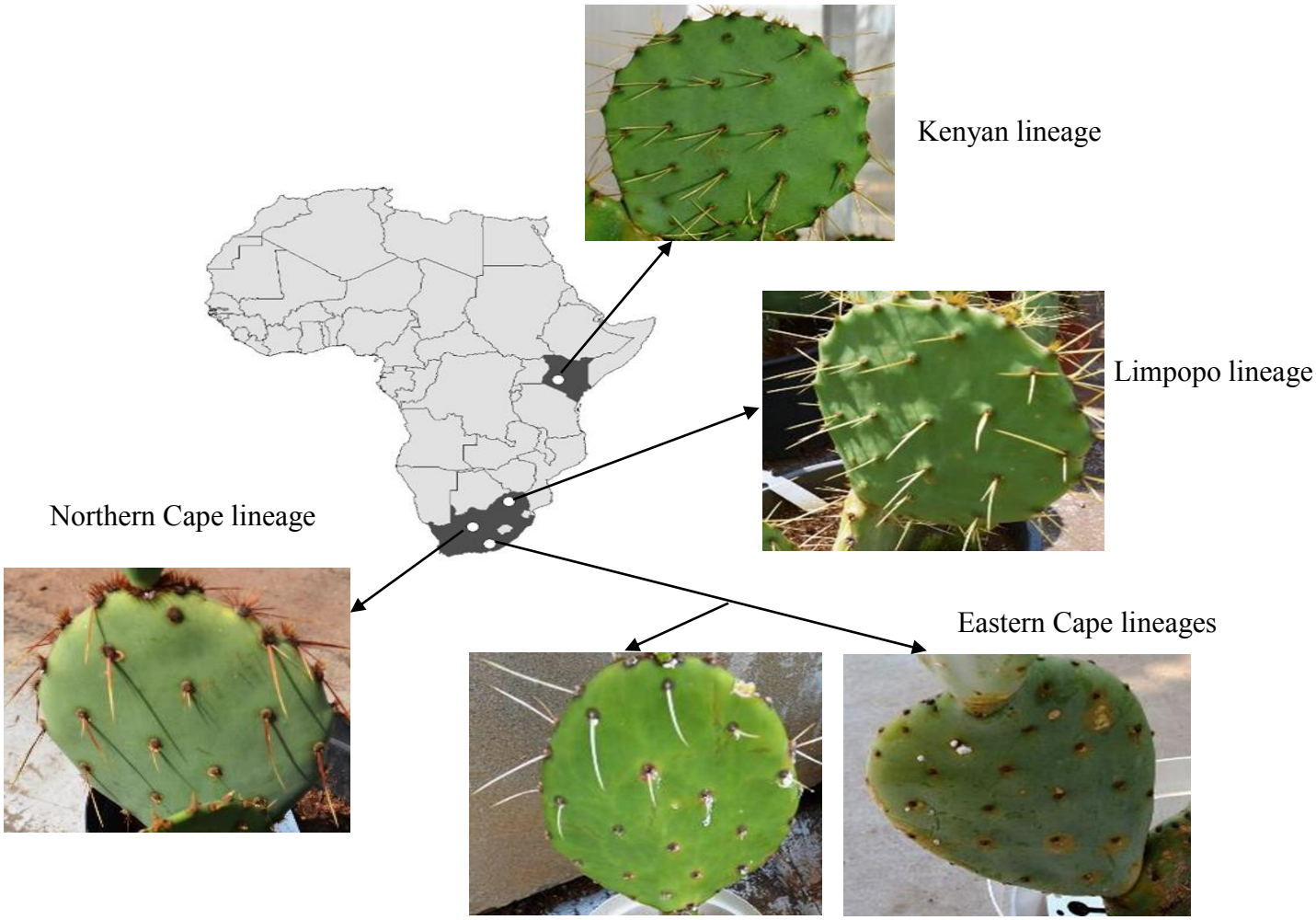
A morphometric and population genetic investigation of *Opuntia engelmannii* Salm-Dyck (Cactaceae: Opuntioideae) lineage variation in Africa.



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This dissertation is submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in the School of Animal, Plant and Environmental Science.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

APES

DECLARATION

I, Siphosenkosi Mbonani, declare that this dissertation is my own, unaided work. It is submitted for the degree Master of Science at the University of the Witwatersrand. It has not been presented before for any degree or examination to any other University.

(Signature of candidate)

On this 10th day of July, 2019 in Braamfontein, Johannesburg

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DEDICATION

I dedicate this work to my parents - Phindile and Makhosi, and Grandparents – David and Constance Mbonani and the rest of my immediate and extended family.

To God be the Glory

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List of abreviations

°C- degrees Celsius

µl- microlitre/s

µm- micrometer

AFLPs- Amplified Fragment Length Polymorphisms

AMOVA- Analysis of Molecular Variance

ANOVA-Analysis of Variance

Bp- Base pair/s

cm- Centimetre/s

CARA - Conservation of Agricultural Resources Act

DEA – Department of Environmental Affairs

dH_2O - distilled water

DNA- Deoxyribonucleic acid

F_{st}- Fixation index

g- grams

H- Shannon-Wiener genetic diversity index

H_0- Null Hypothesis

HCL-Hydrochloric acid

He- Expected Heterozygosity

ml - millitre

mm – millimetre

NEM:BA- National Environmental Management: Biodiversity Act

ng- nanogram/s

PCR- Polymerase Chain Reaction

PhiPT- Measure of genetic differentiation between populations

Rpm- Rate per minute

SNPs- Single Nucleotide polymorphisms

uHe - unbiased expected Heterozygosity

ABSTRACT

Opuntia engelmannii, commonly known as 'small round-leaved prickly pear' is native to the Americas (North, Central and South America) but is highly invasive in South Africa and Kenya. Moreover, it is a priority for biological control in South Africa as it is NEM:BA category 1b invader. Different *O. engelmannii* found in different geographic locations in South Africa and Kenya are variable in their morphology and are hypothesized to be genetically different. *Dactyolopius opuntiae* has been used in South Africa to control invasive cactus for over 80 years and has been considered a successful biocontrol agent on some *Opuntia* species. The success of *O. engelmannii* biological control will depend on *D. opuntiae* biotype compatibility to *O. engelmannii* genetic diversity and on the insects host specificity in both countries.

Firstly, we investigated the validity of observed morphological differences among *O. engelmannii* lineages and a sister taxon, *O. stricta*. Morphological data were used to test the extent of variability among lineages to see which lineages differ morphologically. Diagnostic characters such as spine, cladode, areole and fruit were compared among the lineages to quantify these observed differences. A Principal Component Analysis (PCA) of spine morphology showed that the Limpopo and Kenyan lineage are most similar, while the Eastern Cape (spines) is more similar to *O. stricta*. The Northern Cape lineage is different in its spine morphology to all the other *O. engelmannii* lineages, but it is also different to *O. stricta*. Furthermore, a Nonmetric MultiDimension Scaling (NMDS) of morphology data showed that spine morphology, more than cladode, areole and fruit morphology is a more useful diagnostic character to confirm *O. engelmannii* lineage identity.

Secondly, we investigated genetic structure and differentiation among the lineages to complement the morphological data. The results showed that Eastern Cape (spineless), Limpopo and Kenyan lineages had less genetic differentiation among them ($F_{ST} < 0.05$), and are therefore genetically similar to each other. The Eastern Cape (spines) lineage was genetically differentiated from the other *O. engelmannii* lineages (F_{ST} 0.05 — 0.15) and it was genetically similiar to the *O. stricta* population. Moreoever, we found that these observed genetic differences are not because of the geographical locations of the putative lineages, therefore refuting the isolation by distance hypothesis.

There is some congruency between morphology and molecular identity, the morphometric NMDS and the molecular data PCoA both suggest that the Eastern Cape (spines) is more similar to the *O. stricta* lineage than the other *O. engelmannii* lineages. Both morphometric and molecular data lead to a better understanding of the underlying reasons for successful biocontrol of *Opuntia* using cochineal insects. The Eastern Cape (spines) lineage is more similar to the plant lineage (*O. stricta*) on which the *D. opuntiae* 'stricta'- biotype is an effective biocontrol agent. The use of *D. opuntiae* 'stricta biotype' might be effective in the control of the Eastern Cape (spines) population because both plant lineages have a similar genetic structure. A new 'biotype' of *D. opuntiae*, that has not been released in South Africa was recently collected from the USA. It has successfully established in quarantine where it has shown a high reproductive output on the Kenyan lineage of *O. engelmannii*. Since there is a little genetic differentiation between the Kenyan and the Limpopo lineage, the same insect biotype could potentially be used to successfully control both the Kenyan and Limpopo lineages.

Keywords: alien invasive plants, biological control, diagnostic characters, lineages, morphology, molecular characterisation, *Opuntia engelmannii*.

CHAPTER 1: PROBLEM STATEMENT, GENERAL INTRODUCTION AND LITERATURE REVIEW.

PROBLEM STATEMENT

Opuntia engelmannii is a weed of pasture land and has invaded the Savanna, Grasslands, and the Karoo biomes in South Africa. The weed is abundant in the Northern, Eastern and Western Cape and Limpopo provinces in South Africa (Klein *et al.*, 2015a). The *Opuntias* have spines which can injure people, livestock, and wildlife, thus restraining the movement of grazing animals (Klein *et al.,* 2015b). As with most invasive plants which displace native species, it is listed as a category 1b invader as per the National Environmental Management: Biodiversity Act (DEA, 2014) regulations of South Africa. Plants in this category should be removed and or destroyed wherever possible and any form of trade is strictly prohibited. *Opuntia engelmannii* has been listed as harmful to livestock in other parts of Africa, including Loisaba in Northern Kenya.

Opuntia engelmannii is a variable species; there are at least four varieties of *O. engelmanni* that are found in South Africa. One in the Northern Cape (Douglas); another one in Limpopo (Mokopane); two occur in the Eastern Cape (Bedford), and another variety is found in Northern Kenya (Loisaba) (Klein *et al.,* 2015b). All four South African varieties are morphologically different to each other (Klein *et al.*, 2015b). It is not known if the fifth variety (Kenya) is morphologically and or genetically similar to any of the South African lineages. The USA has taxonomically named these varieties of *O. engelmannii* (Weniger, 1978). In Africa, however, the varieties of *O. engelmannii* have not been formally named in any publication and are identified merely by differences in morphology, therefore, the term 'lineage' was used for the African plant varieties. Molecular population genetic investigations were used to determine how genetically similar each of these five lineages of *O. engelmannii* are. The findings of this study will inform the correct selection of the best insect biotype of the biocontrol agent, *D. opuntiae* which will then be used to control the appropriate lineage of *O. engelmannii*.

GENERAL INTRODUCTION AND LITERATURE REVIEW.

Invasive species

Biological invasions currently pose one of the greatest threats to biodiversity after habitat loss and destruction (Chornesky and Randall, 2003; Foxcroft *et al.*, 2017). They have therefore, captured the attention of the scientific community since the 19th century. Invasive Alien Plants (IAPs) in particular, are a major contributor towards the increasing problem of biological invasions (Allendorf and Lundquist, 2003; Pyšek *et al.*, 2008; Downey and Richardson, 2016). Invasive alien plants are non-native plants that have successfully spread outside their native range (Williamson, 1996; 1999; Richardson *et al.*, 2000). Although biological invasions can be a natural event, their frequency and rate of introduction has significantly increased due to various human globalisation activities, in particular international trade (Allendorf and Lundquist, 2003; Pimentel, 2011). Over the past centuries, most invasions have involved species transported directly or indirectly by humans (McKinney and Lockwood 1999, Pyšek *et al.*, 2002). Invasive Alien Plants cause significant environmental problems in ecosystems (Blackburn *et al.*, 2014). Furthermore, they often lead to socio-economic problems and they affect the structure and functioning of ecosystems (Jeschke *et al.*, 2014; Shackleton *et al.*, 2015). Globally, IAPs occupy 3% of the total land surface area (Ricciardi, 2007). Terrestrial IAPs occupy at least 10 million hectares in South Africa and negatively affect grazing lands, crops, fish production, as well as the production of livestock and other animals (Mack *et al.*, 2000; Richardson and Van Wilgen, 2004). The major terrestrial invaders in South Africa are *Acacia*, *Hakea*, *Pinus*, *Eucalyptus*, *Jacaranda mimosifolia* and at least 57 invasive cacti species have been reported (Richardson and van Wilgen, 2004; Kaplan *et al.*, 2017).

Cacti species: Invasive traits, history and invasion in Africa

Cacti are tolerant to most climatic conditions and are considered the most widespread category of plants that are invasive in South Africa (van Wilgen *et al.*, 2012; Kaplan *et al.*, 2017). The South African Plant Invaders Atlas lists cacti as one of the most diverse alien plant taxa that has shown the greatest increase from 2006 compared to other introduced taxa (Figure 1.1). Cacti species' tolerance to most climatic conditions, ease of growth, beautiful flowers, fruits and the positioning of spines are some of the features

that make them irresistible to plant collectors (Kaplan *et al.*, 2017). South Africa's arid regions provide favourable conditions for species that are adapted to drought and this is one of the main reasons why South Africa is a global hotspot for cacti invasions (Kaplan *et al.*, 2017). Moreover, cacti have a strong effect on woody vegetation and displace woody vegetation by competition for space.

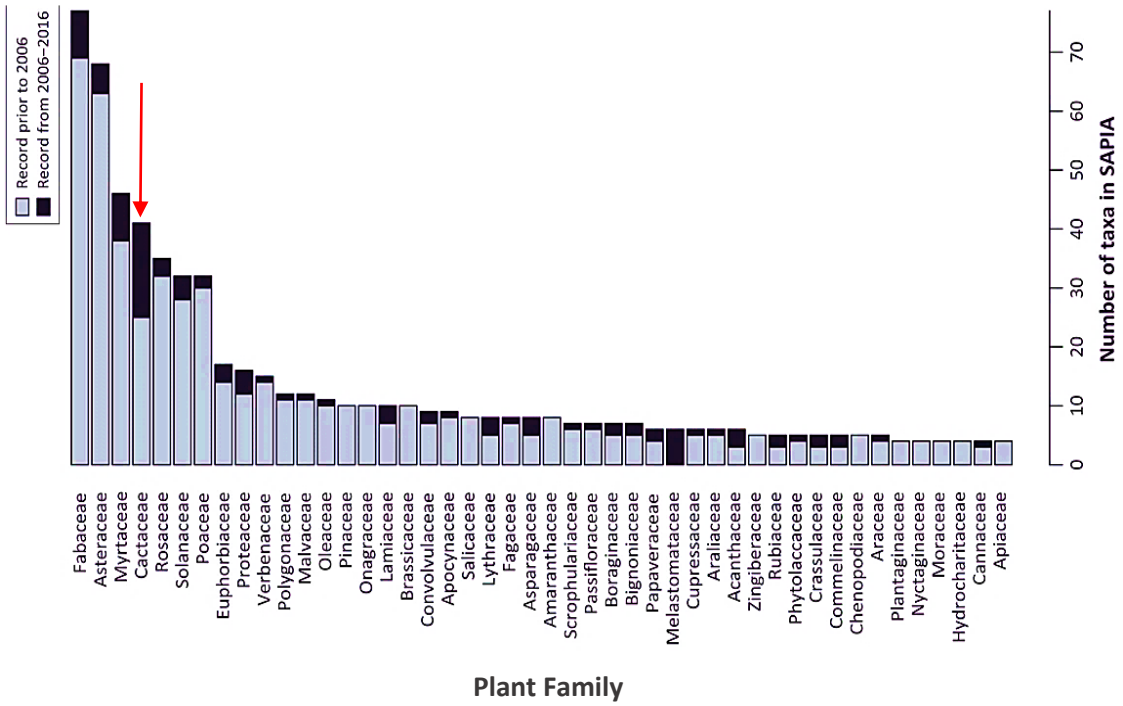

Figure 1.1: The number of alien plant taxa recorded in the South African Plant Invaders Atlas (SAPIA). Families with at least four invasive taxa are shown. The Cactaceae remains in the top five invaders in South Africa (Source: Kaplan *et al.*, 2017).

There are no known native cacti species in Africa but cacti are known for being more invasive in Africa than on any other continent outside their native range (Novoa *et al.*, 2015) (Figure 1.2a). Horticultural trading is the foremost reason for cacti occurring in many non-native countries (Zimmermann *et al.*, 2009), where they are also often grown as either crops or ornamentals (Zimmermann and Moran, 1991; Zimmermann *et al.*, 2009). The Cactaceae has three documented subfamilies: Pereskioideae, Opuntioideae and Cactoideae (Novoa *et al.*, 2015). Of the 1922 Opuntioideae, 57 have been classified as invasive outside of their native range (Figure 1.2b). The family's geographical origin is in America, in particular, Mexico, Eastern Brazil, and much of North America (Paterson *et al.*, 2011; Edwards *et al.*, 2005) (Figure 1.2c).

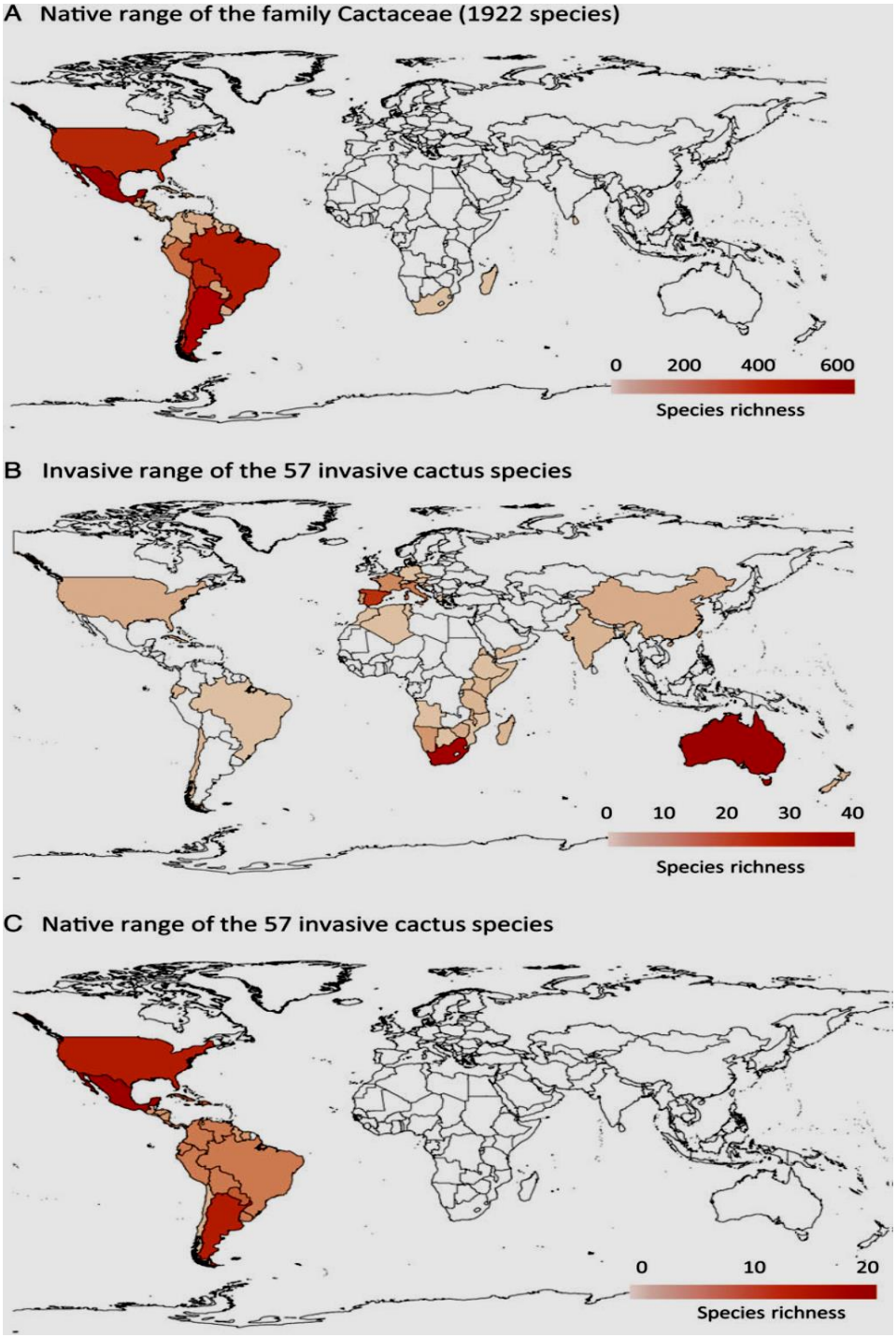

Figure 1.2: (A) Geographic ranges of 1922 species of the Cactaceae across the native range, the (B) invasive range of the 57 cactus species and (C) Native range of the 57 invasive cactus species. Lighter colours show and correspond to less taxa (Source: Novoa *et al.*, 2015).

The Cactaceae and the *Opuntia* genus

The family Cactaceae contains at least 1922 species that belong to 127 genera (Novoa *et al.*, 2015). In the Cactaceae; the *Opuntia* has approximately 180 species and is made up of mainly platyopuntias (Cortázar and Nobel, 1992). Three characteristics differentiate the *Opuntia* from other cacti: (a) growth of cladodes as observably

different jointed segments; (b) presence of spines in the surface of the cladodes and (c) areoles that have short prickles known as glochids (Cortázar and Nobel, 1992). The *Opuntia* genus (Family: Cactaceae; Sub-Family: Opuntioideae) usually have flat pseudostems also referred to as cladodes, flowers with tubular perianths with shorter stamens than the tepals (Samah and Valadez-Moctezuma, 2014). This genus includes atleast 180 species (Cortázar and Nobel, 1992; Reyes-Agüero and Valiente-Banuet, 2006).

Impact and controlling invasive cacti in South Africa

Under the *Opuntia* genus, *Opuntia engelmannii* Salm-Dyck (Cactaceae: Opuntioideae) has invaded pasture land in Northern Cape, amongst other provinces in South Africa (Figure 1.3). Dense infestations of *Opuntia* displace native flora, which results in negative ecological and economic impacts in the country. In an effort to control invading plant species, a number of regulations are employed in South Africa, for example the Conservation of Agricultural Resources Act (CARA). This was introduced in 1984 to regulate a sustainable resolution to environmental problems caused by these invasive species. The CARA regulations have been replaced by the National Environmental Management: Biodiversity Act (NEM:BA) (Act 10 of 2004) – The Alien and Invasive Species (AIS) Regulations were issued on the 1st October 2014. The purpose of NEM:BA is to provide for the sustainable, management and conservation of South Africa's biodiversity within the framework of the National Environmental Management Act (107 of 1998) (Henderson and Wilson, 2017; Zachariades *et al.,* 2017). The National Environmental Management Act does not take into account the morphological variability of *O. engelmannii*. Additionally, 35 *Opuntia* species are listed as invaders under NEM:BA including *O. engelmannii* and *O. humifusa* (Figure 1.4A and B).

Figure 1.3: An example of the infestation level of one of the forms of *Opuntia engelmannii* at Douglas, Northern Cape, South Africa (Photo Credit: K. Musengi).

There are three ways to control and manage cacti: chemical, mechanical, and biological methods. Generally, chemical and mechanical methods are considered to be labour intensive, expensive, unsustainable and are in most cases not environmentally friendly (Moran and Zimmermann, 1991a). Biological control of invasive plants is a sustainable method and environmentally friendly because natural enemies of the target plants are collected from the native range and released where management of the weed is required (Muller-Scharer and Schaffner, 2008; Schwarzländer *et al.,* 2018). In *Opuntia* species for instance, the method chosen usually depends on the location, density and size of the infestation (Kaplan e*t al.,* 2017). *Opuntia engelmannii* may be controlled mechanically by cutting the root 5-10 cm below the soil surface and thereafter stacking all the cladode material to rot. This method was seen as unsatisfactory and leading to further reinfestation as many cladodes became scattered in the process (Henderson, 2007). Moreover, it can also be controlled chemically with the use of herbicides, much work has been done regarding the chemical control of *O. engelmannii* in the past 100 years. Van Wilgen *et al.,* (2012), found that 2, 3, 6-TBA was effective as a foliage spray on *O. engelmannii* and Blair, (1991) reported MSMA to be most effective. The herbicide, Picloram, when applied as an undiluted liquid formulation was found to be the most effective herbicide on *O. engelmannii*, the research showed that most uptake

was through the *O. engelmannii* cladodes. The introduced insect agent, *Dactylopius opuntiae* Cockerell (Hemiptera: Dactyliiopidae), together with chemical and or mechanical control, was successful in slowing the invasion of *O. engelmannii* in South Africa (Moran and Zimmermann, 1991b).

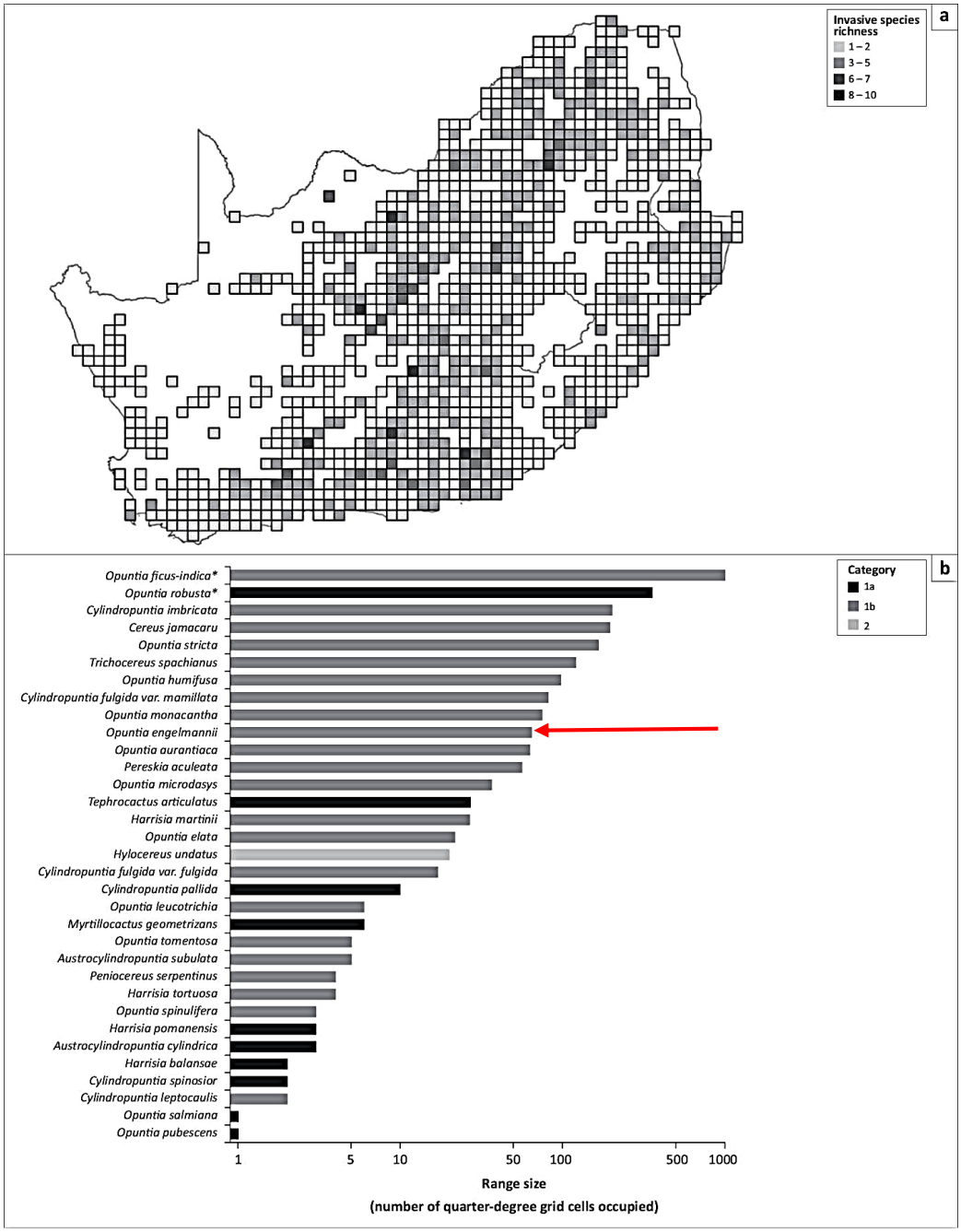

Figure 1.4: The extent of invasion of cacti in South Africa per quarter degree square (A) the species richness and distribution and (B) the range sizes of invasive cacti in South Africa. *Opuntia engelmannii* (shown with the arrow) is one of top 10 invading cacti in South Africa according to NEM:BA (Source: Kaplan *et al.*, 2017).

In an effort to control cacti, South African biologists released the first biological control agent, the cochineal insect, *Dactylopius ceylonicus* against *Opuntia monacantha* in 1913 (Zimmermann, 2010), which was highly invasive in the Eastern Cape and the Karoo at that time (Moran *et al.,* 2013). This program was highly successful and provided hope for other similar biocontrol programs (Zimmermann, 2010). In Australia, two biological control agents were also released thereafter, namely, the cactus moth, *Cactoblastis cactorum,* and the cochineal insect, *Dactylopius opuntiae.* Both these agents provided spectacular control of *O. stricta* and *O. dillenii* between 1929 and 1934 (Zimmermann, 2010). The two released agents, *C. cactorum,* and *D. opuntiae* were therefore assumed to have the potential to control all *Opuntia* species.

Host specific insect biotypes

There are nine known cochineal (*Dactylopius*) species that are extremely host specific (De Lotto, 1974; Kaplan *et al.,* 2017). Some plant lineages have host-specific insect herbivore biotypes, which can narrow down the specificity to a single species. This clarifies why some introductions of insect biotypes were unsuccessful despite the fact that the insect was recorded on that host in its country of origin. A few studies ultimately revealed the existence of such host-specific cochineal biotypes within *D. opuntiae* and *D. tomentosus* (Mathenge *et al.,* 2009; Volschansky *et al.,* 1999; Zimmermann *et al.,* 2009; Paterson *et al.,* 2011). The significance of these findings was the release of two host-specific insect biotypes that provided successful biological control of two new cactus invaders. *Opuntia engelmannii* is inadequately controlled using *D. opuntiae 'stricta'* and *'ficus'* biotypes that have provided successful biological control of some cacti in South Africa i.e *O. stricta* and *O. monacantha* (Table 1.1).

Opuntia engelmannii: **Invasion status in South Africa**

Opuntia engelmannii has heavily invaded the Eastern and Northern Cape regions in South Africa (Figure 1.5 A) and remains a priority for biocontrol in these provinces (Figure 1.5 B). Moreover, the Southern African Plant Invaders (SAPIA) database lists *O. engelmannii* as one of the top five invasive plants that have shown the greatest increase in range and has reached very high local abundancies from 2000-2016 (Henderson and Wilson, 2017).

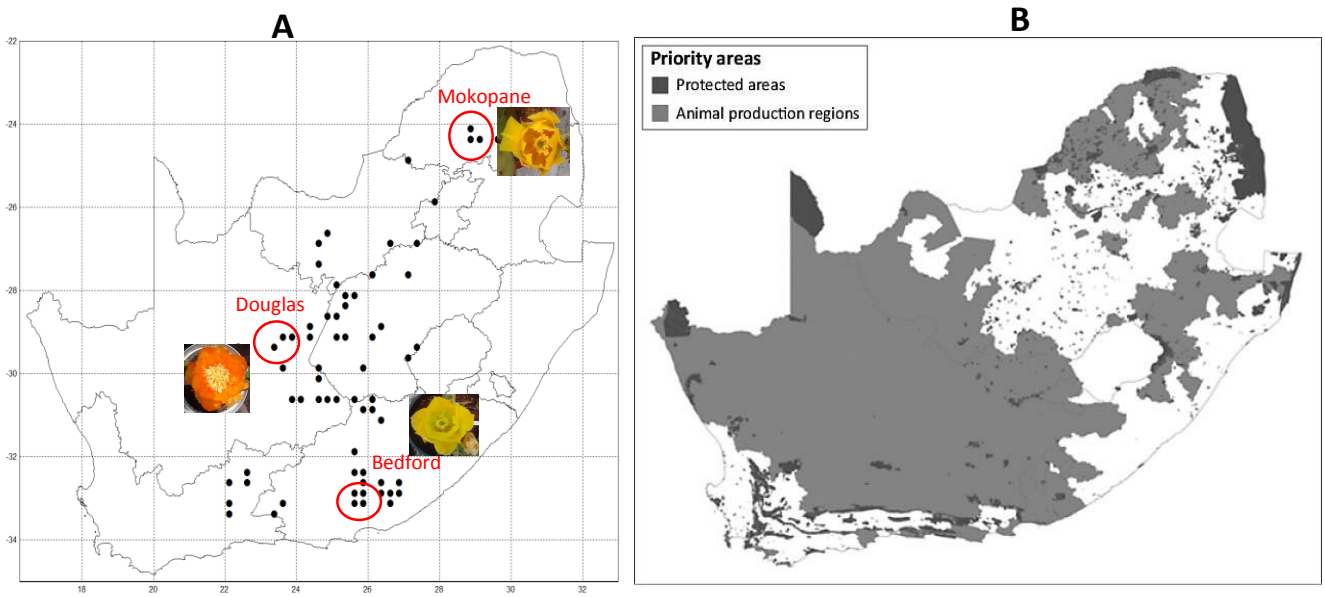

Figure 1.5: A) The occurrence of *Opuntia engelmannii* in South Africa (Source: Henderson, 2016). B) South African map with priority areas for cactus management highlighted in darker shades. The priority areas include sample collection sites (marked in red circles) from the study i.e. Limpopo, Eastern and Northern Cape (Source: Kaplan *et al.*, 2017).

Table 1.1: Status of control of some *Opuntia* species in South Africa (Klein, 2015a; Kaplan *et al.*, 2017; Rule and Hoffmann, 2018).

Species	Control methods	Agent	Status of control
Opuntia aurantiaca	Chemical Biological	*D. austrinus*	Excellent biological control
Opuntia ficus-indica	Chemical Biological	*D. opuntiae 'ficus'*	Good biological control
Opuntia engelmannii	Chemical Biological ineffective (Limited damage)	*D. opuntiae 'stricta'*	Inadequate control
Opuntia humifusa	None effective	*D. opuntiae 'stricta'*	Adequate control
Opuntia monacantha	Biological	*D. ceylonicus*	Good biological control
Opuntia stricta	Biological Limited chemical control	*D. opuntiae 'stricta'*	Excellent biological control

***Opuntia engelmannii*: A morphologically variable species**

Opuntia engelmannii can grow up to 1.5 m in height. Most *O. engelmannii* lineages have cladodes which are usually green-grey and egg-shaped and have spines that are yellow to white (Henderson, 2016). Spines are found mainly in the top half of the cladode and are 1−7 cm long and rigid. *Opuntia engelmannii* is a morphologically variable species, and can be found in four currently recognised morphologically different lineages in South Africa (Figure 1.6). This variability has led to the use of the term 'lineage' in this dissertation based on their appearance and the province where they are found in South Africa. Another lineage has also been reported in Kenya (Klein, 2015b). These five lineages of *O. engelmannii* differ from each other and in their suitability as hosts of the cochineal biotypes that could be introduced against them (Musengi, 2018). In South Africa, one lineage of *O. engelmannii* is found in Limpopo (Mokopane), two occur in the Eastern Cape (Bedford)-the Eastern Cape (spines) and Eastern Cape (spineless) lineages and one in Northern Cape (Douglas) (Klein, 2015a). In Kenya, and many other East african countries, *O. engelmannii* is spreading rapidly and threatening rangelands (Witt *et al.,* 2018), the IAP reduces rangeland access and productivity by forming dense impermeable thickets, Moroever the presence of spines and glochids injure livestock and wild herbivores, thus restraining the movement of grazing animals (Githae, 2018).

The presence or absence of spines and their lengths are useful morphological characteristics for plants in the Cactaceae (Valdez-Cepeda *et al.*, 2003; Samah and Valadez-Moctezuma, 2014). In contrast, Felker *et al.,* (2005) suggested that the absence of spines should not be considered the basis for taxonomic classification, because this character has simple inheritance [seen in one of the South Africa lineages, *O. engelmannii*-Eastern Cape (spineless)]. In this regard, several features of the spine such as length, thickness, angle, colour and form, may be partially dependant on environmental conditions, such as moisture and availability of nutrients (Rebman and Pinkava, 2001). For these reasons, some spineless *O. engelmannii* species have been previously classified as *O. ficus-indica* and others with spines as *O. megacantha*, *O. stricta* and *O. amyclaea,* based on presence and absence of spines (Reyes-Agüero *et al.,* 2005). Other times, *O. engelmannii* species with spines have been classified as *O. ficus-indica* (Felker *et al.,* 2005). Kiesling, (1998) considered *O. amyclaea*, *O. megacantha* and *O. stricta* as synonyms of *O. ficus-indica*, the latter species was later split into two

botanical forms: a) *O. ficus-indica* var. *Amyclaea*, with presence of spines; b) *O. ficus-indica* var *Ficus-indica*, spineless. This further showed that essentially, the presence of spines on the cladodes alone is an inadequate feature to classify *Opuntia* species (Felker *et al.*, 2005). Caruso *et al.*, (2010) reported that spinescence might have been developed multiple times during the evolution of the genus, and that it might have been selected out of different populations by humans. Therefore, the morphological variation within *Opuntia engelmannii* alone must not be considered a good tool to arrive at species or lineage classification.

Figure 1.6: Flower differences among three lineages of *Opuntia engelmannii*. A: Eastern Cape (spines) lineage- flowers are deep yellow, B: Limpopo lineage- faint yellow colour and C: Northern Cape lineage- has orange flowers.

Molecular identification of *O. engelmannii* lineages

Molecular markers based on Polymerase Chain Reaction (PCR) amplification of specific genomic sequences have been suggested in recent years as a direct and real tool to estimate interspecific and generic relationships (Moon *et al.*, 2016). Furthermore, a study by Labra *et al.*, (2003) argued that molecular analyses can be useful in cultivar identification and knowledge of *Opuntia* duplicate ocurrences in collections. *Opuntia* species have been characterized molecularly by non-coding chloroplast genetic regions (e.g., *rpII6*, *trnL-trnF*, and *psbA-trnH*) and one non-coding nuclear region (*ppc*) (Downie and Palmer, 1994; Fehlberg *et al.*, 2013) and the family's monophyly has been maintained in molecular phylogenetic studies across different loci (Applequist and Wallace, 2001; Cuénoud *et al.*, 2002; Nyffeler, 2002). Nevertheless, the continuous morphological and molecular variation, the lack of clear descriptors for each species, high phenotypic plasticity and ploidy variation numbers have led to problems in species

definitions and genotypes assignation in different *Opuntia* species (Caruso *et al.*, 2010). As a result of incorrect assignments, the same lineages are often classified as belonging to different species, and in other cases they are considered to be hybrids among unknown parentals. Studying the taxonomy of a variable species like *O. engelmannii* is therefore important as classification of this species has been largely based only on morphological characteristics such as fruit and cladode variation (Weniger, 1978).

Population genetics as a useful tool for studying invasive plants

Molecular data is increasingly becoming important in invasion biology, alien species status and for the monitoring and reporting of biological invasions (Darling and Mahon, 2011). Molecular studies especially in species with similar morphologies, can be used to typify and differentiate species (Schoch *et al.*, 2012; Yahr *et al.*, 2016). These studies have used molecular techniques involving DNA extraction and amplification to effectively solve taxonomic and phylogenetic problems (Ivanova *et al.*, 2007; Schindel and Miller, 2005). DNA analyses have been used at different taxonomic levels, from individuals, populations and communities of bacteria, yeast, fungi, animals and plants (De Cock *et al.*, 2011). High-grade DNA extraction is an essential first step to conduct molecular studies. This can be performed using conventional methods or commercial kits (Chacon-Cortes *et al.*, 2012).

Molecular population genetics can detect genetic variation within and among populations and can also detect hybridization patterns between closely related species or lineages (Prentis *et al.*, 2008; Zenni *et al.*, 2017). Molecular population genetics were able to identify the invasion of *Tamarix* species in South Africa. *Tamarix ramosissima*, *Tamarix chinensis* and introgressed individuals which form a genetic continuum between *T. ramosissima* and *T. chinensis* were genetically identified in South Africa to investigate the extent of hybrids and hypothesize the origin of the alien invasive *Tamarix* (Mayonde *et al.*, 2015). The accurate identification of alien invasive *Tamarix* species and their hybrids is important for an effective biological control programme using host-specific agents. Hardig *et al.*, (2000) documented an analyses of molecular and morphological data from two other invasive species, *Salix sericea* and *Salix eriocephala* which were geographically distinct to their naturally occurring hybrids. The study showed that although *S. sericea* and *S. eriocephala* are considered members of different sections of the *Salix* genus (Dorn, 1976; Argus, 1997), they are relatively

similar in their vegetative appearance and at a molecular level, the two species were isolated by distance (Hardig *et al.*, 2000). In other research, *Opuntia* lineages have been differentiated and described using molecular markers such as Random Amplification of Polymorphic DNA (RAPDNA) markers (Bendhifi *et al.*, 2013); Amplified Fragment Length Polymorphisms (AFLPs) (Luna-Paez *et al.*, 2007, Valadez-Moctezuma *et al.*, 2015); and Single Sequence Repeats (SSR) (Caruso *et al.*, 2010).

Using relationships among genes, the geographic locations from which those genes are sampled to learn about dispersal in geographically structured populations has become popular (Slatkin, 1993). The term isolation by distance describes patterns of population genetic variation that are derived from spatially limited gene flow. Isolation by distance essentially means a decrease in the genetic similarity betweem populations as the geographic distance between them increases (Perez *et al.*, 2018). A Mantel permutation technique was used in previous studies to test for genetic isolation by distance between related plant species (Sharbel *et al.*, 2000; Pusadee *et al.*, 2009). Genetic isolation by distance for data sets is usually tested for samplesof individuals contained in each geographical region and geographical contrasts are then tested by grouping accessions from pairs of regions (Diniz-Filho *et al.*, 2013). *Opuntia engelmannii* lineages found in different geographic locations in South Africa and Kenya could in fact be isolated by distance.

Early investigation of *O. engelmannii* in South Africa used only qualitative morphological characters (Klein, 2015a). However, this early identification was considered ambiguous (Klein, 2015a). Therefore, the need to investigate the genetic similarity of *O. engelmannii* lineages using Amplified Fragment Length Polymorphisms (AFLPs) as a tool to characterize *Opuntia* lineages in South Africa. Amplified Fragment Length Polymorphisms (AFLPs) were developed by Vos *et.al.*, (1995), AFLPs are an innovative molecular fingerprinting technique that can be applied to DNA of any kind (Blignaut *et al.*, 2013; Simpson, 2017; Kalendar *et al.*, 2019). Amplified Fragment Length Polymorphisms allow one to sample DNA from the entire genome of the different species or lineages. Genomic DNA is digested using two restriction enzymes, generally *EcoRI* and *MseI* (Maughan *et al.*, 1996). Nucleotide adaptors are ligated to the DNA fragments to serve as primer binding sites for PCR amplification (Mueller and Wolfenbarger, 1999). Then, primers complementary to the

adapter and restriction site sequence, with additional nucleotides at the 3-end, are used as selective agents to amplify a subset of ligated fragments. There are different parameters that are regularly encountered in genetic variation studies with AFLPs. These parameters fall under the band-based or the allele frequency-based approaches. The band-based metrics can be directly predicted from the AFLP profiles and include various coefficients of, for example the Shannon diversity index and the less employed, nucleotide diversity (Lynch and Milligan, 1994; Borowsky 2001). Some factors of relatedness developed for dominant markers could also qualify for example, the Nei's gene diversity (Nei 1973; Nei 1978) is an example of allele frequency-based. Amplified Fragment Length Polymorphisms data can be used in population genetics to measure genetic diversity, genetic differentiation between species, population assignments, reconstruction of unresolved phylogenies and identifying hybrids (Blair and Hufbauer, 2010; Gaskin *et al.*, 2011). Amplified Frgament Length Poplymorphism markers in this study are used to test the genetic diversity, differentiation, variation and population assignments between lineages of *O. engelmannii*.

There are no reports specifying whether *O. engelmannii* introduction arose into South Africa from a single or multiple events. Furthermore, there currently has been no attempt to study the quantitative morphological differences and molecular variation among *O. engelmannii* lineages found in Africa. This study combines morphology and molecular data to investigate the level of variation among *O. engelmannii* lineages in South Africa and Kenya. Selection of agents is important in biological control. The understanding of these morphological and molecular differences will have implications for the way agents are selected for the biological control of *O. engelmannii*. New associations between herbivore insect species (e.g., *Dactylopius* agents) and target host plants (e.g., invasive *O. engelmannii*) have been reported to have a strong potential in weed biocontrol because they are as damaging as old associations and their occurrence in agriculture show that they can easily be established in weed biocontrol (Dennil and Moran, 1989).

RESEARCH AIM, OBJECTIVES AND KEY QUESTIONS.

Aim:

The main aim of the study is to investigate the similarity and or dissimilarity among lineages of *O. engelmannii* found in South Africa and Kenya. The ability to morphologically and genetically distinguish between the lineages will help in the effective management and control of the invasive *O. engelmannii* in both countries.

The objectives of the study are:

- To examine the extent of morphological variability among *O. engelmannii* lineages in Africa using multivariate analysis of diagnostic characters (**Chapter 2**).
- To investigate whether there are key morphological 'diagnostic traits' that can be used to confirm lineage identity **(Chapter 2)**.
- To identify morphological differences between *O. engelmannii* and *O. stricta* **(Chapter 2)**.
- Examine how genetically similar the *O. engelmannii* lineages are to each other using Amplified Fragment Length Polymorphism data **(Chapter 3)**.
- To test whether there is a relationship between geographic and genetic distance of focal *O. engelmannii* lineages **(Chapter 3)**.
- To provide a recommendation on the use of morphometric and molecular data for the selection of agents for effective biocontrol of the invasive *O. engelmannii* **(Chapter 4)**.

The key questions associated with these objectives are:

- Are the five lineages of *O. engelmannii* found in Africa morphologically distinct?
- What extent of morphological differentiation is present among the lineages?
- What key morphological "diagnostic characters," if any, can be used to distinguish *O. engelmannii* lineages in Africa?
- Is any *O. engelmannii* lineage morphologically more similar to *O. stricta* than to other *O. engelmannii* lineages*?*
- How genetically differentiated the *O. engelmannii* lineages are to each other?
- Is hybridization occurring among the African *O. engelmannii* lineages?
- How congruent are morphometric and molecular data in defining *Opuntia* lineages?

DISSERTATION STRUCTURE

The dissertation comprises four chapters: an introductory and background chapter (1), central chapters (2) to (3) and a concluding chapter (4). Chapters two and three are written as scientific papers, to be submitted to peer reviewed journals. The aims outlined above are each addressed in one of the two central chapters:

Chapter 1: The present chapter provides a general introduction, literature review, problem statement and the broader aim and objectives of the research.

Chapter 2: Title: An analysis of morphological trait variation among five *O. engelmannii* lineages in Africa. In this chapter, a multivariate analysis is used to evaluate whether the five lineages of *O. engelmannii* in South Africa and Kenya are morphologically similar.

Chapter 3: **Title: Towards a molecular characterisation of *O. engelmannii* lineages in Africa:** In this chapter, population genetics using AFLPs are used to investigate the level of dis/similarity of five *O. engelmannii* lineages in Africa.

Chapter 4: A general discussion to synthesize the overall findings of the research and the implications for biocontrol. Molecular data is used to compare the results from the morphology chapter to further corroborate if there are separate lineages in the country.

CHAPTER 2: AN ANALYSIS OF MORPHOLOGICAL TRAIT VARIATION AMONG FIVE *O. ENGELMANNII* LINEAGES IN AFRICA.

Research output from this chapter

Conference proceedings.

Oral presentation (3min speed talk)

S.E. Mbonani., M.J Byrne and K.L Glennon (2018) - Cryptic cacti: combining molecular and morphological data to solve a biocontrol problem. 44th Annual Conference of the South African Association of Botanists. 9-12th January 2018.

Poster presentation

S.E. Mbonani., M.J Byrne and K.L Glennon (2018) - Cryptic cacti: combining molecular and morphological data to solve a biocontrol problem. 44th Annual Conference of the South African Association of Botanists. University of Pretoria, Hatfield, Gauteng, South Africa. 9-12th January 2018.

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ABSTRACT

Opuntia engelmannii (Cactaceae: Opuntioidae), or small round-leaved prickly pear, is a succulent invasive shrub native to North and Central America. The weed was introduced to South Africa as an ornamental plant more than 300 years ago. At least four lineages of *O. engelmannii* occur in South Africa: one in the Northern Cape, one in Limpopo, and two in the Eastern Cape. A fifth lineage occurs in Northern Kenya. The five lineages vary considerably from each other in several morphological traits. The presence of multiple, variable lineages complicates biocontrol efforts because the agent, *Dactyolopius opuntiae* (Hemiptera: Dactylopiidae), is extremely host specific and is only effective when the correct plant species and insect biotype are matched. This study uses morphological data to test the variation present within and among lineages. Morphological characters such as spine length, spine angle, spine diameter, spine form, area and diameter of the cladodes suggests that these are distinct lineages. Spine morphology shows that the Limpopo and Kenyan lineage are similar, while the Eastern Cape (spines) is more similar to a sister taxon, *O. stricta*. The Northern Cape lineage is different in its morphology to all the *O. engelmannii* lineages tested but also different to *O. stricta*. Multivariate analysis shows that spine morphometry is a useful diagnostic character in identifying different putative *O. engelmannii* lineages in the field. Spine characters also show that the Kenyan lineage matches the Limpopo lineage. Such comparisons will simplify searches for suitable biocontrol agents. Collectively, this information will be used as baseline data for molecular analysis and will assist in selecting an appropriate biotype of *D. opuntiae* that can be used to control the relevant lineage(s) of *O. engelmannii* in South Africa and Kenya.

Keywords: alien invasive plant, diagnostic characters; lineages, morphology, *Opuntia engelmannii*, variation.

2.1 INTRODUCTION

Identification and characterization of species has largely been based on differences in the plants' morphological features for example the colour, size and shape of leaves and flowers (Gunnell and Gubler, 1992; Cope *et al.,* 2012; Waldchen *et al.,* 2018). Furthermore, classification of plant species is an important step for managing collections efficiently and is the basis for cultivating any plant species (Bonos *et al*., 2002). The first step in the classification of germplasm is morphological characterisation (Asudi *et al.,* 2010). Morphological characteristics are therefore essential because they offer baseline observations and are cost effective; colour, feel, taste and odour are some of the useful diagnostic traits that have been used to predict the identification of a species (Applequist, 2006; Waldchen *et al.,* 2018). Evaluation of morphological traits contributes valuable information to the assessment of genetic relatedness and variability of any plant species (Laurentin, 2009). This allows for full description, identification and differentiation of species (Dehling *et al*., 2016).

Characterisation of *Opuntia* genotypes has been largely based only on morphological characteristics, especially fruits and cladodes variation and the species determination is based on taxonomic keys comparing few wild individuals (Martinez-Gonzalez *et al.,* 2015). Three characteristics differentiate the *Opuntia* species from other cacti: (a) growth of cladodes as observably different jointed segments, (b) presence of spines on the surface of the cladode and (c) areoles, which have short prickles known as glochids (Cortázar and Nobel, 1992). The genus *Opuntia* has flat, rounded cladodes with spines. The cladodes are usually green and can become darker and dehydrated in winter. There are usually three to five spines per areole and a number of glochids per areole (Kattermann, 2012; Piovan *et al.,* 2015). *Opuntia* species are an important taxon culturally, ecologically, economically, and medicinally worldwide (Majure *et al.,* 2012). Nevertheless, they are notorious for their taxonomic difficulty due to polyploidy, interspecific hybridization and a great level of morphological variability (Majure *et al.,* 2012).

Evolutionary relationships in these stem succulents have been insufficiently studied and therefore the biogeographic history and phylogeny of most *Opuntia* species remain unresolved. (Majure *et al.,* 2012). The morphological variation and the limited morphological descriptors for cultivar differentation make it difficult to achieve clear

Opuntia classification (Labra *et al.,* 2003; Griffith, 2004; Caruso *et al.,* 2010; Valadez-Moctezuma *et al.,* 2015). These difficulties in morphological interpretation have therefore led to a publication of a large number of binomials, many of which are homonyms, synonyms and false attributions within the *Opuntia* genus (Gibson and Nobel, 1986).

Wislizenus discovered 'the Engelmann Prickly-pear' in northern Chihuahua in 1846. Duplicate samples were presented as synonyms to Salm-Dyck and Engelmann (Parfitt and Pinkava, 1988). The name, *O. engelmannii* has been in varied usage for the conspicuous and large prickly-pear of the Southwest USA. Benson and Walkington, (1965) initially found that the type represents a spiny individual of *O. megacantha* of the cultivated, usually spineless species, *O. ficus-indica* (L.) Miller. They then placed *O. engelmannii* (Salm-Dyck) and *O. megacantha* (Salm-Dyck) in synonymy under *O. ficus-indica* and applied *O. phaeacantha* Engelm. var. *discata* (Griffiths) to the wild material, which continued to bear the common name, Engelmann Prickly-pear (Parfitt and Pinkava, 1988).

Newly revealed morphological characters, especially unique glochid arrangement within areoles, and the restudy of the publication dates by Salm Dyk demonstrates the correct name for the Engelmann Prickly-pear to be *Opuntia engelmannii* -Salm-Dyck ex Engelm (Parfitt and Pinkava, 1988). Other taxa were found to share a similar glochid arrangement, additionally to other characters, necessitating revised synonymy and new combinations that currently include: *O. engelmannii* var. *lindheimeri*, *O. engelmannii* var *linguiformis*, *O. engelmannii* var. *flavispina* were formed (Parfitt and Pinkava, 1988). The following characters were used distinguish *O. engelmannii* from its synonymy with *O. ficus-indica*; areoles on pericarp 12-35, glochids (within each areole on the middle of a stem- segment) conspicuous, 3-5 or more mm long. The discovery of some of these distinguishing and diagnostic characters was important in the recognition of *O. engelmannii* as a species distinct from *O. phaeacantha, O. stricta* and *O. ficus-indica* but are not specifically distinct from *O. lindheimeri.* (Parfitt and Pinkava, 1988). *Opuntia engelmannnii* has typical bluish-green cladodes that become darker in winter. Glochids and three to five spines are present in the areoles; one spine is longer than the other (at least 1.5 cm) which is usually the central spine (Figure 2.1A and B). In young cladodes, the spines have brown and light-green stripes, in the oldest

cladodes they are usually brown (Piovan *et al.*, 2015). Many species of *Opuntia* including *O. engelmannnii* produce edible and highly flavoured fruits known as cactus pear (Figure 2.1C). Some of these diagnostic characters were therefore used in this study to quantify morphological differences among *O. engelmannii* lineages. Weniger (1978), wrote a key for cacti species of the Southwest in the USA, with a few *O. engelmannii* lineages reported in the guide including: *O. engelmannii* var. *engelmannii* having spines with a deep brown at their base, becoming white towards the tip. This description is similar to the Kenyan lineage of *O. engelmannii*.

Introduced to South Africa as an ornamental plant more than 300 years ago and later transported to the interior of the subcontinent (Kiesling and Metzing, 2017). Spiny forms of *O. engelmannii* resulted in dense thickets in some regions, especially the Eastern Cape Province, South Africa (Klein *et al.*, 2015). *Opuntia engelmannii* exists as a morphologically variable species, and can be found in four currently recognised morphologically different lineages in South Africa. This variability has led to the use of the term 'lineage' based on their appearance and the province where they are found in South Africa. Another lineage has also been reported in Kenya (Klein, 2015b). These five lineages of *O. engelmannii* differ from each other and in their suitability as hosts of the cochineal biotypes that could be introduced against them (Musengi, 2018).

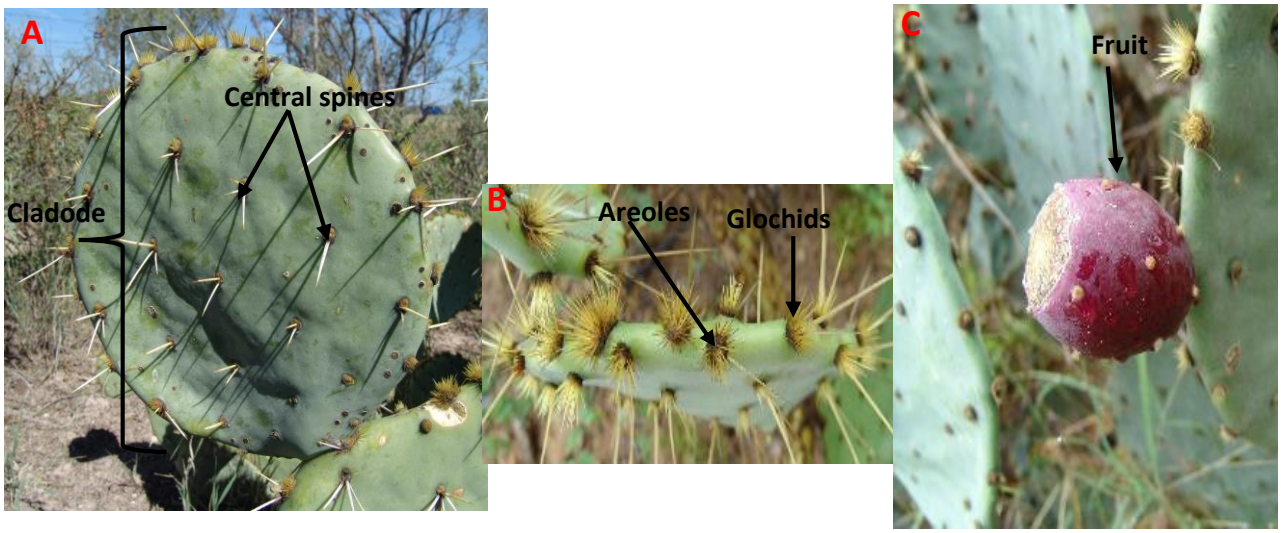

Figure 2.1: a) The main morphological characteristics of a typical cactus including spines and cladodes b) structure and arrangement of areoles on a cladode and (c) fruit on a cladode (photo source: Nic Venter).

It is easy to confuse *Opuntia stricta* with two lineages of *O. engelmannii* namely; *O. engelmannii* var. *texana* and *O. engelmannii* var. *alta,* in Texas (Weniger, 1978). A comparison of species' morphological features are necessary because they provide valuable information about individual accessions, the relationship among the characters, provide basis for analysis of sister relationships and they give clarity on the morphological structure of the species (Khoury *et al.,* 2010). Cluster analysis has been used in several studies to identify and differentiate species from each other and group accessions of plants based on their morphological similarities (Travis *et al.,* 1996; Dikshit *et al.,* 2014; Dikshit and Sivaraj, 2015).

The morphological similar sister taxon, *O. stricta,* was first documented from the Kruger National Park in South Africa in the early fifties but it was only considered an invasive problem from 1989 onward, when the populations had increased drastically. Chemical control was unable to stop the invasions (Foxcroft *et al.,* 2004). After testing the host-specific cochineal insect, "*Stricta D. opuntiae* biotype", it was released on *O. stricta* infestations in the Kruger National Park in 1999 after previous attempts to establish the "*Opuntia*" *D. opuntiae* biotype had failed. The resulting control was remarkable (Foxcroft *et al.,* 2004; Foxcroft *et al.,* 2017). An integrated control effort was successfully trialed, chemically treating isolated plants along the boundary of the 70 000 ha infestation and a constructing mass-rearing facility that offers cochineal redistribution for biological control, is currently in place. Other infestations of *O. stricta* in other parts of the country are also controlled biologically (Paterson *et al.,* 2011; Kaplan *et al.,* 2017). The Australian 'stricta' biotype thrives and is damaging on *O. stricta* but is unable to develop on *O. ficus-indica,* while the opposite is true for the South African 'ficus' biotype, which thrives on *O. ficus-indica* but the development is poor on *O. stricta* 'ficus' biotype, the 'stricta' biotype is currently thriving on *O. stricta* in release sites and, has had considerable damage to the weed (Volchansky *et al.,* 1999). No phylogeny has been shown or studied between *O. engelmannii* and *O. stricta,* nevertheless, *O. stricta* was used in the present study because of its (i) morphological resemblance to the study species *O. engelmannni* and (ii) it is currently under adequate control in South Africa using the 'stricta' biotype of *D. opuntiae*. If any of the *O. engelmannii* lineages are found to be similar to *O. stricta*, the 'stricta' biotype of *D. opuntiae* could potentially be used to biologically control that lineage.

Aim, objectives and key questions

The aim of this chapter is to provide a morphometric analysis of the differences among the lineages of *O. engelmannii* and a closely related, morphologically similar species, *O. stricta* found in Africa.

The objectives of this chapter are:

- To examine morphological differences among *O. engelmannii* lineages in Africa using multivariate analysis.
- To investigate whether there are key 'diagnostic traits' that can be used to predict lineage identity.
- To identify morphological differences between *O. engelmannii* and *O. stricta*.

This chapter will answer these key questions:

- Are the five lineages of *O. engelmannii* found in Africa morphologically distinct?
- What morphological differences are present among lineages?
- What key 'diagnostic characters' if any, can be used to distinguish among *O. engelmannii* lineages in Africa?
- Is any *O. engelmannii* lineage morphologically more similar to *O. stricta* than to other *O. engelmannii* lineages?

2.2 MATERIALS AND METHODS

A total of 25 individuals that represent each of five *O. engelmannii* lineages and an additional 25 from a single population of *O. stricta* were used. Cladodes from separate plants in the same population were collected from Loisaba in Kenya (0˚22'37.70"N 36˚47'18.40"E), Mokopane in Limpopo (24˚10'57.12"S 29˚00'50.04"E), Douglas in the Northern Cape (29˚06'55.70"S 23˚45'04.98"E) and Bedford in the Eastern Cape, for both "spines" and "spineless" lineages (32˚41'16.37"S 26˚06'23.04"E) (Figure 2.2). Cladodes were then planted in pots in the Insectary at the University of the Witwatersrand, Johannesburg, South Africa for further experimental use.

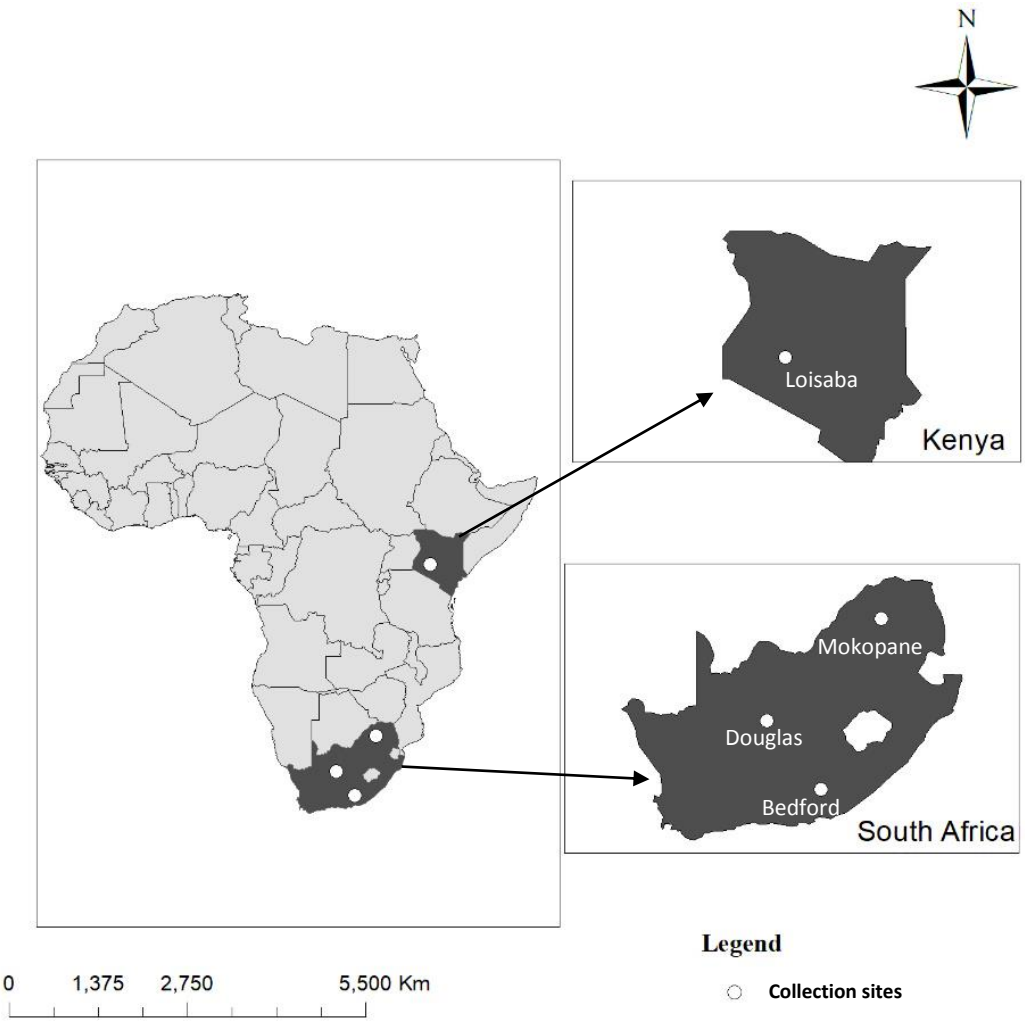

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Figure 2.2: Collection sites of four *Opuntia engelmannii* lineages shown as white dots from Loisaba-Kenya, Limpopo, Northern Cape and Eastern Cape-South Africa.

Spine morphology

Spines, cladodes, areoles and fruits are an important, informative character for *Opuntia* species. Therefore, we used methods similar to previous work (Mosco, 2009; Peharec *et al.*, 2010). The methodology was adapted from similar *Opuntia* morphology studies, which used one or two spines per plant for qualitative differences such as colour and at least 17 spines for quantitative morphological studies such as length. Measurements were conducted on four *O. engelmannii* lineages (without the Eastern Cape- spineless lineage) and the sister taxon, *O. stricta*. For qualitative differences, six spines were used in each individual potted plant and noted differences in spine colour among the lineages and these were tabulated. In this study, the spine's shape was not taken into account as previous studies (Mauseth, 2006; Peharec *et al.*, 2010), whereby they considered spine shape as a least variable character, as it was mostly circular. A dissecting microscope

(MW3-L9) and a camera (Zeiss SLR Model) was used to examine outer appearance and gross anatomy of the individual spines. The individual spines were placed in Petri dishes with a matte-black surface and an image taken. For quantitative measurements the spine lengths were measured using digital callipers (ACCUD®). A total of 10 spines per cladode on the three uppermost (oldest) cladodes were measured per individual plant, totalling to 30 spine measurements per plant. The spine lengths were measured from the base of the spine to the tip of the spine. The average spine length per individual plant and an average of each lineage (n=25) was calculated, this was then compared to the other lineages (n=125) by box plots. The diameter of individual spines was measured at the middle 'widest part' of the spine using digital callipers. The total number of spines per cladode were counted on three uppermost cladodes in each individual plant. This was then averaged per lineage and compared among lineages. Spine angle was obtained by measuring the ventral angle between the surface of the cladode and the spine. The spine angle was measured on the three uppermost cladodes. Ten spine angles were recorded per cladode and binned per individual of each lineage. This was compared among individuals of the same lineage and thereafter compared among the different lineages.

Cladode morphology

Cladode, areole and fruit morphology measurements were conducted on all *O. engelmannii* lineages including the 'Eastern Cape (spineless) lineage' (n=125) and the sister taxon, *O. stricta* (n=25). Cladode colour was observed and images of the cladodes were taken for each lineage. The number of areoles per 30cm on each cladode was averaged for three cladodes per individual plantand then compared among lineages. The area of each cladode was measured per individual using digital callipers. The area of each cladode was calculated using the equation:

$$\text{Area(mm\^2)} = \text{Length(mm)} \text{ X } \text{width(mm)}$$

Additionally, each cladode was measured for thickness per individual plant using digital callipers. The average cladode thickness per individual was recorded per lineage and this was compared among the lineages.

Areole arrangement

Differences in the number of spines per areole were counted. The spines per areole were harvested and viewed using a Zeiss camera. With respect to previous studies, Mosco, 2009 and Peharec *et al.,* 2010, only fully developed areoles (these look like raised cushion-disc and covered by small hairs) were studied. Ten areoles per cladode were counted per individual plant. Within an areole, the number of glochids produced per areole was counted for each individual. The multiple measurements were averaged among individuals of the same plant and compared among the lineages.

Fruit morphology

The number of fruits per plant was counted on each plant, multiple fruit measurements were averaged among individuals of the same plant and compared among lineages. Fruit length was measured from the base of the fruit to the uppermost part of the fruit using digital callipers. Fruit diameter was measured at the widest part of the fruit, multiple fruit measurements were averaged among individuals of the same plant and compared among the lineages. Lastly, the following features (bracts and or spines) were noted on the fruits and each individual of the five lineages was assessed and catergorised as per the features they displayed. As such, one individual could display more than one of these feature.

Data analysis

All morphometric data were tested for normality and homogeneity using R software (V3.4.4).

a) An ANOVA test was performed to test whether there was a significant difference for each spine, cladode, areole and fruit characters or trait, a Tukeys post hoc test was used to see where the difference lies among the lineages.

b) A Principal Component Analysis for spine, cladode and fruit diagnostic characters was used to establish the morphological similarity among the lineages. A Nonmetric MultiDimension Scaling-A (NMDS-A) with all 18 quantitative and qualitative morphometric dataset for *O. engelmannii* lineages and the sister taxon, *O. stricta* was performed on all the data to test whether these characters grouped the lineages. An additional NMDS-B with 12 quantitative and qualitative morphometric characters (without spine traits) for *O. engelmannii* lineages and the sister taxon,

O. stricta was performed on all the data to test whether there were clear groupings of lineages without including the spine traits.

2.3 RESULTS

Qualitative differences were noted in the spine gross anatomy (the outer appearance of the spine) (Figure 2.3). The Eastern Cape, Limpopo and Kenyan lineage, A, C, D respectively, have a similar spine colour, while the Northern Cape lineage has distinct multi-coloured spines (dark orange at the base of the spine, faint orange mid-spine and powdery white at the tip of the spine).

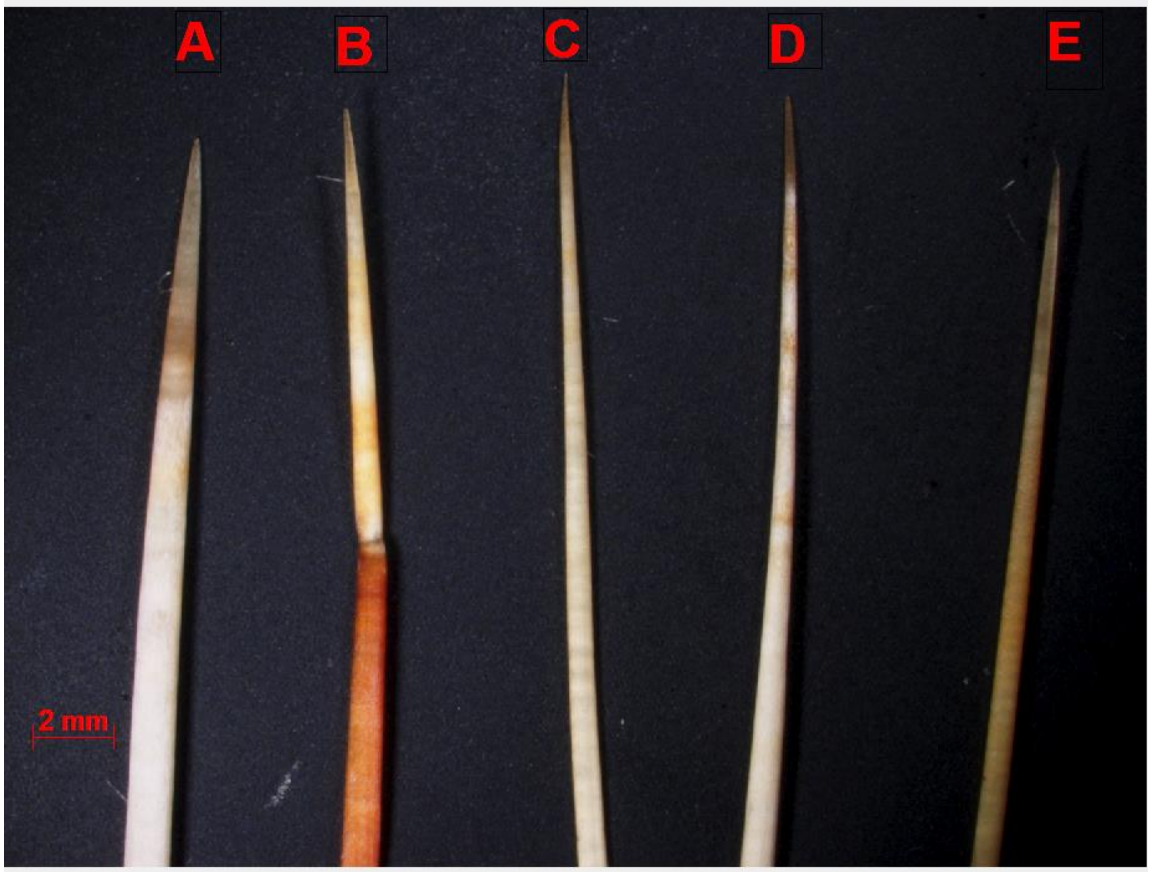

Figure 2.3: Appearance of the spines of *Opuntia engelmannii* lineages studied. A-Eastern Cape, B- Northern Cape, C-Limpopo, D-Kenya, E- *O. stricta*. The Eastern Cape, Limpopo and Kenyan have a similar powdery white colour with some faint bands, while the Northern Cape lineage has a distinctive orange colour. *O. stricta* lineage has a distinctive faint yellow colour.

Spine morphometrics show that the Limpopo lineage has significantly longer spines than the other lineages ($F_{4, 120} = 39.1168$, $p < 0.0001$) (Figure 2.4A). In contrast, the Eastern Cape (spines) lineage has a significantly larger diameter than the other lineages ($F_{4, 120}=36.1890$, $p < 0.0001$) (Figure 2.4B). The Limpopo lineage has significantly

more spines per individual plant than all the other lineages ($F_{4, 120}$=61.5540, p<0.0001) (Figure 2.4C). The Northern Cape lineage has a significantly different (less) spine angle to all the *O. engelmannii* lineages ($F_{4, 120}$ =113.1376, p <0.0001) (Figure 2.4D).

A PCA of spine-related traits showed 94.99% of the variation is explained by principal component 1. The total number of spines and the spine angle can be used to distinguish and account for differences between *O. engelmannii* lineages. The sister taxon, *O. stricta* has similar spine morphology to the Eastern Cape (spines) lineage, while Kenya and Limpopo have a similar spine morphology. The Northern Cape lineage is different in its spine morphology to the other lineages of *O. engelmannii* (Figure 2.5).

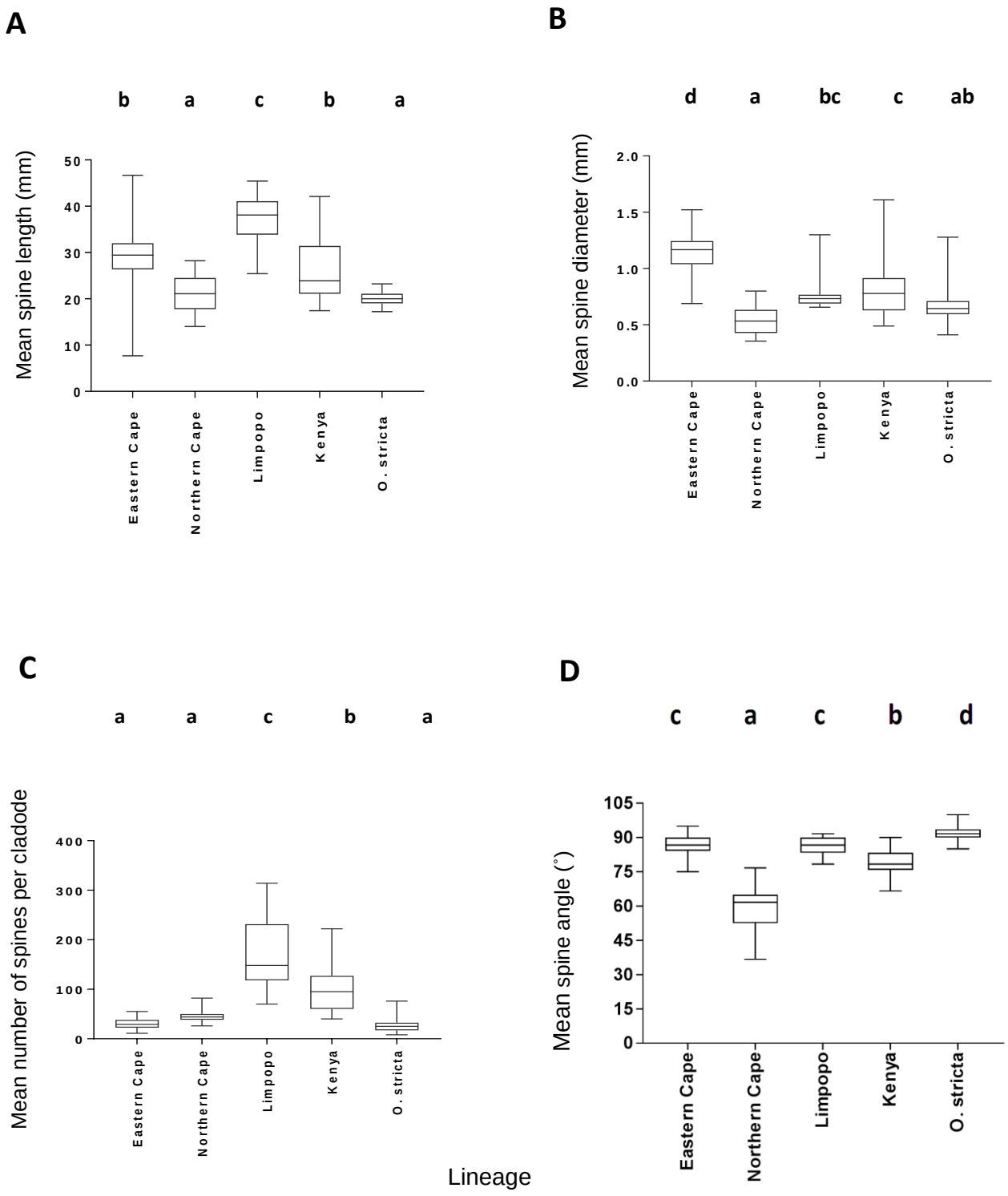

Figure 2.4: The differences in A) spine length, B) spine diameter, C) number of spines and D) spine angle among Eastern Cape (spines), Northern Cape, Limpopo, Kenyan lineages of *Opuntia engelmannii* and the sister taxon (*Opuntia stricta*). Bars represent the standard errors of the mean. Different letters (Tukeys post hoc test) indicate significant differences among the lineages ($P < 0.0001$) n=125.

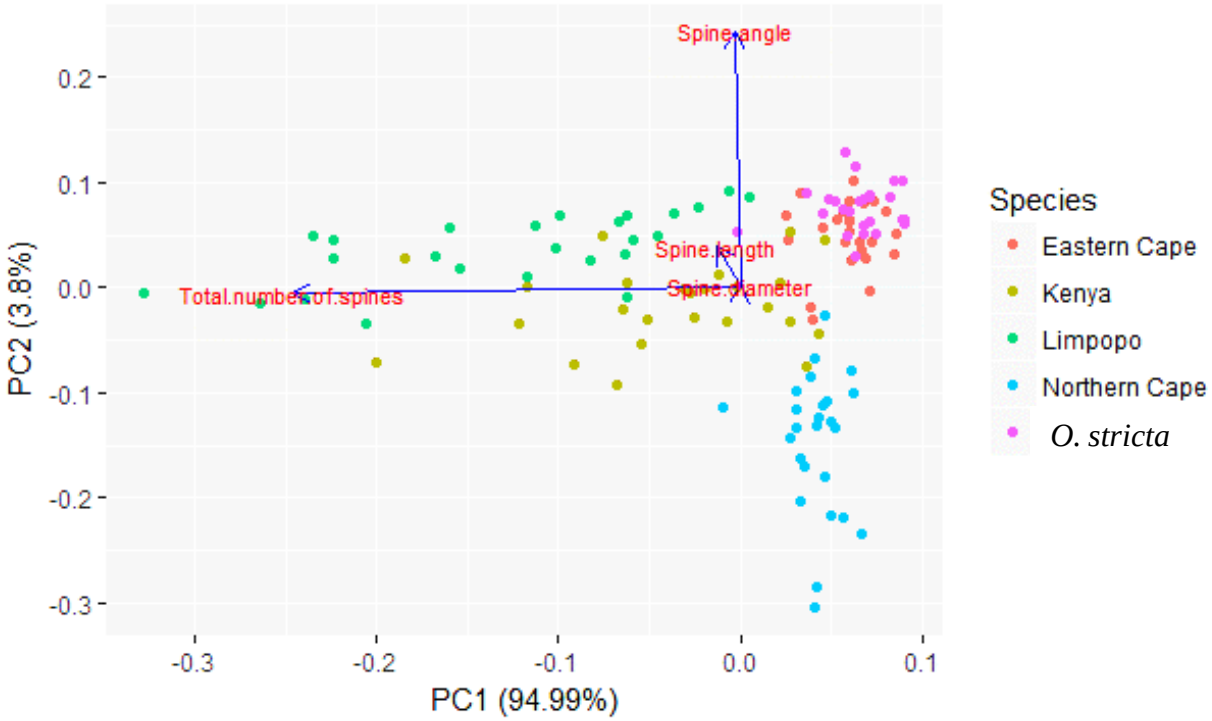

Figure 2.5: Principal coordinate analysis of four spine traits. The total number of spines and the spine angle distinguish between the different *Opuntia engelmannii* lineages.

The areole structure of *O. engelmannii* lineages and the sister taxon, *O. stricta* show that the Northern Cape, Limpopo and Kenyan lineage have more spines per areole than the Eastern Cape and *O. stricta* lineages (ANOVA $F_{5, 145}$=117.9881, $p < 0.0001$) (Figure 2.6 and 2.7B). Areole arrangement morphometrics also show that the Northern Cape has a significantly higher number of glochids per areole, while the Eastern Cape, Kenya, Limpopo do not differ in number of glochids per areole ($F_{5, 145}$=191.3912, $p< 0.0001$) (Figure 2.7A).

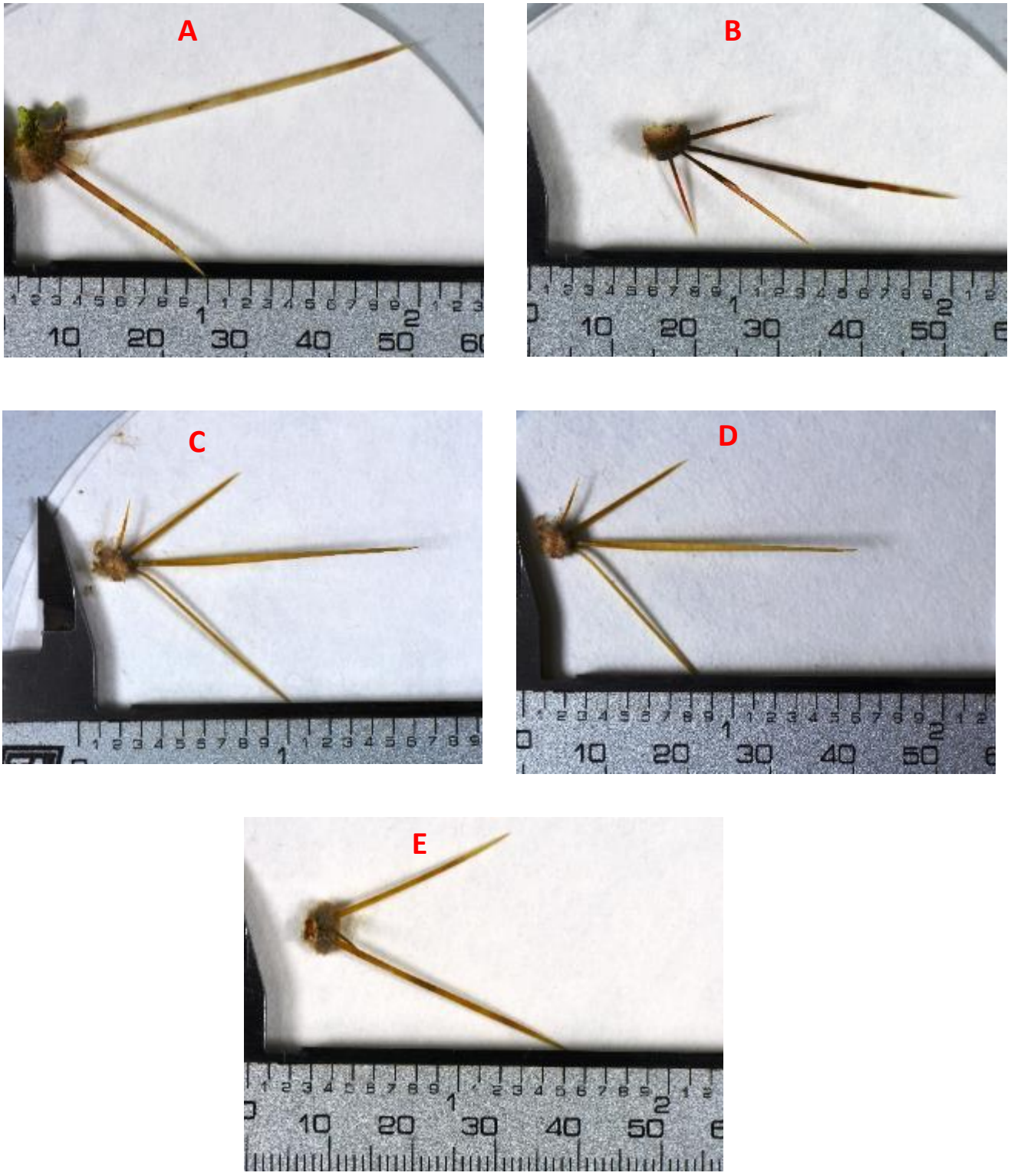

Figure 2.6: The radial arrangement of spines within an areole - showing the number of spines per areole for *Opuntia engelmannii* lineages; A) Eastern Cape (spines), B) Northern Cape, C) Limpopo, D) Kenya, E) *O. stricta*.

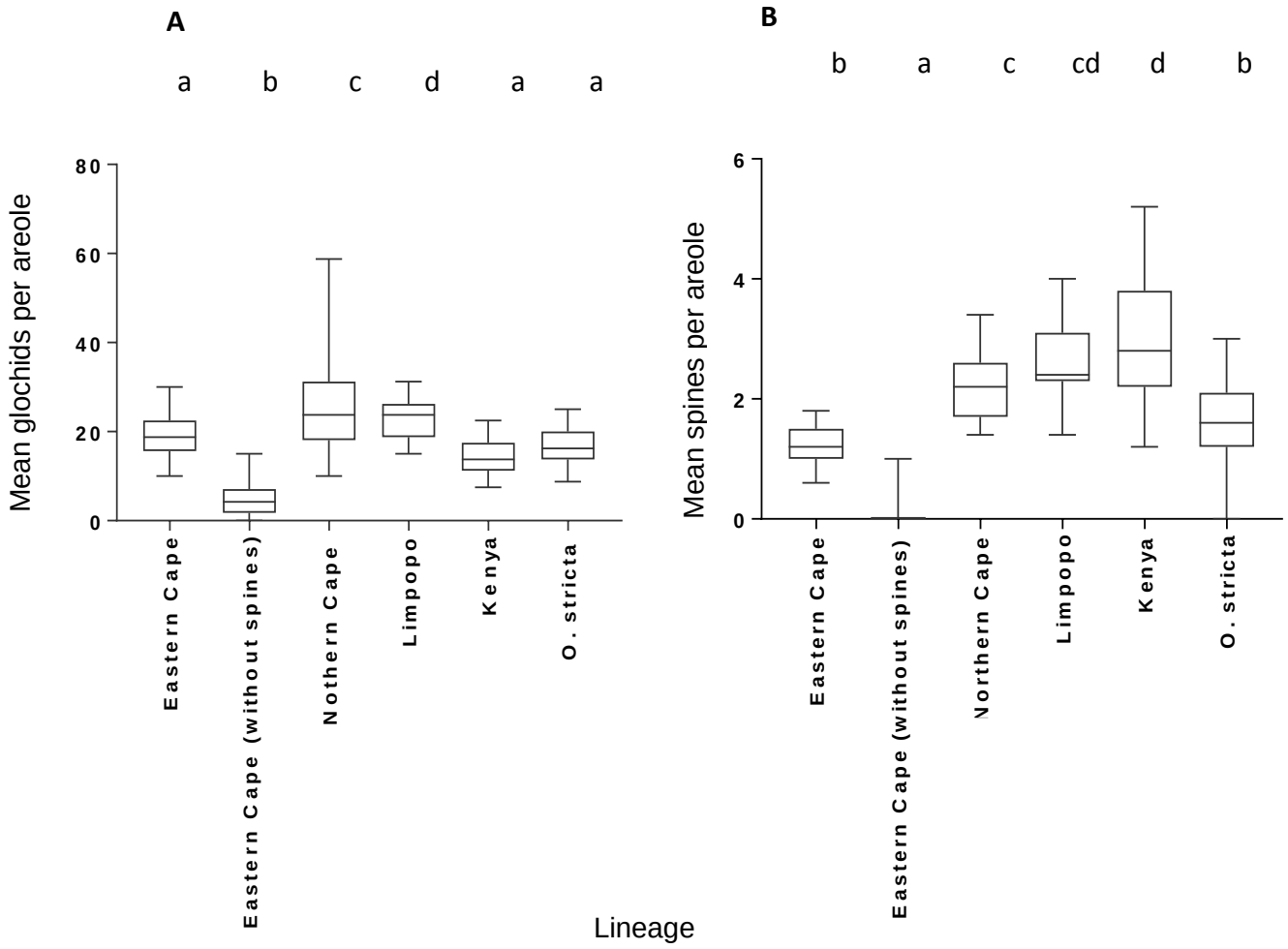

Figure 2.7: A) The mean number of glochids per areole of *Opuntia engelmannii* lineages and *Opuntia stricta* and B) mean number of spines per areole on each individual plant among the different *Opuntia engelmannii* lineages and the sister taxon, *Opuntia stricta*. Bars represent the standard error. Different letters (Tukeys post hoc test) indicate significant differences among the lineages ($P < 0.0001$) n=150.

Cladode morphology shows that the Limpopo and Kenyan lineage have a similar cladode colour and form (Figure 2.8) and both have a similar number of areoles on each cladode (ANOVA $F_{5, 145} = 29.7216$, $p<0.001$**)** (Figure 2.9C). The Kenyan lineage has significantly thinner cladodes (ANOVA, $F_{5, 145}= 31.7216$, $p < 0.0001$) (Figure 2.9B) when compared to the Limpopo lineage. In contrast, the Limpopo lineage has a greater cladode surface area (ANOVA, $F_{5, 145}=45.3262$, $p < 0.0001$) (Figure 2.9A) than all the other *O. engelmannii* lineages. Eastern Cape (spines) and Northern Cape have a similar cladode colour, form and thickness (Figure 2.8). The sister taxon, *O . stricta* has a

smaller cladode area and a lower number of areoles counted per cladode (Figure 2.9A and 2.9C) when compared to all the *O. engelmannii* lineages.

A PCA of cladode related traits shows that (i) the Eastern Cape (spineless) lineage doesn't cluster with any of the *O. engelmannii* lineages nor the sister taxon, *O. stricta*, (ii) individuals of *Opuntia stricta* are clustered together, meaning cladode morphology can be used to confirm *O. stricta* species identity. There is a great deal of overlap between The Kenyan, Limpopo, Eastern Cape (spines) and Northern Cape lineage, which means cladode morphology is possibly not the best character for separating *O. englemannii* lineages (Figure 2.10).

Figure 2.8: Cladode morphology shows that the Limpopo amd Kenyan lineage have a similar shape, texture and size while the Northern Cape lineage has smaller cladode area, which are also a darker green colour. The sister taxon, *Opuntia stricta* has significantly a thinner and a more longitudinal cladode morphology. A) Eastern Cape (spines), B) Eastern Cape (without spines), C) Northern Cape, D) Limpopo, E) Kenya and F) *O. stricta.*

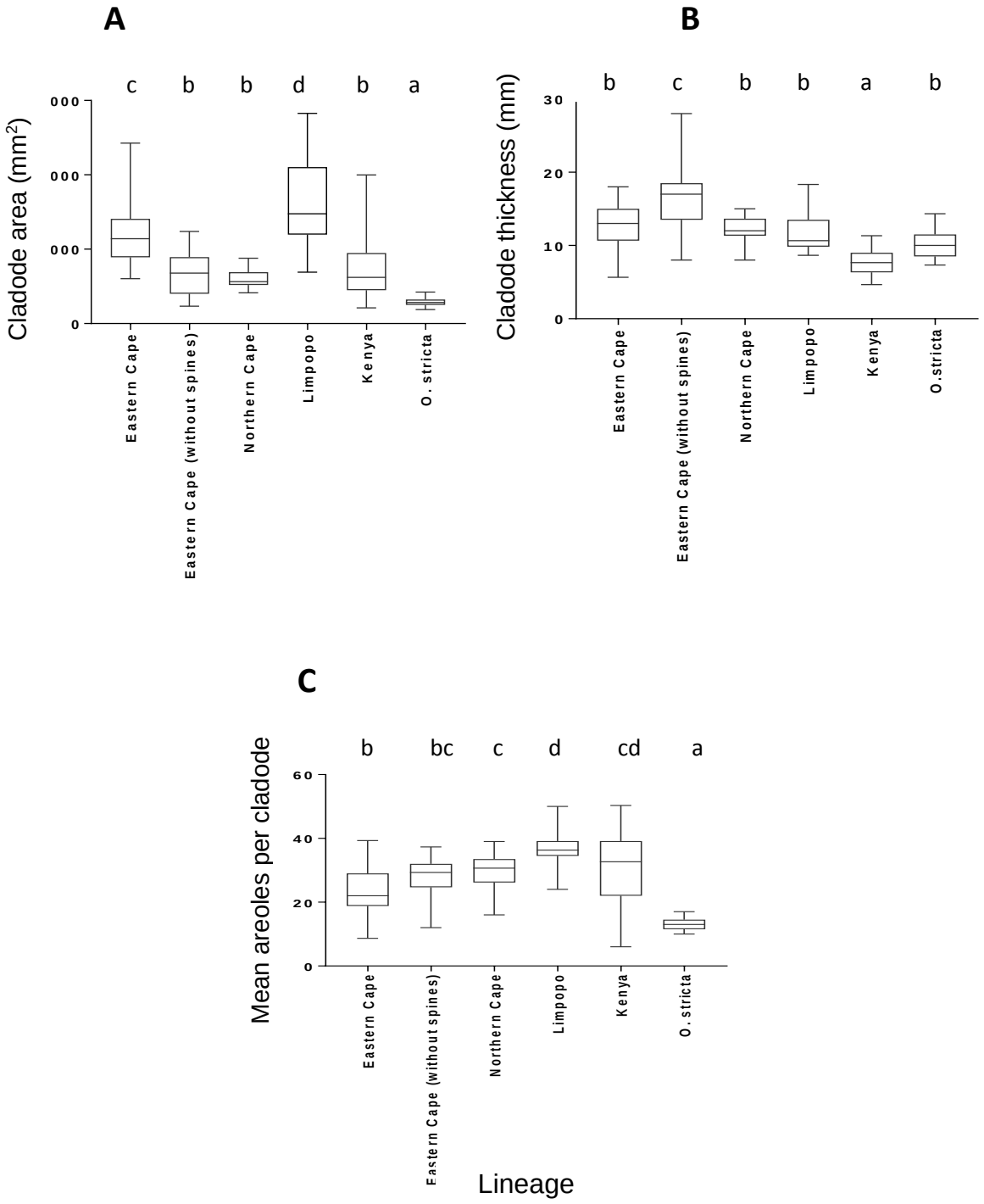

Figure 2.9: A) The average area of the cladode, B) the difference in cladode thickness and C) the average number of areoles per cladode among the different *Opuntia engelmannii* lineages and the sister taxon, *Opuntia stricta*. Bars represent the standard errors. Different letters (Tukeys post hoc test) indicate significant differences among the lineages ($P < 0.001$) n=150.

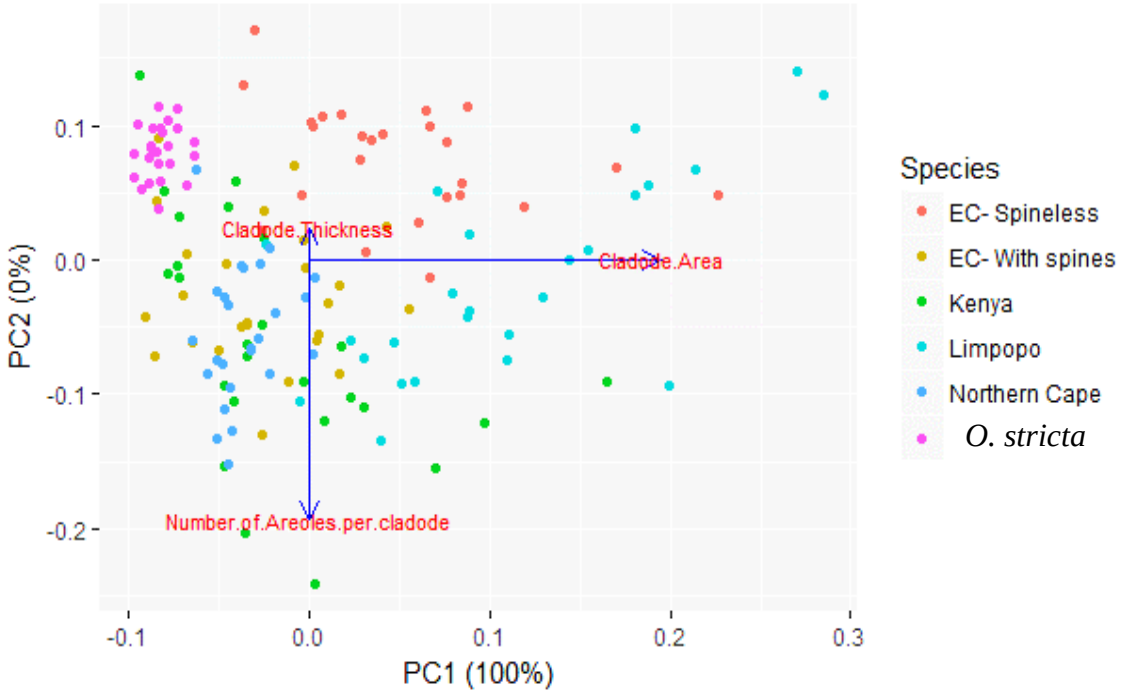

Figure 2.10 : Cladode morphology PCA of the number of areoles per cladode, cladode thickness and area to account for the differences between *Opuntia engelmannii* lineages. Eastern Cape (spines) and Northern Cape have a similar cladode morphology. Likewise, Kenya and Limpopo have similar cladode morphology. The sister taxon, *Opuntia stricta* has a different cladode make up to all the *Opuntia engelmannii* lineages.

The Eastern Cape (spineless) lineage did not produce any fruit throughout the duration of the study, therefore fruit morphometries were conducted on the Eastern Cape (spines), Northern Cape, Kenyan, Limpopo and *O. stricta* lineages. A similar fruit shape (U-shape) was observed in all *O. engelmannii* lineages, with the exception of Eastern Cape (spines), which were different by having glochids at the base of the fruits. This was different to all *O.engelmannii* lineages but similar to *O. stricta* (Table 2.2). The Limpopo lineage produced a significantly higher number of fruits (ANOVA, $F_{5,47}$=16.3909 , p< 0.001) and significantly broader fruits than the other lineages (ANOVA, $F_{5,47}$=16.3909 , p< 0.001) (Figure 2.11 A and C), but the length of the fruit, however, is similar to the Kenyan, and Northern Cape lineage (ANOVA, $F_{5,47}$=109.785, p<0.001) Figure 2.11 B). The Eastern Cape (spines) had an occurrence of both bracts and spines on their fruit, while other lineages had only spines on their fruit (Table 2.2). A PCA of fruit related traits showed no groupings, therefore it is was considered inadequate for separating both *O. engelmannii* and *O. stricta* species (Figure 2.12).

Table 2.2: Fruit morphology of five *Opuntia engelmannii* lineages examining fruit shape, colour, presence of bracts and spines in the fruit and the presence or absence of glochids on the base of the fruit.

Lineage	Fruit shape	Fruit colour	Bracts and or Spines on the fruits?	Glochids at base of the fruit?
Eastern Cape (spines)	V-shaped fruits	Green all over	Bracts and spines	No
EasternCape (spineless)	No fruits	No fruits	No fruits	No fruits
Northern Cape	U-shaped fruits with a more round top	Green with dark pink top	Spines only	Yes
Limpopo	U-shaped fruits	Dark green with a purple top	Spines only	Yes
Kenya	U-shaped fruits but more tubular than Northern Cape and Limpopo	Green all over	Spines only	Yes
O. stricta	long, tube-like fruits	Green with pink base	No bracts and spines	No

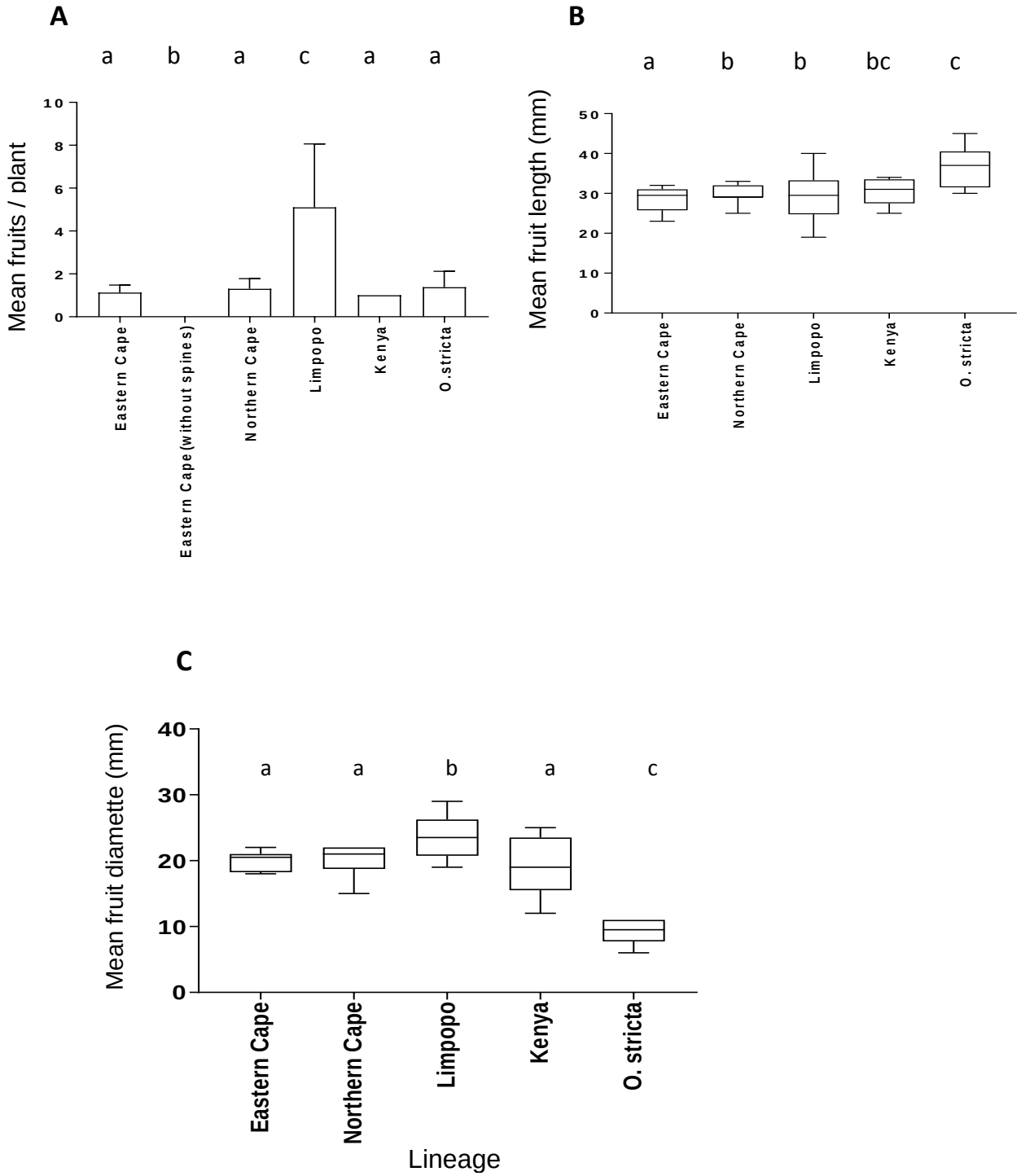

Figure 2.11: A) The mean number of fruits produced per plant. No fruits were produced by the Eastern Cape (spineless) lineage, B) the mean fruit length and C) the mean fruit diameter among the different *OpuntiA engelmannii* lineages, and the sister taxon, *Opuntia stricta*. Bars represent the standard errors. Different letters (Tukeys post hoc test) indicate significant differences among the lineages ($P < 0.001$) n=150.

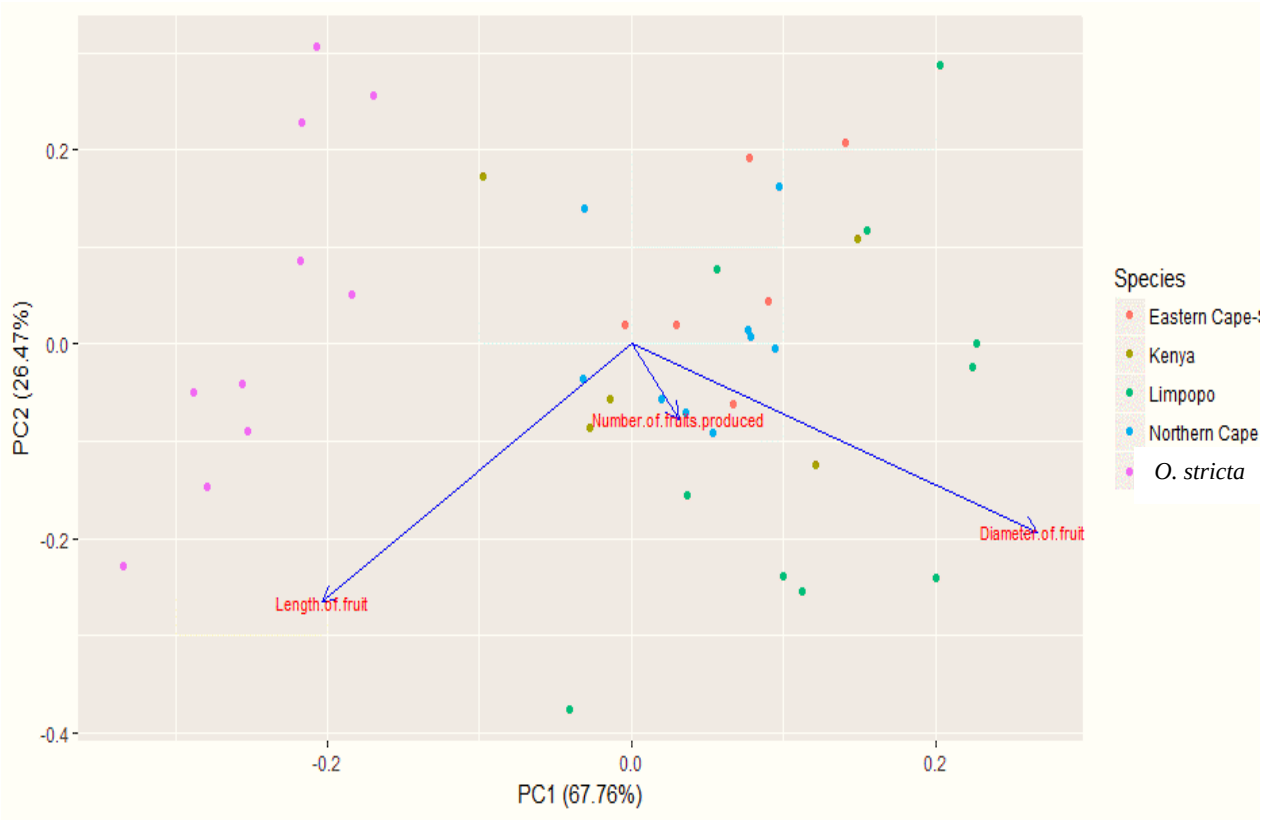

Figure 2.12: Fruit morphology PCA, 67.76% of the variation is explained by principal component 1 and 26.47% of the variation is explained by principal component 2. The length and diameter of the fruit can be used to distinguish and account for variation between *Opuntia engelmannii* and *Opuntia stricta*.

Plant height can be used to distinguish between the lineages (Bowers, 1996). Plants of the Limpopo lineage can grow up to at least 2 m in height, while those of the Northern Cape lineage are low growing and grow less than 1m (Table 2.3).

Table 2.3: South African *Opuntia engelmannii* plant height data (Source: Hildergard Klein, pers observations).

Lineage	Plant height (m)
Northern Cape	>1
Eastern Cape (spines)	<= 1.5
Eastern Cape (spineless)	<= 1.5
Limpopo	< 2

A Nonmetric MultiDimension Scaling (NMDS) ordination method was used to combine l8 quantitaive and qualitative traits of the *O. engelmannii* lineages and *O. stricta*. NMDS-A and B results suggest that both the Eastern Cape lineages and *O. stricta* are different to the other *O. engelmannnii* lineages examined here. There are

clear groupings among the lineages more especially in the NMDS-A with spine morphology (Figure 2.13). There are major similaritites and overlap between the Kenyan and the Limpopo lineage in spine and cladode morphology. While in NMDS-B without the presence of spine data there is some moderate overlap among both the Eastern Cape lineages and the Northern Cape lineage but the groupings are not as significant as NMDS-A (Figure 2.14).

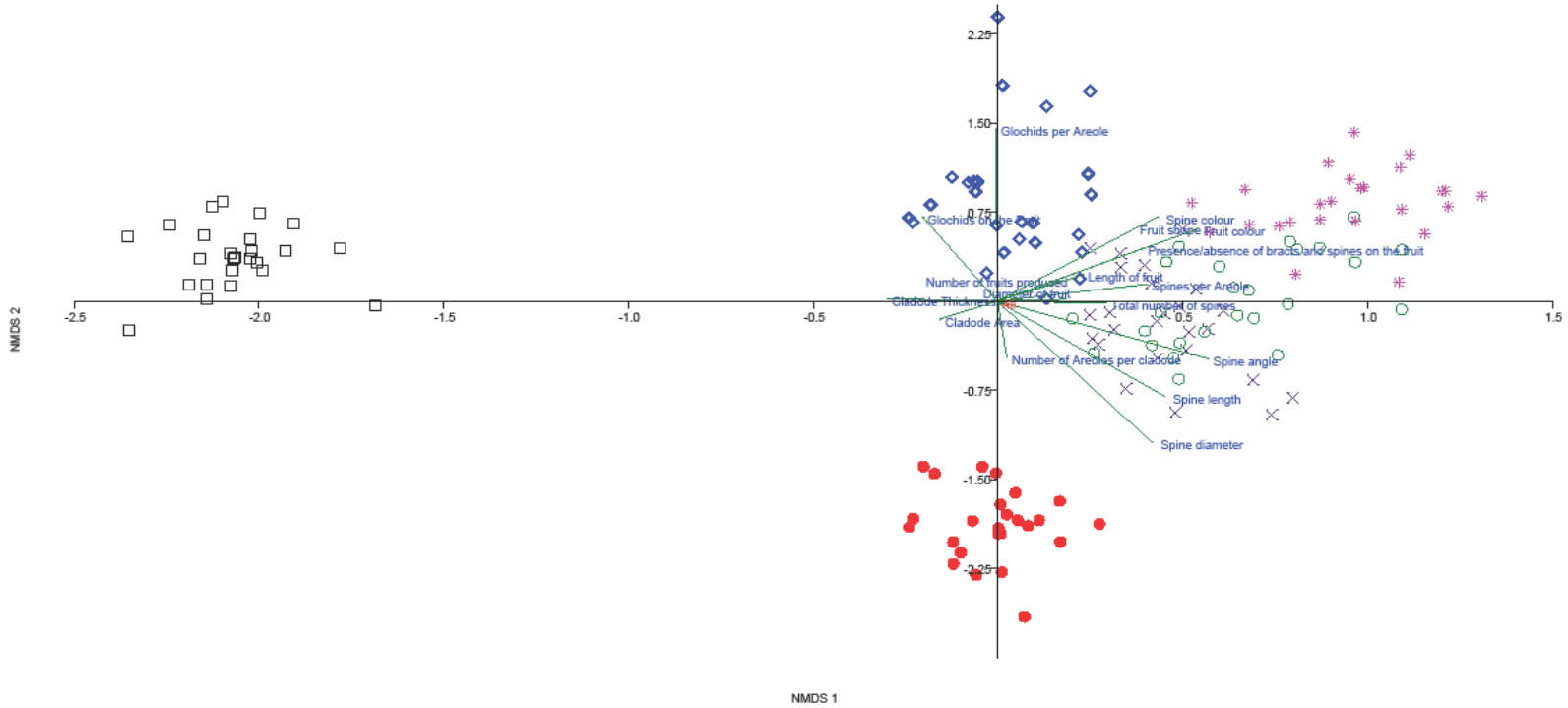

Figure 2.13: Nonmetric MultiDimension Scaling -A (NMDS-A) using 18 quantitative and qualitative morphometric characters for five *Opuntia engelmannii* lineages and *Opuntia stricta* (n=25/taxon). Eastern Cape (spineless) are shown in Black Blocks, Eastern Cape (spines) - Red Circles, Northern Cape – Blue Diamonds, Limpopo- Purple Crosses, Kenya- Green Circles and *Opuntia stricta* -Pink Stars. The Kenyan and Limpopo lineages have a similar morphology, with both the Eastern Cape lineages having a different morphology to Kenya and Limpopo. The Northern Cape lineage also has a different mophology to all the the *Opuntia engelmannii* lineages, which is also different to *Opuntia stricta*.

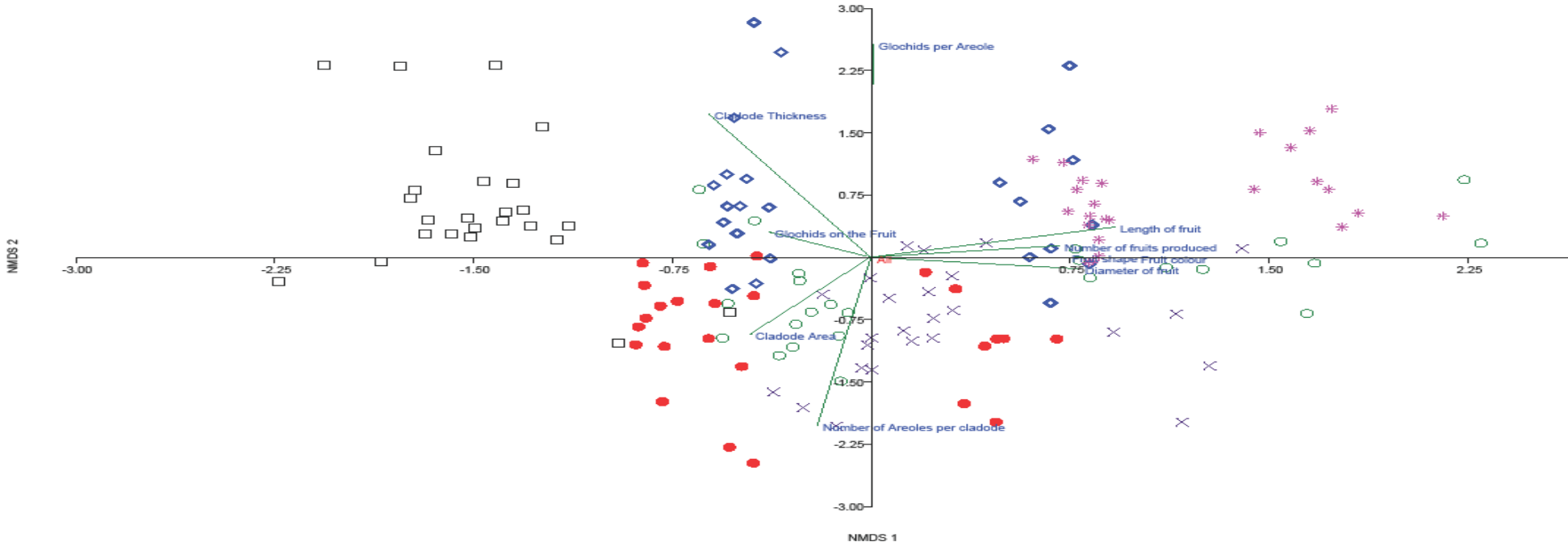

Figure 2.14: Non-Metric Dimension Scaling-B (NMDS-B) using 12 quantitative and qualitative morphometric characters (excluding spine morphology characters) for five *Opuntia engelmannii* lineages and *O. stricta* (n=25/taxon). Eastern Cape (spineless) are shown in Black Blocks, Eastern Cape (spines)- Red Circles, Northern Cape – Blue Diamonds, Limpopo- Purple Crosses, Kenya- Dark Green circles and *Opuntia stricta* - Pink Stars. The Kenyan and Limpopo lineage have a similar morphology, with both the Eastern Cape lineages having a different morphology to Kenya and Limpopo. The Northern Cape lineage also has a different mophology to all the the *Opuntia engelmannii* lineages, which is also different to *Opuntia stricta*.

2.4 DISCUSSION

The question of morphological trait idenfication is an important in a variable IAP like *O. engelmannii* because at least 86% of invasive species can be identified by their morphological traits alone (Devin and Beisel, 2008; Lockwood *et al.*, 2013). For example, spines are an important taxonomic feature in *Opuntia* species (Robinson, 1974; Gibson and Nobel, 1986). Typical *O. engelmannii* spines are 'yellow' or 'white' (Smale, 1976; Weniger, 1978), and these features are particularly evident in both Limpopo and Kenyan lineages. Spine colour show that the Eastern Cape (spines), Limpopo and Kenyan lineage have a similar 'yellow' spine colour. Whereas, the Northern Cape and sister taxon, *O. stricta* have different spine colour to the other *O. engelmannii* lineages. The Northern Cape lineage has a different spine colour than all the *O. engelmannii* lineages and is also different to *O. stricta*.

In addition to spine colour, a quantitative morphometric analysis of five *O. engelmannii* lineages in Africa highlighted the variability of *O. engelmannii* traits among these lineages. There were clear groupings when spine morphology data were included. The groupings were not as clear when spine morphology data were omitted. There was a clear variation in morphologies among the lineages of *O. engelmannii*. The Kenyan and Limpopo lineage have similar spine morphology and the Northern Cape lineage is different to both the *O. engelmannii* lineages and *O. stricta* species. Spine length, diameter and total number of spines were all found to be significantly different among the 'spiny' lineages of *O. engelmannii*. The Limpopo lineage had significantly longer spines and produced significantly more spines than the other lineages ($F_{4, 120} = 61.5540$, $p < 0.0001$), while the Northern Cape and *O. stricta* lineages had significantly shorter spines and produced less spine per plant ($F_{4, 120} = 39.1168$, $p < 0.0001$). These results were further substantiated by the spine diameter results. The Northern Cape and *O. stricta* lineages had significantly thinner spines than the other lineages of *O. engelmannii*. The Eastern Cape (spines) lineage, however has significantly thicker spines ($F_{4, 120} = 36.1890$, $p < 0.0001$). Spine angle results showed that: (a) Northern Cape lineage had a significantly lower average spine angle from the surface of the cladode to the spine and (b) *O. stricta* had a higher spine angle. Again the Northern Cape lineage is significantly different to the other *O. engelmannii* lineages but is also different to the sister taxon ($F_{4, 120} = 113.1376$, $p < 0.0001$). In a similar study, spine length and thickness were also found to be the most effective character for species

classification in 30 *Aylostera* and *Rebutia* (Cactoideae) hybrids (Mihalte and Sestras, 2012), these results are in line with the present study and show spine morphology as more informative in confirming lineage identity more than cladode morphology and areole structure. Spines have a number of important functions in *Opuntia* species, such as providing defense against herbivores (Norman and Martin, 1986). In the present study, the number of spines per areole was either between two or three; most individuals had less than three spines per areole. A similar morphometric study on *Gymnocalycium kieslingii* (Cactaceae) found that the number of spines per areole in *G. kieslingii* ranged between three to seven (Gebauer, 2016). Physiological functions of spines are partially known. According to Gibson and Nobel (1986) spines have been reported to reduce plant transpiration and protect stems from excessive solar radiation and harmful electromagnetic waves. In contrast, Nobel, (1988) and Drezner, (2011) suggested that spines affect plant growth because they seem to reduce plant interception of photosynthetic active radiation and carbon dioxide capture. These findings suggest that Limpopo lineage would have a limited plant growth and reduced transpiration but would be more protected from electromagnetic waves since it had the highest number of spines produced than the other studied *O. engelmannii* lineages. Comparisons of plant growth and height among the different lineages of *O. engelmannii* in the field will be useful in confirming lineage identity.

A major distinction between the two Eastern Cape lineages is the presence or absence of spines. Felker *et al.,* (2006) indicates that spinelessness is relatively simply inherited and it has been suggested that it is possible to obtain spineless individuals if the parents yield fertile progeny and if one of the parents were spineless (Felker *et al.,* 2006). Felker *et al.,* (2006) suggests that human cultivation of *Opuntia* is perhaps responsible for the transition from spiny to spineless lineages because spineless *Opuntias* are extremely palatable to domestic stock, but also wildlife. A similar study on *Opuntia* 'varieties' carried out a cluster analysis with 24 morphological characteristics on 29 cactus varieties showed that the spread of certain *Opuntia* varieties could be attributed to 'desirable' traits of fruits, young cladodes used as a vegetable and reduced number of spines in the plant which are all desirable characteristics in *Opuntia* trade and domestication process (Gallegos–Vásquez *et al.,* 2012). Furthermore, most studies that differentiate *Opuntia* species groups have used spine, fruit and cladode morphology as basis for differentiation (Reyes–Agüero *et al.,* 2005; Gallegos–Vásquez *et al.,* 2012).

Similar studies have used morphometric approaches such as leaf morphology and more comprehensive approaches such as the whole architecture of the plant to differentiate between species belonging to the same genus, like plant branching patterns in *Crambe abyssinica, C. hispanica* and *C. glabrata* (Warwick and Gugel, 2003). Characterisation of *Opuntia* lineages to date has been largely based only on morphological characteristics, like fruits and cladodes variation (Caruso *et al.,* 2010). Typical of all *Opuntia* species, *O. engelmannii* grows by producing new cladodes, cladodes are therefore an important diagnostic character to the platyopuntias. The pale-green cladode colour was similar among all *O. engelmannii* lineages, in-line with Bobich and Nobel, (2001) who stressed that most *Opuntia* cladodes are usually pale green. Peña-Valdivia *et al.,* (2008) showed that cladode dimensions i.e. cladode surface area and thickness might also be a ploidy indicator. For example, greater sized fruits, cladodes and stomata are more common in polyploid (6n, 8n) than diploid *Opuntia* plants. The Eastern Cape (spineless) lineage had the thickest cladodes, while the Kenyan lineage had the greatest surface area, which suggests that both of these lineages could be polyploids. *Opuntia stricta* had the smallest surface area relative to the Eastern Cape and Kenyan *O. engelmannii* lineages. Additionally, it also had the least number of areoles per cladode when compared to the *O. engelmannii* lineages, further substantiating evidence by Majure and Puente, (2014), that *O. stricta* is a diploid species while all the other *O. engelmannii* cladode traits indicate that these are possibly polyploids.

The Limpopo lineage cladodes had significantly greater surface areas than the Kenyan and Northern Cape lineages (ANOVA, $F_{5, 145}$=45.3262, $p < 0.0001$) and it also had significantly thicker cladodes than the Kenyan lineage too (ANOVA, $F_{5, 145}$= 31.7216, $p < 0.0001$). This would also explain why the Limpopo lineage would grow to at least 2m in the field. A study by Cortázar and Nobel, (1992) showed a linear positive relationship between the cladode surface area and the thickness the cladode. Increasing cladode thickness is especially evident for larger cladodes like that of Eastern Cape (spineless) and Limpopo lineages. There is a great deal of overlap among The Kenyan, Limpopo, Eastern Cape (spines) and Northern Cape lineage in cladode morphometrics, which means cladode morphology is possibly not the best to confirm *O. engelmannii* lineage identity. The results are similar with those pointed out by Valdez–Cepeda *et al.,*

(2003) who reported that spine, areole and fruit traits are important for classification of cactus pear varieties, as opposed to cladodes, which are mostly shaped by seasonal effects and the local climate. Morever, cladode differences between species has been reported as a reflection of environmental conditions during cactus growth (English *et al.*, 2012; Menezes *et al.*, 2015). A study on the comparative morphology of *Sarracenia purpurea* 'varieties' had results similar to the present study by supporting morphological differentiation of *S. purpurea* subsp. *venosa* var. *burkii* from *S. purpurea*, using morphological characters such the shape of the pitcher hood, the ratio of pitcher length to the diameter of the pitcher opening and the presence or absence of hairs on the outside of the pitcher and flower colour (Ellison *et al.*, 2004). However, similar to cladodes, the size and shape of pitchers were reported to change naturally in response to environmental conditions such as the change in seasoning and the availabity of nutrients (Mandossian, 1966; Ellison and Gotelli, 2002).

Fruit characters have been used to differentiate and assess patterns of divergence between *Gambelia* species (Elisens and Nelson, 1993). However, results for the present study show that fruit was not a great predictor of lineage identity for both *O. engelmannii* lineages and *O. stricta* individuals. The Eastern Cape (spineless) lineage did not produce any fruit throughout the duration of the study most individuals in this lineage were placed under shade at the Wits Plant Nursery. Cortazer and Nobel, (1992) found that fruits of *O. ficus-indica* generally do not occur on shaded cladodes. These findings could perhaps explain why the Eastern Cape (spineless) lineage had no fruits through the duration of the experiments conducted for this dissertation.

2.5 CONCLUSION

The study of 150 cactus individuals showed that spine, cladode, areole and fruit diagnostic characters are different among cacti lineages. There were clear morphological groupings amongst the lineages. Spine data rather than cladode, areoles and fruit data is important in distinguishing among the studied *O. engelmannii* lineages and confirming lineage identity. The Eastern Cape (spines) lineage was found to be more similar to *O. stricta* in its morphology than the other studied *O. engelmannii* lineages. Morphological data suggests that there are at least four distinct lineages of *O. engelmannii*. The Kenyan and Limpopo lineages are morphologically similar, while the Northern Cape, Eastern Cape (spines) and Eastern Cape (spineless) are all

morphological different lineages. In some studies species divergence on the basis of morphological data could not be differentiated when using molecular data, the opposite is also true (Gemeinholzer and Bachmann, 2005). Morphological data can be closely dependent on environmental conditions. However, the use of multiple morphological diagnostic characters limits problems associated with the environmental influence as all traits are unlikely to be similarily affected by the environmental conditions. Human activities have played a major role in selecting *Opuntia* plants with bigger fruits, fewer areoles and spines.These are typical in the process of domestication of *Opuntia species*, these activities have an impact in morphologically differentiating and diverging *Opuntia* species belonging to the same species (Reyes–Agüero *et al.,* 2005; Gallegos–Vásquez *et al.,* 2012). Field data in the form of plant height needs to be studied and quantified as plant height is also an important morphological attribute. No data on plant height has been studied for the different *O. engelmannii* lineages and these could be useful in distinguishing among the lineages. The use of plant height data and cladode pH is recommended as cladode pH has been reported to vary between and within the *Opuntia* genus and therefore can be used to differentiate *O. engelmannii* lineages in Africa (Musengi *et al.,* 2018).

CHAPTER 3: TOWARDS A MOLECULAR CHARACTERISATION OF *O. ENGELMANNII* LINEAGES IN AFRICA.

Research output from this chapter

Oral presentation (15 min Oral presentation) - Best student oral presentation

S.E. Mbonani., K.L Glennon and M.J Byrne (2018) - Towards a molecular characterisation of *O. engelmannii* lineages in Africa. 45th Annual Research Symposium on the Management of Biological Invasions in Southern Africa. University of Venda, Thohoyandou, Limpopo, South Africa. 3–6 July 2018.

ABSTRACT

The invasive cactus *Opuntia engelmannii* is a morphologically variable species that has invaded pasture lands in South Africa and Kenya. Morphometric data suggests that geographically isolated *O. engelmannii* lineages are distinct from each other, but the Eastern Cape (spines) lineage has a similar spine and cladode morphometry to *O. stricta*. Molecular data play an important role in supporting or refuting observed differences among lineages and help direct searches for biocontrol agents to the most appropriate host. Little is known with regards to the genetic characteristics of *O. engelmannii* taxa in Africa. This could limit biological control efforts because the cochineal insect agent *Dactyolopius opuntiae* is extremely host specific and only effective when matched to the correct plant species or lineage. We molecularly characterised five *O. engelmannii* lineages in Africa using Amplified Fragment Length Polymorphisms (AFLPs) and estimated genetic similarity and population genetic structure among lineages. The Limpopo and Kenyan lineages had relatively little genetic differentiation between them in terms of their genetic structure. The Eastern Cape (spines) lineage had moderate differentiation (F_{ST} 0.05−0.15) when compared to all other *O. engelmannii* lineages. Eastern Cape (spines) lineage had a genetic structure which was closer to the *O. stricta* population. The study found that the observed genetic variations and differences are not because the putative lineages are isolated geographically. These results will simplify the search for suitable biocontrol agents by directing future collections of the appropriate biotype of *D. opuntiae* to only relevant lineages of *O. engelmannii* in its home range. In addition, given the genetic similarity of the Eastern Cape (spines) lineage to *O. stricta*, we recommend the efficacy of *D. opuntiae* '*stricta*' biotype be tested on Eastern Cape (spines) lineage.

Keywords: AFLPs; biological control; cactaceae; invasive species; genetic differentiation; population genetics

3.1 INTRODUCTION

Identifying Invasive alien plants presents a challenge for invasion biologists because some species are difficult to distinguish based on morphological traits alone (Wiens and Penkrot, 2002; Mayonde *et al.*, 2016; Mayonde *et al.*, 2019). In South Africa, plants in the Cactaceae are among the top five invasive alien plants and often present a unique challenge due to the morphological variability observed among populations. *Opuntia* species, in particular, show a variety of morphology lineage divergence (Schaal *et al.*, 1998). The morphological variation within the *Opuntia* highlight that phenotypical and morphometric characteristics alone may not provide enough information for lineage classification. The extremely variable *Opuntia engelmannii* (Cactaceae: Opuntioideae) has invaded pasture lands in South Africa and displaced native plant species. Characterizing the invading populations has been a challenge, which in turn results in difficulty when identifying agents for biocontrol.

Opuntia engelmannii lineages in Africa are found to be morphologically different from each other with numerous diagnostic characters. Spine, areole, cladode and fruit characters are some diagnostic characters which are variable among lineages. There are clear groupings in the spine morphology. There is an overlap between the Kenyan and Limpopo lineages and these could potentially be the same lineage. Both the Eastern Cape lineages have a different spine morphology or lack of spines (Eastern Cape-spineless) to the other *O. engelmannii* lineages. Without the presence of spine data, *O. engelmannii* groupings are not as clear, and some overlap is seen between *O. engelmannii* lineages (Northern Cape, Eastern Cape-spines) and the *O. stricta* lineage. Spine diagnostic traits, rather than cladode, areoles and fruit diagnostic traits are important in distinguishing the studied *O. engelmannii* lineages and confirming lineage identity. While morphological identifications have been useful (Chapter 2), molecular data can test additional hypotheses regarding introductions and how dispersal among such populations may have occurred (Gaskin *et al.*, 2011). Morphological statistical methods, including principal component analysis and cluster, can be used as effective tools to assess variability (Chessa, 2010; Khoury *et al.*, 2010). Morphological trait analysis is a valuable tool as it is based on the genetic differences reflected in the individual's phenotype. However, morphology alone may not always reflect the actual genetic variation because phenotype is also dependant on some environment interactions of individuals and therefore lineages (Smith and Smith, 1992).

Due to environmental influences on the stability of morphological traits and phenotypic plasticity, which influence adaptation and ecological range of species, morphological traits alone are not always adequate for reliable differentiation between species (Photita *et al.,* 2005; Cai *et al.,* 2009; Ecker e*t al.,* 2018). As a complement to morphological data, molecular markers have been suggested in recent years as a direct tool to estimate interspecific and generic relationships (McDonald *et al.,* 2007). Morphological identification of lineages is complicated and molecular work was suggested to help identify lineages and duplicate occurrences in collections and among lineages (Wang *et al.,* 2004). Amplified Fragment Length Polymorphisms (AFLPs) are an innovative molecular fingerprinting technique that can be applied to DNA of any kind (Janssen *et al.,* 1996). Amplified Fragment Length Polymorphisms allow one to sample DNA from the entire genome. Genomic DNA is digested using two restriction enzymes, generally *EcoRI* and *MseI* (Maughan *et al.,* 1996).

Nucleotide adaptors are ligated to the DNA fragments to serve as primer binding sites for PCR amplification (Mueller and Wolfenbarger, 1999). Then, primers complementary to the adapter and restriction site sequence, with additional nucleotides at the 3'-end, are used as selective agents to amplify a subset of ligated fragments. There are different parameters that are regularly encountered in genetic variation studies with AFLPs. These parameters fall under the band-based or the allele frequency-based parameters. The band-based metrics can be directly predicted from the AFLP profiles and include various coefficients for example, the Shannon's diversity index (Wang *et al.,* 2010; Ravikiran *et al.,* 2018) and the less employed, nucleotide diversity (Borowsky 2001; Polanco and Ruiz, 2002). Some factors of relatedness developed for dominant markers can also qualify, for example, the Nei's gene diversity (Nei 1973; Nei 1978) is an example of allele frequency based parameter.

Genetic diversity, differentiation and genetic structure are important tools that are used to draw recommendations for host specificity testing of agents in invasion biology (Gaskin *et al.,* 2011). The fixaton index (F_{ST}) is a measure of population differentiation of individuals, often estimated from genetic polymorphism data such as AFLPs (Gaudeul *et al.,* 2000). The genetic variation at the level of the individual is measured in terms of heterozygosity (He) (Brown, 1978; Buza *et al.,* 2000). The number and

frequency of alleles at a locus and the amount of inbreeding all determine the level of heterozygosity within a lineage (Buza *et al.,* 2000). An F_{ST} of less than 0.05 will generally be interpreted as low and this may mean that differentiation among *O. engelmannii* lineages is weak. Multi-locus DNA markers offer genome wide data and have been reported to be successful in detecting polymorphic loci, heterozygosity and genetic diversity in lineages, therefore, they are suitable for investigating patterns of hybridization and genetic differentiation (Vos *et al.,* 1995) among *O. engelmannii* lineages.

The genetic differences observed among individuals of the same species from different lineages might be because of their geographic separation. The lineages may be different because they are found in different geographic locations. The term "isolation by distance" was first used by Sewall Wright (Wright, 1940; Bohonak, 2002) to define patterns of genetic variation and differentiation that originate from spatially limited gene flow between distantly separated lineages. The genetic similarity among population pairs is plotted as a function of the geographic distance between those population pairs (Neigel, 1997; Bohonak, 2002). This trend could potentially explain the morphological diffences observed among *O. engelmannii* lineages. Alternatively, gene flow has been reported as an important mechanism for transferring genetic diversity among morphologically similar populations or lineages (Rieseberg and Soltis, 1991; Shao *et al.,* 2018). If the rate of gene flow is high between two lineages then these lineages could be considered to have an equivalent genetic diversity (Shao *et al.,* 2018).

Genetic structuring of a population is influenced by the pattern of gene flow between and within populations. For example, in taxa such as Oaks (*Quercus spp*.) where a lack of reproductive barriers helps facilitate gene flow between populations of the same species. Intraspecific population structure in Oaks continues to be influenced by interspecific genetic exchange following the phenotypic deviation of species (Schaal *et al.,* 1998). In species such as Dandelions (*Taraxacum officinale*) each lineage within a population is genetically isolated from every other lineage possibly through apomixis. Thus, separate phylogenetic lineages are well established within populations of Dandelions (Schaal *et al.,* 1998).

The molecular analysis of *O. engelmannii* lineages will be useful in determining whether plant lineages that are closely related to the biological control agents (old associations) are more effective, or if more distantly related plant lineages to the biological control agents (new asssociations) are more effective. The management of *Opuntia* lineages is necessary, and requires morphological, physiological, biochemical, genetic, and taxonomic research to expand the information and knowledge about the species diversity (Rathore *et al.*, 2005).

AIM, OBJECTIVES AND KEY QUESTIONS

The main aim of this chapter is to investigate the level of genetic similarity or dissimilarity betweeen lineages of *O. engelmannii* and *O. stricta* that occur in South Africa and Kenya. This knowledge will help in the biological control of the invasive *O. englemannii* as the correct plant lineage (*O. engelmannii*) and insect biotype (*D. opuntiae*) have to be matched for a successful biological control programme.

The objectives of this chapter are:

- Examine how genetically different the five *O. engelmannii* lineages are to each other using Amplified Fragment Length Polymorphism data.
- To test whether there is a relationship between geographic and genetic distance for the five *O. engelmannii* under consideration.

The key questions affiliated with these objectives are:

- How genetically differentiated the *O. engelmannii* lineages are from each other?
- Is there hybridization occurring among the African *O. engelmannii* lineages examined in this study?

3.2 MATERIALS AND METHODS

i. Sample preparation

A total of 150 plants (25 for each of the six lineages) were used for molecular population genetics. Cladode cuttings were obtained from the different collection sites (Figure 2.2). The cladodes were planted and grown in pots at a plant nursery at the University of the Witwatersrand, Johannesburg, South Africa. Approximately 3 g of the cladode samples were collected and dried in silica gel and sealed in a zip lock plastic bag to help facilitate drying and preservation of the DNA, the samples were then stored at −20°C prior to molecular anaysis. The plant material was cut into smaller pieces then crushed in liquid nitrogen using a mortar and pestle for DNA extraction.

ii. DNA extraction

Genomic DNA was extracted from 150 cladode samples using Qiagen DNEasy plant kit (Southern Cross Biotechnology, Germany) (Appendix 3.1) with modifications from Fehlberg *et al.,* (2013). Lysis buffer was added to the cladode material in a 1.5 ml microtube and incubated at 65°C for 30 min prior to extraction time to soften the cladode material. 600 µl of Buffer AP1 and 6 µl of RNAse A were added to the cladode material, the lysate was vortexed and incubated at 65°C for 30 min. The tubes were inverted three times during the incubation. Buffer P3 was then added to the tubes and the lysate was incubated for 30 min on ice. The lysate was centrifuged at 14 000 rpms for five minutes. The lysate was transferred into QIAshredder spin column placed in a 2 mL collection tube, the lysate was centrifuged at 14 000 rpms for 5 minutes at 14 000 rpms. The flow-through was transferred into a new collection tube and 1.5 volumes of Buffer AW1 was added to the mixture, because of the viscous nature of *Opuntia* tissue, a spatula was used to mix the solution. 650 µl of the mixture was transferred into a new DNeasy-Mini spin column in 2 mL collection tube. The mixture was centrifuged for five minutes at 8 000 rpm and the flow-through was discarded - this step was repeated with the remaining sample. The spin column was placed in a new 2 mL collection tube, 500 µl of Buffer AW2 was added into the spin column, this was then centrifuged for 10 min at 8 000 rpm, the flow-through was discarded once again. The same amount of buffer AW2 was added and this was centrifuged at 14 000 rpms for 10 minutes. Care was taken to ensure that when removing the spin column from the collection tube the column does not come into contact with the flow-through. The spin column with the DNA was then transferred into a new 2 mL microcentrifuge tube. The DNA was eluted

with 75 µl of Buffer AE, the mixture was incubated at room temperature for 5 minutes, then centrifuged for 5 min at 8 000 rpm. The quantity of the extracted DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, USA). Successful DNA extraction comprised of at least 200ng/µl of DNA when measured on the NanoDrop measurement. Samples with less 200ng/µl of DNA were concentrated using a high capacity vacuum concentrator machine (Thermo Fisher Scientific Inc, USA).

iii. Amplified Fragment Length Polymorphism genotyping

Restriction-digestion

AFLP genotyping followed Blignaut *et al.*, (2013) with some modifications (Appendix A4). Genomic DNA was digested using Fast digest *EcoR*I and *MseI* enzymes. 025 µl of 5 units *EcoRI* and 2 µl of 1X *EcoRI* was added to 2 µl of the extracted DNA, 15.75 µl of dH_2O was added to make up a final volume of 20 µl. This mixture was incubated for 2 hours at 37°C. Following EcoRI digestion, 10 µl of MSeI restriction mix was added containing 0.5 µl 5 units of MseI, 3 µl of 1X Cutsmart buffer and 6.5 µl of dH_2O to make up a final volume of 30 µl. The reaction was incubated for 2 hours at 37°C. After incubation the enzymes were inactivated by further incubation at 65°C for 30 minutes.

Ligation

A 10 µl ligation mixture was added to the *EcoRI/ MseI* restriction mix using 0.025 µl of 1 unit T4 DNA ligase, 4 µl 1X T4 DNA ligase Buffer, 1 µl of a 50 µM *MSeI* adaptor, 1 µl of a 5 µM *EcoRI* adaptor and 3.975 µl of dH_2O. The digestion-ligation mix was incubated overnight at 4°C.

Pre-Selective amplification

Following ligation the digestion-ligation mix was diluted 1:5 with dH_2O and this was the template used for pre-selective amplification. A 15 µl pre- selective PCR reaction was made with 2.5 µl of the diluted digestion ligation reaction mix, 1.5 µl of 1 µM *MseI*+0 primer, 1.5 µM *EcoRI*+0 primer, 7.5 µl Readymix buffer and 2 µl dH_2O. The pre-selective reactions were ran on a PCR machine with 23 cycles of denaturation at 94°C for 30 seconds, 23 cycles of annealing at 56°C for 30 seconds, one cycle of extension at 72°C for 30 seconds and a final cycle of extension at 60°C for 30 minutes.

The pre-selective PCR products were held at 20°C. Five microlitres of the PCR product were run on a 1% agarose gel and the presence of a smear between 100-500 bp confirmed successful pre-selective amplification.

Selective amplification

Following pre-selective amplification, the mix was diluted 1:19 with dH_2O and this template was used for selective amplification. For each sample and each fluorescently labelled primer a 20 µl selective PCR reaction was made with 5 µl of the diluted pre-selective reaction mix was made with the following: 0.5 µl of 0.25 µM of fluorescently-labelled *EcoRI-ATG* (Green dye- HEX ®)/ *EcoRI-CAT* (Black dye-FAM®)/ *EcoRI - AAT* (Blue dye-NED®), 2 µl of 1 µM unlabelled *MseI*+CTT, 10 µl PCR mastermix and 2.5 µl dH_2O. The selective amplification reactions were run on a PCR machine with 30 cycles of denaturation at 94°C for 30 seconds, 30 cylces of annealing at 56°C for 30 seconds, one cycle of extension at 72°C for 30 seconds and a final cycle of extension at 60°C for 30 minutes. The selective PCR products were held at 20°C. Five microlitres of the PCR product was run on an agarose gel, a smear between 100-500 bp confirmed successful selective amplification.

iv. Fragment analysis

A total of 450 samples (150 individuals per primer: *ATG/MseCTT*, *E+CAT/MseCTT*, *E+AAT/MseCTT*) were sent to a Central Analytic Facilities (CAF) in Stellenbosch University for further processing. LIZ 600 was used as internal size standard for all the samples and chromatographs were sent from CAF for further interpretation and analysis. Chromatographs were scored manually in GeneMarker 2.6.7 with either "0" for absence of peaks and "1" for presence of peaks. The binary data matrix of the data was then analyzed using GENALEX 6.51b1 and STRUCTURE 2.3.4.

v. Data analysis

a) Genetic diversity of geographically separate *O. engelmannii* lineages

To test if all lineages were genetically diverse and if they shared any fragment, the scored genotypes, the number of polymorphic loci and the allele frequency across all three primer pairs were calculated. Polymorphism, F_{ST}, heterozygosity and Shannon's genetic diversity index were used to compare the genetic diversity and variability

among the *Opuntia* lineages. An AMOVA was used to test whether there is a difference in genetic variation among the lineages.

b) Genetic structure of geographically separate *Opuntia* lineages

A principal Coordinate Analysis (PCoA) was used to test the genetic variability and clustering as a means of genetic differentiation among the *Opuntia* lineages using Principal Components matrices-PC1 and PC2. Spatial autocorrelation between the genetic and geographical distance was tested. An autocorrelation plot between geographical and genetic distance was used to test for isolation by distance between elements from two matrices. *Opuntia* lineage clustering and assignment tests were performed using STRUCTURE ver. 2.3.4 (Pritchard *et al.,* 2000; Falush *et al.,* 2007). To determine the number of clusters (K) represented in the *Opuntia* samples from both South Africa and Kenya, the dominant AFLP data were diploidized (i.e. homozygous alleles doubled to be treated as homozygous diploids for each locus; Falush *et al.,* 2007). A 100 000 run burn-in (α stabilized at approximately 1 000 runs) and a 1 000 000 run length were used. The analyses also calculated 95% probability intervals around the average assignment value of each sample using the 100 000 runs performed after burn-in. The number of clusters was tested as K=1–10. Ten clusters with 10 replicates per K were tested so as to detect any cryptic population structure present in our samples. The best number of clusters (K=2) was selected using a web-based programme, STRUCTURE HARVESTER (Evanno *et al.,* 2005), which calculates the optimal value of K (ΔK) as per Pritchard *et al.,* (2000). The average posterior probability values of samples assigned to each of the clusters (across the 10 replicate runs of the selected K value of three) was calculated. An individual assigned with > 90 % average posterior probability to one of the parental clusters is considered an unmixed genotype for that species.

3.3 RESULTS

The five *Opuntia engelmannii* lineages and the *O. stricta* population were found to be genetically different and AFLPs were successful in amplifying the DNA (Figure 3.1) and detecting polymorphic loci among lineages (Table 3.1). The three primer combinations resulted in 92 unambiguous polymorphic loci among the 150 samples. The fluorescently labelled primer, *E+CAT/MseCTT* (Black dye)-FAM® was successful in detecting more than 90% of polymorphic loci across all studied *Opuntia* lineages. The Kenyan and Limpopo lineages had a similar percentage polymorphic loci in each primer combination (Table 3.2) and across all three primer combination pairs (Table 3.3). Both the Eastern Cape lineages and *O. stricta* species had a similar percentage polymorphic loci across all three primer combination pairs. The Kenyan and Limpopo lineage had no shared fragments among individuals with 100% polymorphic loci for both *E+ATG/MseCTT* and *E+CAT/MseCTT* primers.

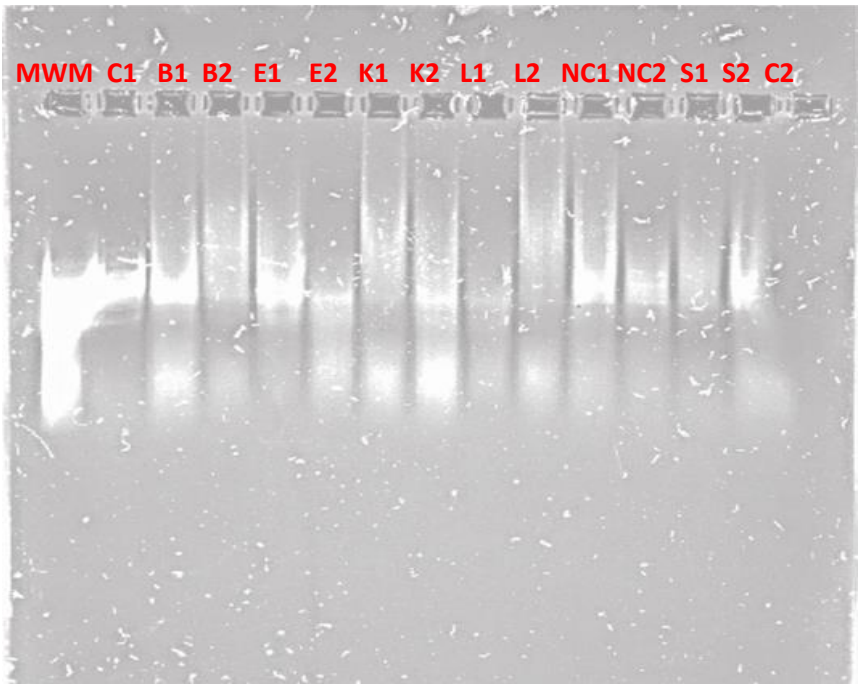

Figure 3.1 : Successful selective amplification of *Opuntia engelmannii* lineages and *Opuntia stricta* individuals with *E+ATG/MseCTT, E+CAT/MseCTT, E+AAT/MseCTT* was confirmed by running a 1% agarose gel on all the *Opuntia* samples (n=150). Gel smears were observed between 50-500bp, successful PCR smears confirms the extraction of good quality DNA. MWM-Molecular weight marker, C1- Positive control (non-amplified DNA), B-Eastern Cape (spines) lineage, E-Eastern Cape (spineless) lineage, K-Kenyan lineage, L-Limpopo lineage (n=25), N-Northern Cape lineage, S- *O. stricta* and C2-Negative Control (Master mix without DNA).

Table 3.1: Total number of polymorphic loci across three primer combination pairs.

Primer pair	Total number of polymorphic loci
***E+ATG/MseCTT* - HEX®**	22
***E+CAT/MseCTT* -FAM®**	40
***E+AAT/MseCTT* -NED®**	30

Table 3.2: The percentage of polymorphic loci in each primer combination per *Opuntia engelmannii* lineage and *Opuntia stricta*.

	Polymorphic loci (%)		
Lineage	***E+ATG/MseCTT***	***E+CAT/MseCTT***	***E+AAT/MseCTT***
EasternCape-spines	90	100	38
EasternCape-spineless	82	97	35
Kenya	100	100	62
Limpopo	100	90	60
Northern Cape	95	93	95
O. stricta	91	100	70

Genetic diversity estimates indicated differences among the *Opuntia* lineages. The Northern Cape lineage had a greater percentage of polymorphic loci across all three primer combination pairs, a higher number of expected and observed heterozygosity and a significantly greater genetic diversity in its alleles. Both the Kenyan and Limpopo lineages had significant differences in heterozygosity between each other but both had a similar genetic diversity index. The Eastern Cape (spineless) lineage had the lowest genetic diversity (Table 3.3).

Table 3.3: Genetic diversity estimates for *Opuntia engelmannii* lineages and *Opuntia stricta*. Different letters indicate significant differences among the lineages in each column.

Lineage	Polymorphic loci %	Mean He	Mean uHe	Mean Shannon's diversity Index
Eastern Cape (spines)	68.48[a]	0.228[a]	0.233[a]	0.430[a]
Eastern Cape (spineless)	66.30[a]	0.134[b]	0.136[b]	0.224[b]
Kenya	84.78[b]	0.202[a]	0.206[a]	0.33[a]
Limpopo	83.70[b]	0.193[b]	0.197[b]	0.316[a]
Northern Cape	98.91[c]	0.327[c]	0.334[c]	0.508[c]
O. stricta	77.17[ab]	0.258[a]	0.264[a]	0.388[a]

The PCoA followed the same trend as the spine morphology and the combined NMDS with all morphology data (Chapter 2): The Kenyan and Limpopo lineages were genetically similar, the Eastern Cape (spines) lineage did not cluster with any of the other *O. engelmannii* lineages (Figure 3.2). *Opuntia stricta* clusters with some individuals of the Northern Cape and Eastern Cape (spineless) lineages, but doesn't cluster with any individuals of the Kenyan, Limpopo and Eastern Cape (spineless) lineages. The Eastern Cape (spineless) somewhat overlaps with the Kenyan lineage. Eastern Cape (spines) showed more genetic variability and differentiation compared to the other *O. engelmannii* lineages (Figure 3.2). The Northern Cape lineage had some overlap with both the Kenyan and Limpopo lineages and also had some individuals overlapping with the *O. stricta* lineage (Figure 3.2). The data indicated differences in molecular variance among the *Opuntia* lineages (Table 3.5).

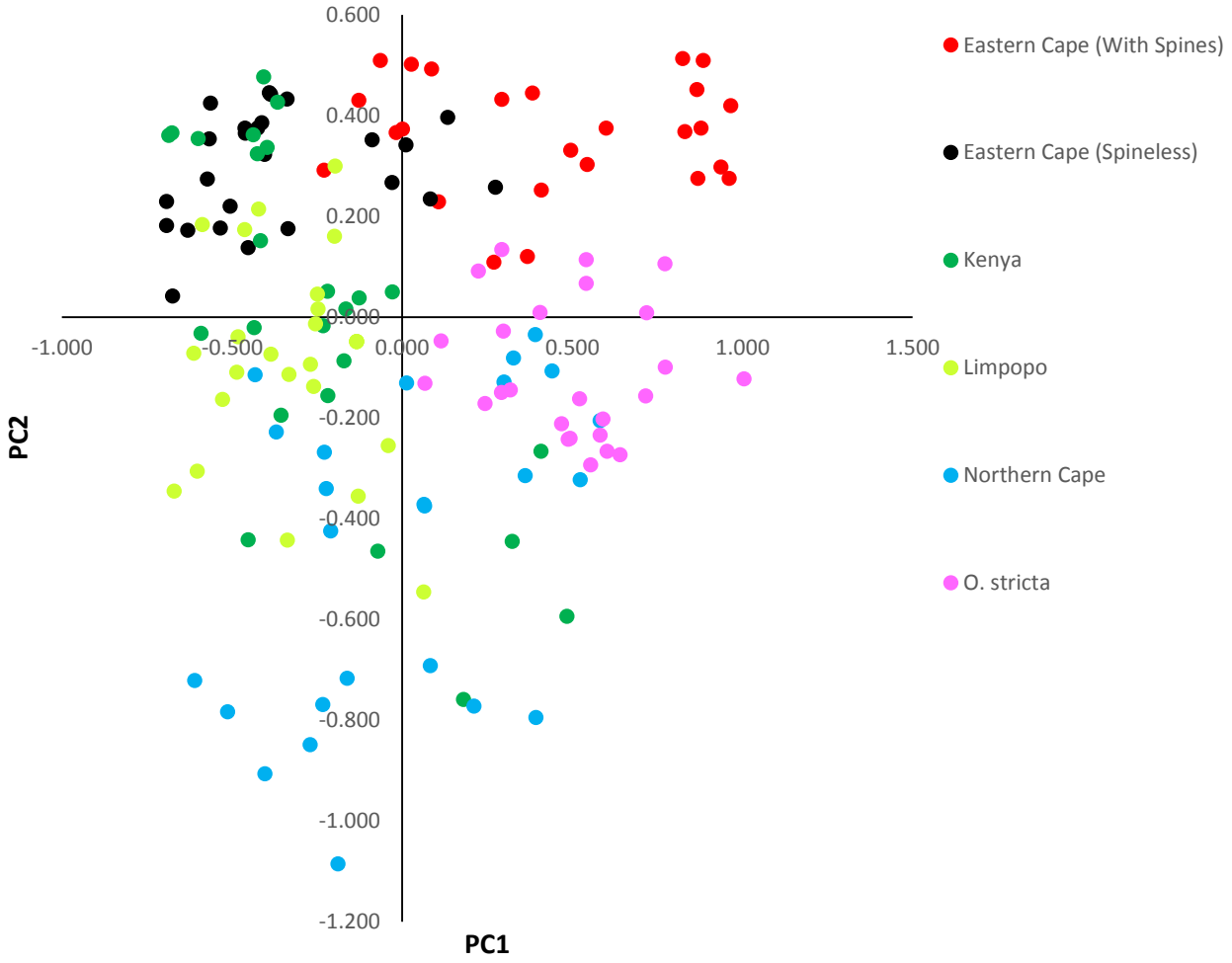

Figure 3.2: A Principal Component Analysis (PCoA) of 92 variable Amplified Fragment Length Polymorphism Fragments that show the genetic variability among focal *Opuntia engelmannii* lineages (n=125) and *O. stricta* (n=25). The Eastern Cape (spines) lineage does not cluster with any of the *Opuntia engelmannii* lineages. The Northern Cape lineage also clusters separately with some individuals overlapping with *Opuntia stricta.*

Table 3.4: Pairwise F_{ST} values among *Opuntia engelmannii* lineages and *Opuntia stricta* population based on Nei's genetic distance after genotyping with AFLP markers, where 0=No genetic difference between lineages and 0.15-0.25= Great genetic difference between lineages.

	Eastern Cape-spines	**Eastern Cape-spineless**	**Kenya**	**Limpopo**	**Northern Cape**	***O. stricta***
Eastern Cape-Spines	—					
Eastern Cape-Spineless	0.075	—				
Kenya	0.078	0.014	—			
Limpopo	0.095	0.015	0.015	—		
Northern Cape	0.097	0.043	0.029	0.025	—	
O. stricta	0.05	0.156	0.086	0.083	0.073	—

The 'isolation by distance' hypothesis was tested using GenAlex 6.51b1. It is expected that lineages that have a smaller geographic distance between them would also have a lower genetic distance. The data from the *O. engelmannii* lineages do not support this hypothesis as there is a smaller geographic distance between the two Eastern Cape lineages (<50 km) but there is a great genetic distance between the two lineages relative to the genetic distances among other lineages (F_{ST}=0.075) (Figure 3.3). There was possibly moderate gene flow between the Northern Cape and Limpopo lineages.

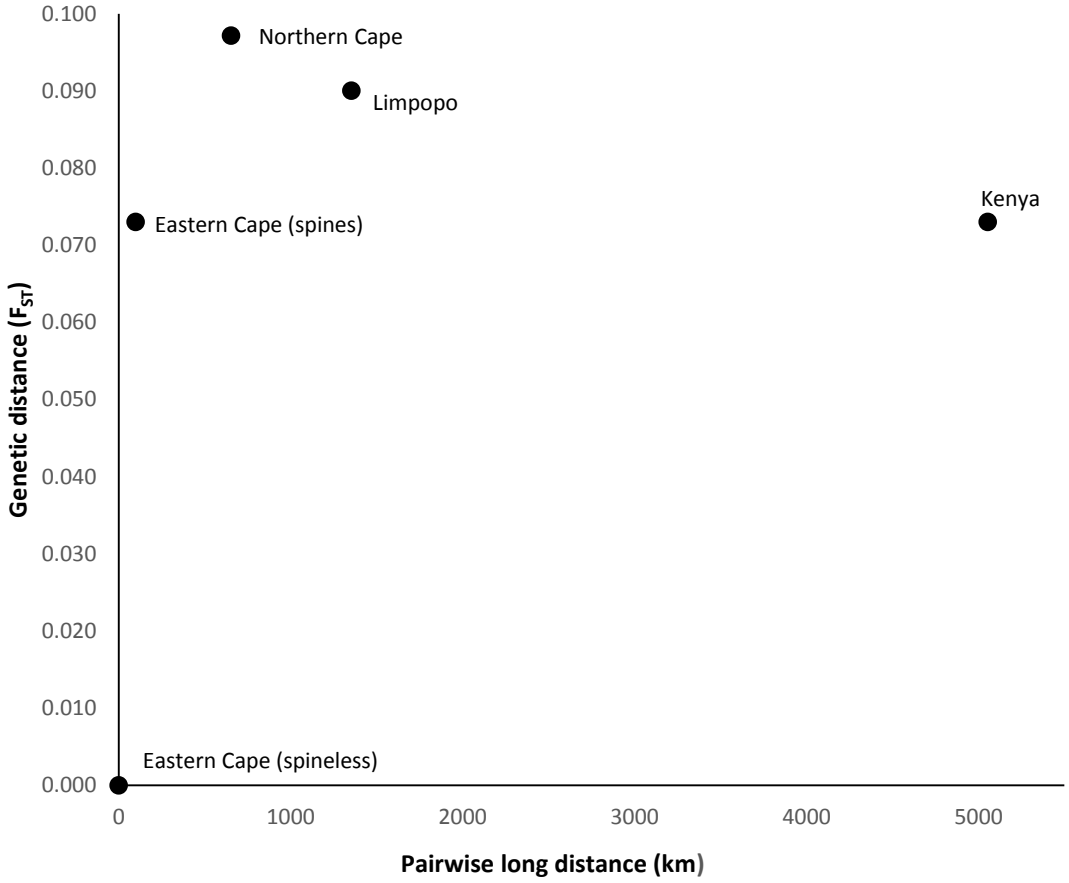

Figure 3.3: The geographical location and pairwise genetic distance among *Opuntia engelmannii* lineages showing no relationship between geographic distance and genetic distance as only 6% of the genetic variation (F_{ST}) among *O. engelmannii* lineages can be explained by the pair wise geographic distance (km).

The ΔK method of Evanno *et al.*, (2005) resulted in an optimal K = 2 for two distinct clusters (*Opuntia engelmannii* and *O. stricta* lineages) as indicated by the average mean posterior probabilities (Figure 3.4). Each specimen was assigned to one of the two species, either *O. engelmannii* or *O. stricta* using STRUCTURE (Figure 3.5).

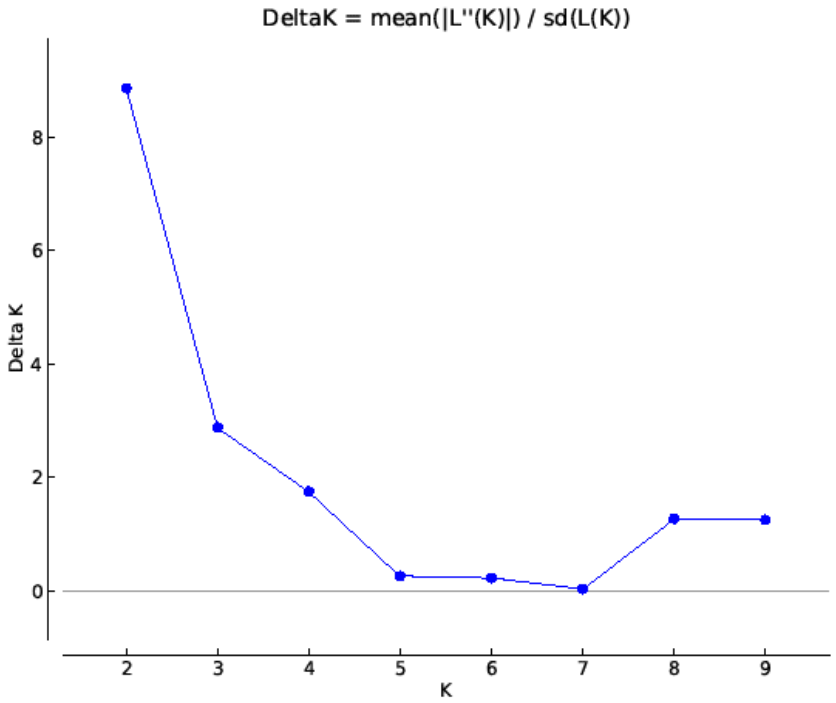

Figure 3.4: Graph of delta K used to estimate the maximum number of clusters of sampled *Opuntia engelmannii* lineages and *Opuntia stricta* population using Evanno *et al.*, (2005) method. One to 10 clusters with 10 replicates each were used to estimate delta K. The most probable number of clusters is suggested to be two clusters.

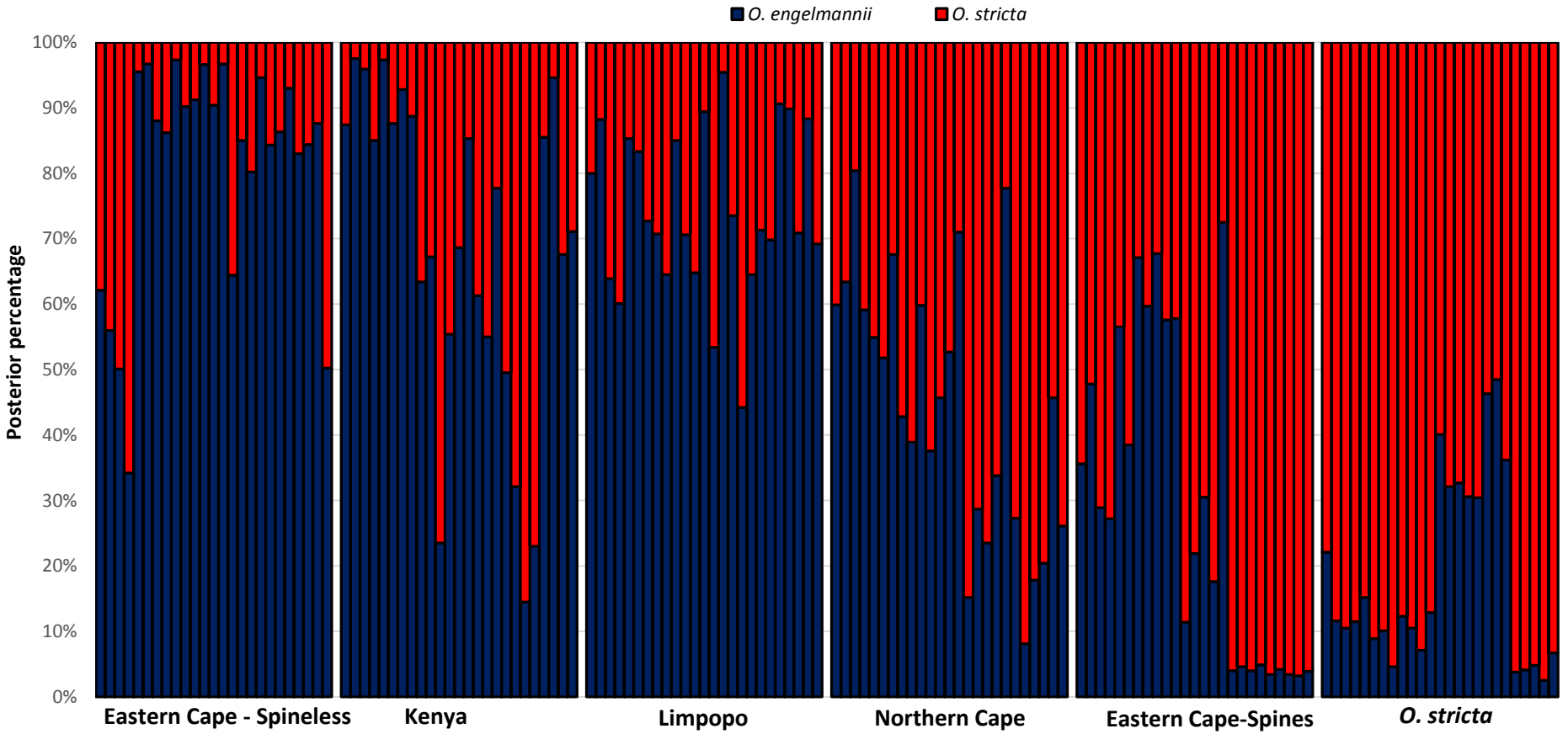

Figure 3.5: Cluster analysis of 150 *Opuntia* individuals that comprise six different morphological and geographic lineages. Each vertical bar represents the genetic clusters assigned to one individual. The individuals are grouped per *Opuntia* lineage based on their geographical identities. The percentages on the x-axis show the percent affiliation with the two proposed genetic clusters. Each cluster tentatively represents *Opuntia engelmannii* and *Opuntia stricta.*

3.4 DISCUSSION

The molecular variations and differentiation between lineages of *O. engelmannii* and individuals of *O. stricta* that occur in South Africa and Kenya will have implications for the way agents are selected for the biological control of *O. engelmannii*. The molecular data PCoA showed a similar trend to the morphology data. The Kenyan and Limpopo lineages were found to be similar in their genetic diversity estimates as well in their genetic similarity. Eastern Cape (spines) lineage did not cluster with any of the other *O. engelmannii* lineages, but some individuals overlapped with *O. stricta*. Eastern Cape (spines) lineage and *O. stricta* were more genetically differentiated compared to the other *O. engelmannii* lineages.

The Kenyan and Limpopo lineages had a lower genetic differentiation between them relative to the other *O. engelmannii* lineages. Additionally, Eastern Cape (spineless) overlaps with some individuals from the Kenyan lineage and therefore, has some similarity with the Kenyan lineage. The Northern Cape lineage overlapped with the Limpopo, Kenyan and *O. stricta* lineages and has both clusters representing *Opuntia engelmannii* and *O. stricta* on an equal ratio, which suggests that it might be a hybrid of the two species. Hybridization involving two or more ancestral species has long been documented as an essential mechanism of speciation in plants (Ellstrand and Hoffman, 1990; Schaal, 1998). Hybridization between lineages was also observed by Mayonde *et al.*, (2015) and a PCoA was used to reveal genetic differences between the *Tamarix* parental species (alien and indigenous), and South African hybrids were revealed and placed in a continuum between alien and indigenous *Tamarix* species.

The polymorphic loci of *O. engelmannii* lineages *(P* =79%) were relatively high, similar to that of other introduced IAPs i.e. *Pueraria lobata* (*P* =75%) and *Flaveria bidentis (P* = 79%) (Pappert *et al.*, 2000). Both the Eastern Cape lineages had a relatively low percentage polymorphic loci (*P*=68%) when compared to the other *O. engelmannii* lineages (*P*= 89%), further supporting PCoA evidence that both the Eastern Cape lineages could possible be a separate taxa to each other. The heterozygosity of *O. engelmannii* (H_e =0.22) was similar to another IAP with a related introduction history (introduced as an ornamental) e.g *Pueraria lobata* (H_e = 0.28) and the mean genetic variation within lineages of *O. engelmannii* (H_e = 0.36) was also

similar to *P. lobata* (H_e = 0.26) (Schierenbeck *et al.,* 1995). This could mean multiple introductions have occurred in both species. This further substantiates work done by Helsen *et al.,* (2007) where *Opuntia* species were reported to have a high level of genetic polymorphism and heterozygosity with a number of effective alleles ranging from six to 53. High genetic diversity in IAPs is usually caused by multiple introductions (Mayonde *et al.,* 2019; Ellstrand and Schierenbeck, 2000). This high genetic polymorphism and hetorozygosity is also indicative of high levels of genetic diversity within the *Opuntia* genus.

The overall mean heterozygosity indicated a moderate genetic diversity among the lineages (H_e = 0.22, range 0.13 – 0.34 SE = 0.01). This observed value was different to another South American IAP, *Campuloclinium macrocephalum* (H_e= 0.18, and range 0.10 — 0.25), which was introduced fairly recently and has no agricultural importance (Gitonga *et al.,* 2015); this low heterozygosity was indicative of a low genetic variability in this species contrary to *O. engelmannii.* This comparison further supports that *O. engelmannii* is a genetically variable IAP and this variability is possibly through repeated human intervention possibly for agricultural reasons (Henderson, 2012). There was high, but significant, molecular variance between *O. engelmannii* lineages (PhiPT = 0.215; P < 0.001). Based on Wrights' qualitative indices for F_{ST} (Nei, 1986), these results indicate 'moderate' genetic differentiation among the *O. engelmannii* lineages suggesting that they are possibly different taxa. The likelihood of gene flow between the Kenyan and Limpopo lineages is high and these could effectively be a single population more likely introduced from South Africa to Kenya by the Succulent society for horticulture and agricultural reasons. These two lineages are likely to be recently separated. The introduction of new alleles through gene flow increases genetic differentiation between populations and makes new combinations of traits possible (Halewood *et al.,* 2018), for example, the differences in spine colour between the Kenyan lineage (yellow spines with brown-base) and the Limpopo lineage (uniform yellow glossy) spines.

Pairwise F_{ST} values calculated for all lineages found slight genetic differentiation between three *O. engelmannii* lineages, *viz.* Kenya, Limpopo, and Eastern Cape (spineless) (F_{ST} < 0.05) and these could possibly be the same taxon even though the Eastern Cape (spineless) is morphologically different to both Kenya and Limpopo

(Chapter 2). A moderate genetic variation was found between *O. stricta* and Eastern Cape (spines) lineages, and a greater genetic variation between *O. stricta* and Eastern Cape- spineless (F_{ST} 0.05 — 0.15). There was slight genetic difference observed in all the *Opuntia* lineages sampled (F_{ST} > 0.25). The grouping of individuals from the different *O. engelmannii* lineages was not exclusive to one species, but contained individuals from other *Opuntia* species, i.e *O. stricta*. For example, individuals from *O. engelmannii* lineages were found grouping with some *O. stricta* individuals. Based on the Bayesian STRUCTURE assignment analysis, several individuals could have migrated from province to province in South Africa. Roadside cutters and contamination of vehicles used for mechanical control (e.g Henderson *et al.*, 2007) may account for long-distance dispersal i.e. from Eastern Cape to Northern Cape. Furthermore, Birds such Hornbills (*Buceros bicornis*) and Common ravens (*Corvus corax*) have been reported to eat and also disperse *Opuntia* fruits (González-Espinosa and Quintana-Ascencio, 1986; Dean and Milton, 2000; Padrón *et al.,* 2011). These birds could also account for long-distance dispersal of *O. engelmannii* from province to province as both species are powerful fliers that have been reported to disperse seeds of *Opuntia* far from the mother plants (Dean and Milton, 2000).

Isolation by distance was tested to possibly explain the genetic differentiation of *O. engelmannii* lineages. The results show that there is a weak relationship between genetic distance and the geographic location of the *Opuntia* lineages. Spatial autocorrelation indicated a weak positive correlation (r = 0.07, P = 0.089; 999 permutations) correlation between genetic and geographical distances. Presumably, the migration of *O. engelmannii* individuals from province to province could has also led to the lack of a weak phylogeographic signal and non-significant isolation by distance (R^2 = 0.0677, P = 0.075). Specifically, we would expect the two Eastern Cape lineages to have a lower genetic distance and similar genotypes as they have a shorter geographic distance between them. However, the results from this study show that there is a moderate genetic difference between the two lineages. Additionally, we would expect the Kenyan lineage to have a greater genetic distance relative to all the other lineages as per the geographic location, but the Kenyan lineage is more genetically similar to the Limpopo lineage, an alternative hypothesis could be that humans introduced the Kenyan lineage to Limpopo and then it underwent local adapation. In a similar study on *C. macrocephalum* and *Flaveria bidentis*, both American weeds, the authors found

that long-distance seed dispersal, *via* national roads, contributed to a weak geographical genetic structure and rapid range expansion in Northern China (Ma *et al.*, 2011). This trend may explain the genetic patterns of the various *Opuntia* lineages. Heterozygosity is an important measurement of gene diversity (Wang *et al.*, 2004). There was a weak positive relationship ($R^2 = 0.2614$) between the heterozygosity and genetic distance among the lineages because the level of differentiation and genetic distance between groups is significantly influenced by the level of heterozygosity for highly variable species like *O. engelmannii*.

The ΔK method of Evanno *et al.*, (2005) resulted in an optimal K = 2 for two distinct clusters (*O. engelmannii* and *O. stricta*) as indicated by the average mean posterior probabilities with an indication of sub-structuring. STRUCTURE results revealed that all lineages showed genetic admixture between two genetic clusters of *O. engelmannii* and *O. stricta*. Additionally, the different proportions of admixture in individuals found in STRUCTURE analyses could indicate that individuals varying in their levels of genetic admixture were introduced into South Africa multiple times. There were eight individuals of the *O. stricta* species that showed evidence of admixture with *O. engelmannii* and these have possibly continued to spread rapidly through seeds that are possibly apomictic, a trend common in *Opuntia* species i.e *O. stricta* and *O. ficus indica* (Reyes-Agüero and Valiente-Banuet, 2006).

A high heterozygosity and consequently, a high genetic diversity was observed with regards to the Northern Cape lineage, futher indicating that it might be a possible hybrid as hybridization affects the genetic diversity of a population by increasing heterozygosity (Abbott *et al.*, 2013). Interestingly, in the present study a very strong positive correlation between heterozygosity and the genetic diversity index was observed among all the lineages ($R^2 = 0.9193$), which proves that genetic diversity has a positive correlation with heterozygosity (Gregorius, 1978; Ellstrand and Elam, 1993) even among *Opuntia* species. Hybridization has long been thought to contribute to biodiversity, often leading to the formation of new taxa (Smith and Daly, 1959; Stebbins 1959; Hovick *et al.*, 2014; Mayonde *et al.*, 2015). Rieseberg *et al.*, (1993), showed that hybrids are a mixture of both parental and intermediate morphological characters rather than just intermediate characters and that a large proportion of first (64%) and later generation hybrids (89%) exhibit extreme or novel characters.

Alternatively, cladistic identification of hybrid taxa is difficult and may not be possible based on only morphological data only (Rieseberg *et al.,* 1993). Therefore, molecular data, are thought to be more informative than morphological data for cladistic identification and hybridization studies (Nason *et al*., 1992; Rieseberg *et al.,* 1993; Rieseberg and Wendel 1993; Arnold, 1997). A study on the invasive tree *Salix*, showed that inherent patterns in character variations have biological explanations for the invasive *S. eriocephala* and *S. sericea.* Unique compilations of co-adapted gene complexes and mixing of these complexes *via* hybridization produces individuals demonstrating a range of recombinant traits (Buerkle *et al.,* 2003). This could potentially be true for the Northern Cape lineage where the individuals are admixed with *O. engelmannii* and *O. stricta* genes. The multi-coloured spines and a significantly higher number of glochids per areole (both morphological traits of the Northern Cape lineage) are an example of these recombinant traits.

3.5 CONCLUSION

AFLP data showed that Eastern Cape (spines) and Northern Cape lineages are genetically differentiated from all the other studies of *O. engelmannii* lineages. The Eastern Cape (spines) lineage is in fact more genetically similar to *O. stricta* than the other *O. engelmannii* lineages and could potentially be a new agricultural variety of *O. engelmannii*. The Northern Cape lineage is different to the other *O. engelmannii* lineages but is also differentiated to *O. stricta*. The Limpopo and Kenyan lineages not only show an outstanding morphological resemblance but they are also genetically similar with a great deal of overlap suggesting that these could potentially be a same taxon which is more likely to be recently separated by humans. The low genetic differentiation between the lineages relative to the other *O. engelmannii* lineages suggests that the Limpopo lineage is a descendant from the Kenyan lineage or vice versa as South Africa and Kenya were both colonies of the British. The British cactus and succulent society could perhaps explain the introduction and the migration of *O. engelmannii* in both countries (Smith *et al.,* 2008). Amplified Fragment Length Polymorphism markers are an ideal tool for definition *Opuntia* interspecific relationships, however, AFLP data alone may not provide enough information for species classification and establishing sister group relationships among different species. Better resolution among the closely related lineages may be found by using

Restriction site Associated DNA Sequencing (RAD-Seq). Such data will help deduce on the phylogenetic relationship between the studied *O. engelmannii* lineages.

CHAPTER 4: CRYPTIC CACTI: COMBINING MOLECULAR AND MORPHOLOGICAL DATA TO SOLVE A BIOCONTROL PROBLEM.

Research output from this chapter

Conference proceedings.

Poster presentation

Marcus J Byrne., Siphosenkosi Mbonani., Zanele Machimane., Iain Paterson., Arne Witt and Nic Venter (2018) – Cochineal and Cactus: Are new associations' biocontrol winners? XV International Symposium on Biological Control of Weeds. Switzerland. 26-31st August 2018.

This chapter highlights some of the most important results obtained from this study and their implications for the biological control of *O. engelmannii* in South Africa and Kenya. The specific objectives are outlined below with the major findings and a brief discussion.

1. **To examine the extent of morphological differences among five *O. engelmannii* lineages in Africa.**

   A multivariate approach was used to address this objective based on 125 *O. engelmannii* individuals belonging to the five lineages and 25 individuals belonging to the sister taxon, *O. stricta*. The data set consisted of 17 qualitative and quantitative morphological characters. The morphological characteristics of five *O. engelmannii* lineages suggests that the Northern Cape, the Eastern Cape (spines) and Eastern Cape (spineless) lineages are distinct to the other *O. engelmannii* lineages *viz* the Kenyan, Limpopo lineages. Qualitative and quantitative cladode characters show that the Kenyan and Limpopo lineages are similar to each other. The sister taxon, *O. stricta* and the Eastern Cape (spines) both have a different cladode and fruit morphology to the other *O. engelmannii* lineages studied. The Northern Cape lineage is different in its morphology to all the *O. engelmannii* lineages but is also different to the sister taxon, *O. stricta*.

2. **To investigate whether there are key morphological 'diagnostic traits' that can be used to predict lineage identity amongst five African lineages of *O. engelmannii*.**

   Clear groupings among lineages were observed with spine characters. Therefore, spine morphology seem to be the best in predicting *O. engelmannii* lineage identity and could potentially be used as the best *O. engelmannii* diagnostic character, better than cladodes, areoles or fruits. There were clear groupings in the spine morphology PCA and the NMDS when using spine data. There was an overlap between the Kenyan and Limpopo lineages and these could potentially be the same lineage. Both Eastern Cape lineages have a different spine makeup or lack thereof (Eastern Cape-spineless) to the other *O. engelmannii* lineages. Without the presence of spine data there was an overlap between *O. engelmannii* lineages (Northern Cape, Eastern Cape-spines) and *O. stricta*. Spine diversity within the Cactaceae family is remarkable and spine morphological and anatomical traits are therefore useful tools for taxonomists' interested in the *Opuntia* genus (Gebauer, 2016). Data from this study suggests that spine characters such as

colour, length and angle are best for distinguishing between lineages of *O. engelmannii* in the field.

3. **To identify morphological differences between *O. engelmannii* and *O. stricta.***

   Opuntia engelmannii encompasses at least three morphologically distinct lineages when compared to *O. stricta.* *Opuntia stricta* had clear groupings in spines, cladode and fruit diagnostic characters, while *O. engelmannii* had some overlaps amongst lineages i.e Kenyan and Limpopo lineages. The fruit data PCA shows just how variable *O. engelmannii* lineages are, and that *O. stricta* has a different fruit morphology to *O. engelmannii* lineages. There was similarity between *O. engelmannii*-Eastern Cape (spines) lineage and *O. stricta* in terms of cladode diagnostic characters. These results are in line with some of the earlier observations made by Weniger, 1978, which specify that *O. stricta* is morphogically similar to two lineages of *O. engelmannii* in the USA namely; *O. engelmannii* var. *lindheimeri*, and *O. engelmannii* var. *linguiformis*, which are morphologically similar to the Eastern Cape (spines) lineage. Findings from our present study showed that fruit morphology *viz* fruit shape, colour and presence of glochids on the fruits of *O. stricta* are some of the diagnostic traits that are different to *O. engelmannii.*

4. **Examine how genetically similar the *O. engelmannii* lineages are to each other using Amplified Fragment Length Polymorphism data.**

   Population genetics of *O. engelmannii* comprising of 125 individuals of the Eastern Cape (spines), Eastern Cape (spineless), Kenya, Limpopo, Northern Cape lineages and 25 individuals of the closely related species, *O. stricta* were studied using AFLP. Amplified Fragment Length Polymorphism analysis indicated that Eastern Cape (spines) is genetically different to all the other studied *O. engelmannii* lineages and its genetic make-up is in fact more similar to that of the *O. stricta* lineage. Molecular data also suggests that the Northern Cape lineage is different to the other *O. engelmannii* lineages and is also differentiated to the *O. stricta* lineage. Furthermore, Bayesian clustering data analyses resulted in two distinct genetic clusters. The Northern Cape lineage has its genetic make-up on 50:50 ratio between *O. engelmannii* and *O. stricta*, indicating that it might be a possible hybrid between the two. There was genetic variation between the *Opuntia* lineages suggesting successful sexual reproduction, recent common introduction, multiple introductions and/or allowing gene flow within

and between lineages more specifically between the Kenyan and Limpopo lineages. An objective in each genetic study is to understand individual genotypes against its lineage and possibly clarify individual genotypes into different groups using Bayesian clustering for example (Kumbhar, 2015). *Opuntia engelmannii* lineages used in this study showed a significant amount of genetic diversity relative to *O. stricta*. Classification of genotypes at DNA level with molecular markers is a powerful tool for assessing of genetic divergence (Hashimoto *et al.*, 2004). The Eastern Cape (spines) lineage in particular shows significant morphological and genetic differences to the other *O. engelmannii* lineages. This genetic diversity must be further investigated.

5. **To test whether there is a relationship between geographic and genetic distance of focal *O. engelmannii* lineages.**

   There was a weak positive relationship between geographic and genetic distance among the lineages. Six percent of the genetic variation observed was explained by the geographic locations of the lineages. The genetic difference observed between individuals of the same species but different lineages is therefore not because of the geographic distance between them. The introduction of new alleles through gene flow could explain the genetic similarity between lineages (i.e Kenyan and Limpopo lineages). The isolation by distance hypothesis was therefore rejected to explain the differences in genetic structure among *O. engelmannii* lineages.

6. **To provide a recommendation on the use of morphometric and molecular data for effective biocontrol of the invasive *O. engelmannii* and other weeds**.

   Morphometric findings from this study could be used in identifying different *O. engelmannii* lineages in the field, using specific diagnostic chararcters (i.e spine characters). Upon successful morphological identification, the correct biocontrol agent can be collected off the plant. Both morphology and molecular data suggests that there is there is little difference between between the Kenyan and the Limpopo lineages. Molecular data shows that the Northern Cape lineage is different to all the lineages of *O. engelmannii* examined and also different to *O. stricta*. *Opuntia* are notoriously amongst the most difficult plant taxa to control, either mechanically or chemically, and therefore, the efforts to employ biological control is essential (Julien and Griffiths, 1998). *Dactylopius opuntiae* has been used in South Africa to control invasive cactus for over 80 years and has been considered a success. Furthermore, *O. stricta* has been

substantially controlled in South Africa using *D. opuntiae* 'stricta biotype' and previous studies have reported that the 'stricta' biotype has a potential to damage other *Opuntia* species (Volchansky *et al.*, 1999; Hoffmann *et al.*, 2002). The insect has not been successful in controlling the Kenyan and Limpopo lineage of *O. engelmannii* presumably because there is a great genetic difference between *O. stricta* and the Kenyan and Limpopo lineages of *O. engelmannii*. Nevertheless, the Eastern Cape (spines) lineage has little to moderate genetic similarity to *O. stricta*. Given the genetic similarity between the two, the efficacy of *D. opuntiae* 'stricta' biotype can be tested on Eastern Cape (spines) lineage.

A recent PhD study presented the 'stricta' biotype of *D. opuntiae* as having shown varying degrees of acceptance to the different *Opuntia* host taxa (Musengi, 2018). *Dactylopius opuntiae* were least accepting of *O. engelmannii* lineages from both Limpopo and Kenya but were more accepting of *Opuntia stricta* and *O. humifusa* which supported the development from neonate to a reproducing adult of the 'stricta' biotype. The results from our present study show there was a great genetic difference between *O. stricta* and *O. engelmannii* lineages from both Limpopo and Kenya. These great genetic differences found in the study help to explain why Musengi *et al.*, (2018) found *D. opuntiae* 'stricta' biotype have a low reproductive output on the Kenyan lineage. Together with this dissertation, the work gives prospects and insights for the biological control of the invasive *O. engelmannii* in South Africa and Kenya.

In addition, a recent survey covered a broad range of *O. engelmannii* distribution of the species from the west coast of the United States of America in California to the Gulf of Mexico in Texas (Figure 4.1). Samples of *O. engelmannii* and cochineal insects were collected for molecular analysis. Ten lineages of cochineal were imported into quarantine at Wits University and are currently being tested to determine which is the most effective agent for each of the five lineages of *O. engelmannii* that are problematic in Africa. Four sites in the USA map (site 5, 13, 20, and 21) (Figure 4.1) have *O. engelmannii* with a spine morphology that resembles that of the Kenyan lineage. *Dactylopius opuntiae* collected from these four sites have successfully established and have a high reproductive output on the Kenyan lineage. This candidate biological control agent should therefore be further investigated to test if they can be effective on both the Kenyan and Limpopo lineages.

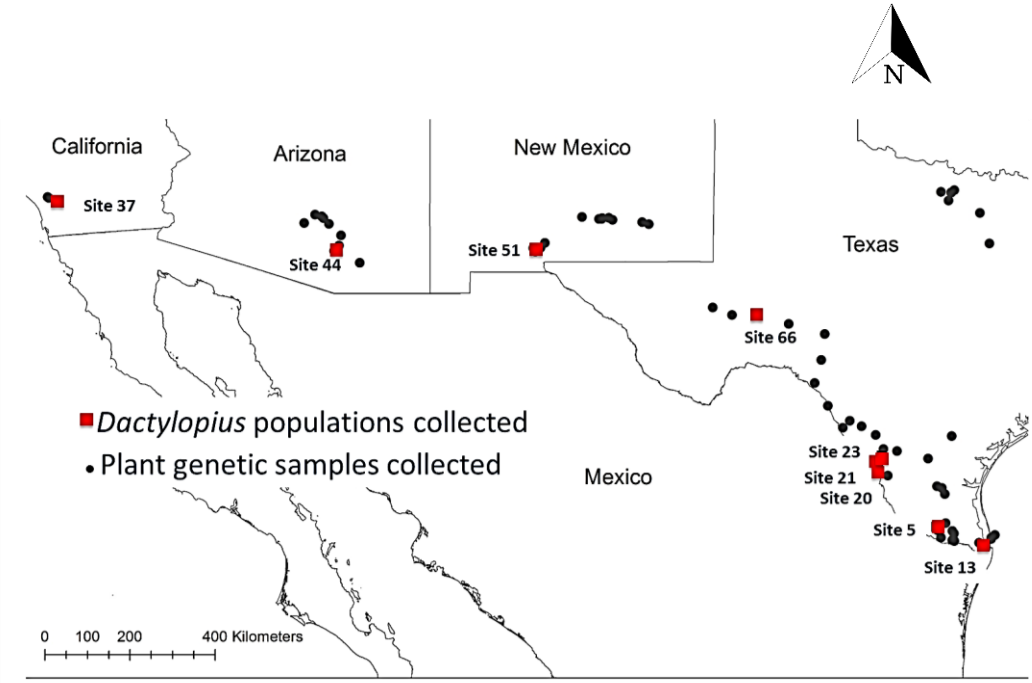

Figure 4.1: The collection sites of the *Opuntia engelmannii* lineages and their agents in the USA. The black dots indicate sites where *Opuntia engelmannii* plant material where collected. The red squares indicate sites where *Dactylopius opuntiae* biotypes where collected late 2017 (Source: Nic Venter).

CONCLUSION

A collective morphological and population genetic study of *O. engelmannii* was undertaken to quantify morphological and genetic similarities among putative lineages of *O. engelmannii* found in South Africa and Kenya. Results from this study will simplify the search for suitable biocontrol agents by directing future collections of the appropriate biotype of *D. opuntiae* to only relevant lineages of *O. engelmannii* in its home range. Collectively, the data show that there are possibly three different taxonomic varieties of *O. engelmannii* in Africa. Specifically, spine characters were the most discriminating relative to cladode, areole and fruit characters. For instance, the three morphological varieties likely comprise of plants from the: (1) Eastern Cape (spines); (2) Eastern Cape (spineless), Kenya and Limpopo; and (3) the Northern Cape lineages. Furthermore, there was observable genetic differentiation associated with this high morphological divergence. There was a genetic overlap between the Kenyan and the Limpopo lineage and these could possibly be the same evolutionary lineage. The Eastern Cape (spines) and the Northern Cape lineages were found to be genetically different to the other *O. engelmannii* lineages *viz* Kenya, Limpopo and Eastern Cape (spineless). Interestingly, there was genetic overlap between the Kenyan, Limpopo and

Eastern Cape (spineless). Domestication may be why the Eastern Cape (spineless) differs morphologically to both the Kenyan and Limpopo lineages. Genetic data suggest that the Northern Cape lineage might be a hybrid between *O. engelmannii* and *O. stricta*. Genetic differentiation between these African *O. engelmannii* lineages is not because of their separate geographic locations but possibly because of lack of gene flow or long-distance dispersal and or the lineages having different source populations. These results suggest that both morphological and molecular data should be used to inform biocontrol decisions surrounding the choice of agents for *O. engelmannii* in South Africa and Kenya.

A firm understanding of lineage diversity can be useful for delineating new or old associations between plant lineages (e.g., invasive *O. engelmannii*) and insect biotypes (e.g., *Dactylopius* agents) can lead to more effective and improved surveys for new agents of other cactus weeds by targeting the most effective agent biotypes. Cochineal insect biotypes (from four sites across the southwestern U.S [5, 13, 21 & 20]) were collected off either *O. engelmannii* var. *texana* or the very similar *O. engelmannii* var. *alta*, in Texas, United States. Preliminary host specificity results suggest that old associations are favourable for biological control because both of these U.S. *O. engelmannii* varieties are morphologically similar to the Kenyan lineage in terms of spine length, angle, colour and cladode form and shape. These cochineal insects collected from either *O. engelmannii* var. *texana* or *O. engelmannii* var. *alta* have a high reproductive output on the Kenyan lineage and little to no development on the Eastern Cape (spines) lineage. Further, genetic evidence from the present study suggest that this lineages is possibly a different variety from other *O. engelmannii* lineages in South Africa. The other *O. engelmannii* lineages that cochineal was collected from in the U.S. were morphologically distinct from the Kenyan lineage and therefore there was little to no cochineal development recorded. Since there is a little genetic differentiation between the Kenyan and the Limpopo lineage, the same insect biotype could potentially be used to successfully control both the Kenyan and Limpopo lineages.

The study contributes (i) to the research gaps that have been identified in *O. engelmanni* lineage identity as no work has been done on morphological traits that can be used to distinguish *O. engelmannii* lineages and the molecular population genetics of *O.*

engelmannii lineages in South Africa and Kenya, (ii) provides baseline molecular data for observed morphological differences and variation among the African *O. engelmannii* lineages and (iii) contribute to future management plans for the control of a category 1b invader of the NEM:BA act (10/2004) in Africa and more importantly to priority areas for cactus management highlighted in Figure 1.5B.

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APPENDICES

Appendix I: DNA extractions using Qiagen protocol with some modifications to accommodate the viscous and succulent nature for *Opuntia engelmannii*. Extraction of genomic DNA from Cactaceae is often contaminated with pectin and mucilage**.**

1. Crush the cladodes with liquid nitrogen using a mortar and pestle. Weigh 0.3 g and transfer the on an Eppendorf tube.
2. Add 600 µL Buffer AP1 and 6 µL RNAse A. Vortex and incubate the mixture for 30 min at 65˚C. Invert the tube 2-3 times during the incubation.
3. Add 130 µL Buffer P3. Mix and incubate for 5 min on ice.
4. Centrifuge the lysate for 30 min at 20 000 x g (Note: this step might have to be repeated at least 3 times.
5. Pipet the lysate into a Qiashredder spin column placed in a 2 mL collection tube. Centrifuge for 20 min at 20 000 x g.
6. Transfer the flow-through into a new tube without disturbing the pellet add 1.5 volumes of Buffer AW1 and mix by pipetting.
7. Transfer 650 µL of the mixture into a DNeasy mini spin column placed in a 2 mL collection tube. Centrifuge for 5 min at 6000 xg. Discard the flow-through. Repeat this step with the remaining sample.Place the spin column into a new 2 mL collection tube. Add 500 µL Buffer AW2 and centrifuge for 5 min at 6000 x g. Discard the flow-through.
8. Add another 500 µL Buffer AW2. Centrifuge for 2 min at 20 000 x g.
9. Transfer the spin column to a new 1.5 mL or 2 mL microcentrifuge tube.
10. Add 100 µL Elution Buffer. Incubate for 5 min at room temperature and centrifuge for 5 min at 6000 x g
11. Repeat the previous step.

EXTRACTION:

1. DNA extraction with Qiagen kit following manufacturer's instructions
2. DNA samples quantified using micro-volume UV-Vis spectrophotometer. Good quality DNA ($A_{260/280}$ ~ 1.8 and $A_{260/230}$ ~ 2.0) diluted to a final concentration of 100ng/µL

Appendix II: AFLP protocol (from Blignaut *et al.*, 2013)

DIGESTION:

For each sample, make a 20 µL reaction containing:

		Volume (µL)
200ng of genomic DNA		X µL
5 units of *Eco*RI		0,25 µL
1X EcoRI buffer	(10X original)	2 µL
dH_20		X µL
		Final volume: 20 µL

3. Incubate the reaction for 2 hours at 37°C
4. After *Eco*RI digestion, for each sample, make a 10 µL reaction containing :

Volume (µL)	
5 units of *Mse*I	0,5 µL
1X Cutsmart buffer (10X)	3 µL
dH_20	6.5 µL
	Final volume: 10 µL

5. Add the reaction to the 20 µl to make a final volume of 30 µL
6. Incubate the reaction at 37°C for 2 hours
7. After incubation, inactivate the enzymes by incubating the reaction at 65 ºC for 30 minutes.

LIGATION:

8. For each sample, make a 10 µL ligation reaction mix containing :

Volume (µL)	
1 unit T4 DNA Ligase	0,025 µL
1X T4 DNA Ligase buffer	4 µL

50µM *Mse*I adaptor 1 µL

5µM *Eco*RI adaptor 1 µL

dH_20 3.975 µL

Final volume: 10 µL

9. Add 10uL directly to the digestion mixture
10. Incubate digestion-ligation mix overnight at 4°C (in the fridge)
11. Following ligation, dilute digestion-ligation mix 1:5 with sterile distilled water – use this diluted mixture as a template for the pre-selective PCR

Pre-selective amplification:

For each sample, make a 15µL pre-selective PCR reaction containing:

	Volume
2.5µL of the diluted digestion-ligation reaction mix	2,5 µL
1µM *Mse*I+0 primer	1,5 µL
1µM *Eco*RI+0	1,5 µL
Readymix buffer	7.5 µL
dH_20	2 µL
Final volume:	15 µL

12. Run the Pre-selective reactions on PCR following the following cycles: 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds for 23 cycles; final step 60°C for 30 minutes – hold at 20C.
13. Run 5µL of the PCR product on a 1% aragose gel and look for a smear between 100 and 500 bp
14. Dilute pre-selective PCR products with sterile distilled PCR-grade water (1:19 dilution)

Selective amplification:

For each sample AND EACH FLUORESCENTLY LABELED *EcoRI* primer, set up a 20µL selective PCR reaction containing:

Volume

(1:19 dilution) selective PCR template 5 µL

1µM unlabelled *Mse*I+CTT 2 µL

PCR Mastermix 10 µL

dH_20 volume 2,5 µL

Final volume: 20 µL

15. Use pre-selective PCR conditions but with 30 repeat cycles instead of 23
16. After amplification, run 5µL of each sample on a 1% agarose gel to confirm successful amplification

Table 1: Pre- selective amplification PCR cycling conditions followed for *Opuntia engelmannii* lineages.

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	30 seconds	23
Denaturation	94°C	30 seconds	
Annealing	56°C	30 seconds	23
Extension	72°C	30 seconds	
Final Extension	60°C	30 minutes	1
Hold	20°C	∞	

Table 2: Selective amplification PCR cycling conditions followed for *Opuntia engelmannii* lineages.

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	30 seconds	30
Denaturation	94°C	30 seconds	
Annealing	56°C	30 seconds	30
Extension	72°C	30 seconds	
Final Extension	60°C	30 minutes	1
Hold	20°C	∞	

Appendix III: Chromatographs of the different *Opuntia* lineages showing successful amplification from the three primer primers/ dyes.

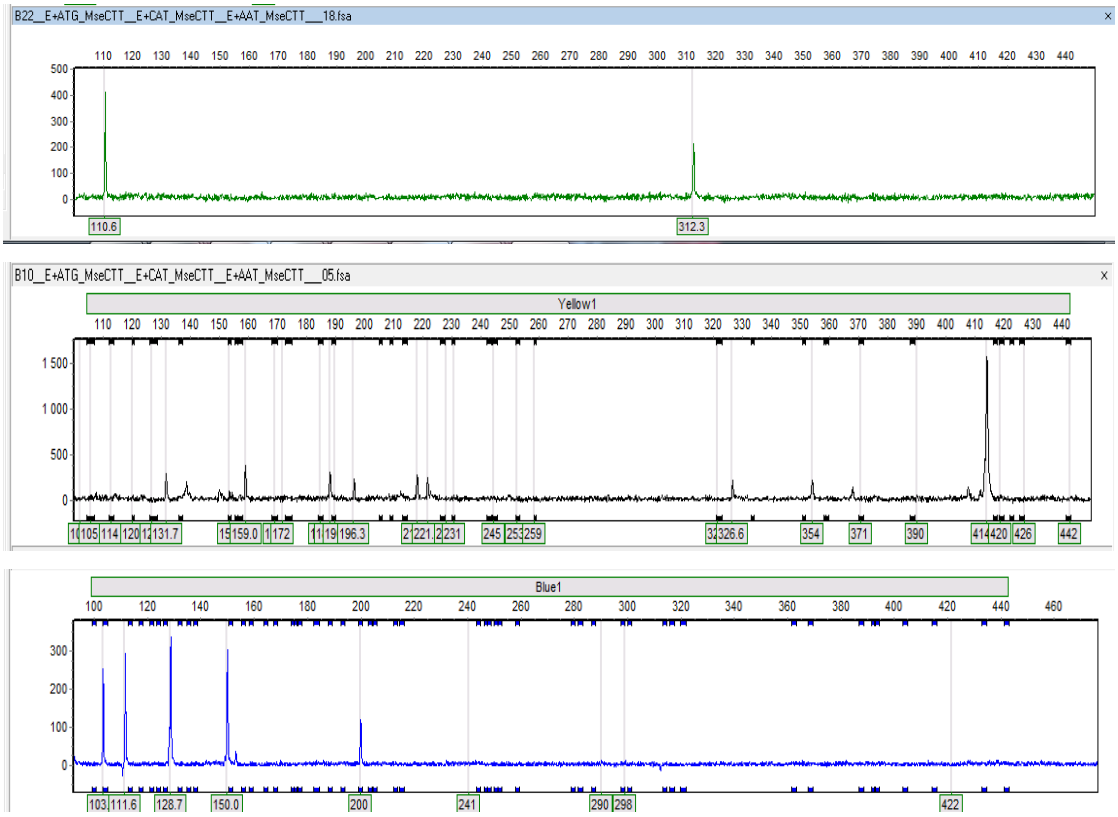

Figure I: Successful chromatographs from the Eastern Cape (spines) lineage of *Opuntia engelmannii*, *E+ATG/MseCTT* (Green dye), *E+CAT/MseCTT* (Black dye), *E+AAT/MseCTT* (Blue dye) primer combination group.

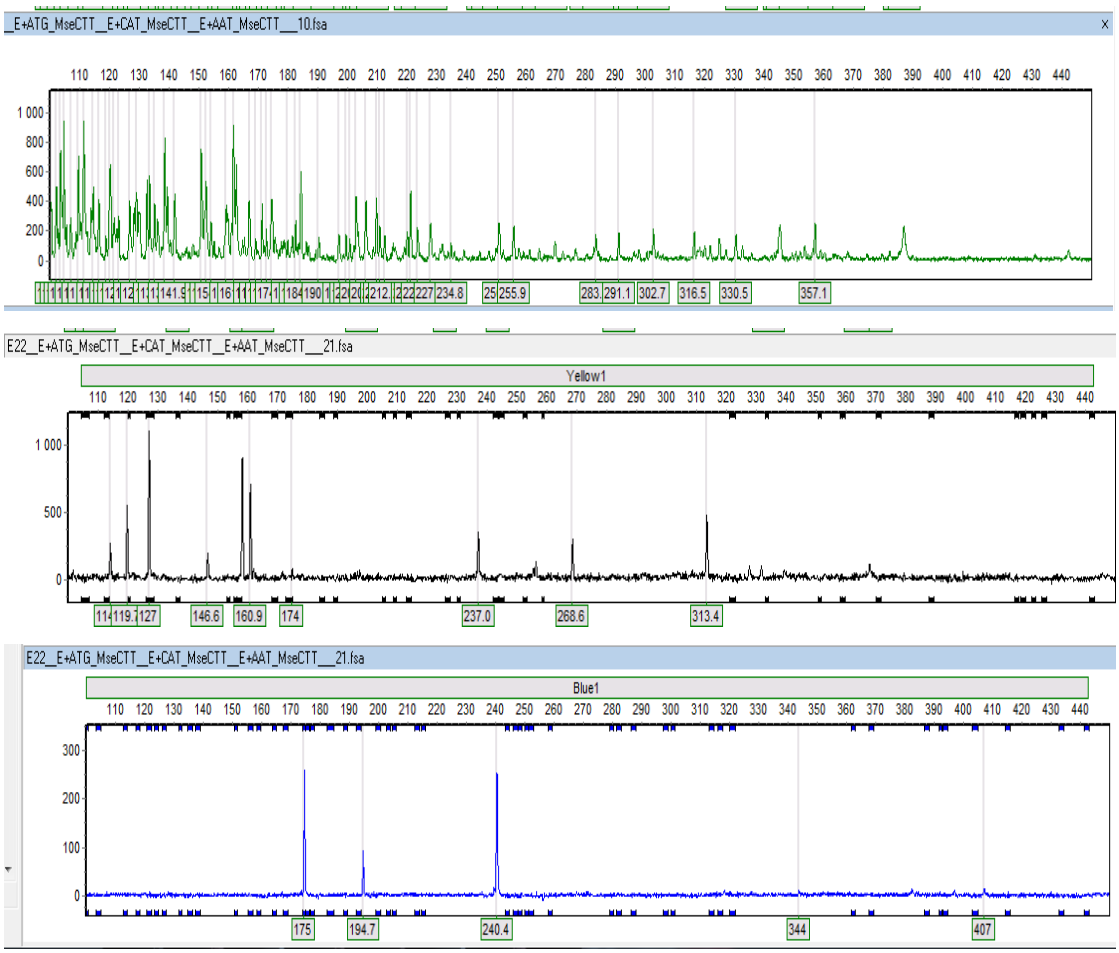

Figure II: Successful chromatographs from Eastern Cape (spineless) lineage of *Opuntia engelmannii*, *E+ATG/MseCTT* (Green dye), *E+CAT/MseCTT* (Black dye*), E+AAT/MseCTT* (Blue dye) primer combination group.

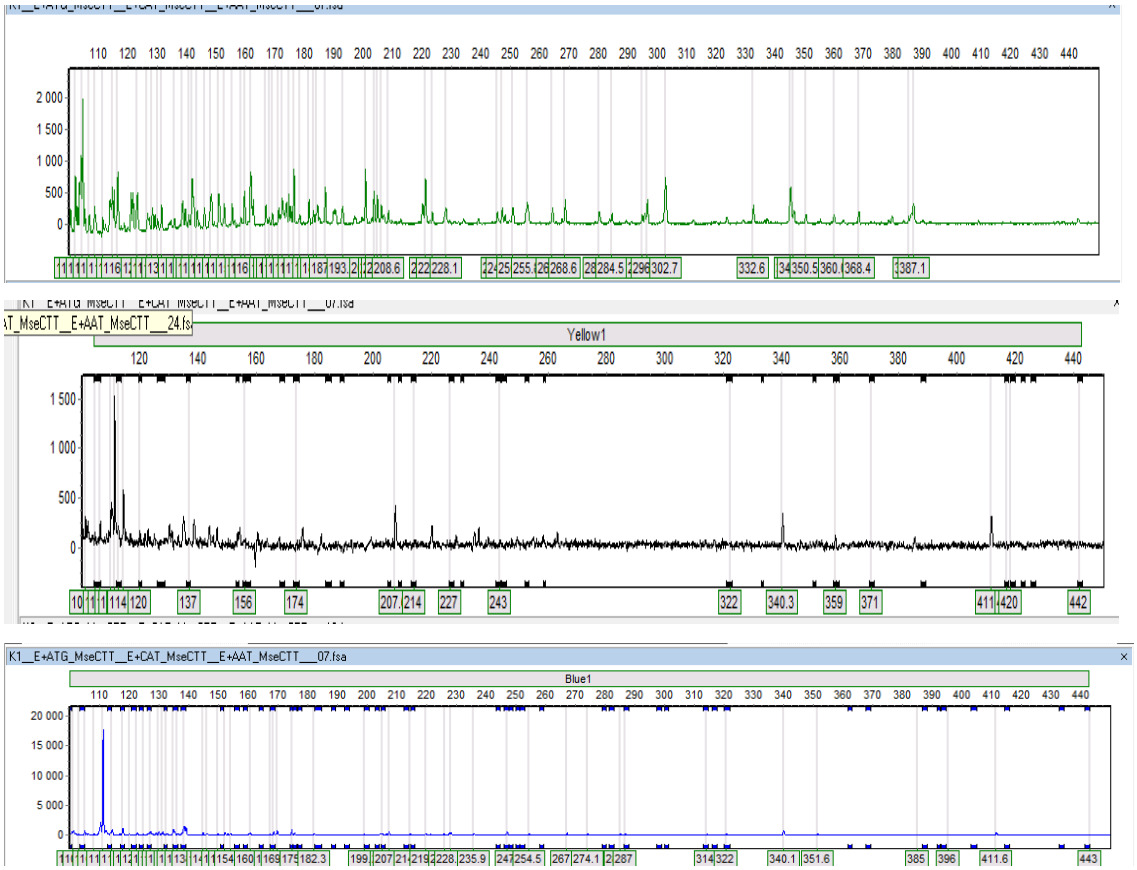

Figure III: Successful chromatographs from Kenyan lineage of *Opuntia engelmannii*, *E+ATG/MseCTT* (Green dye), *E+CAT/MseCTT* (Black dye), *E+AAT/MseCTT* (Blue dye) primer combination group.

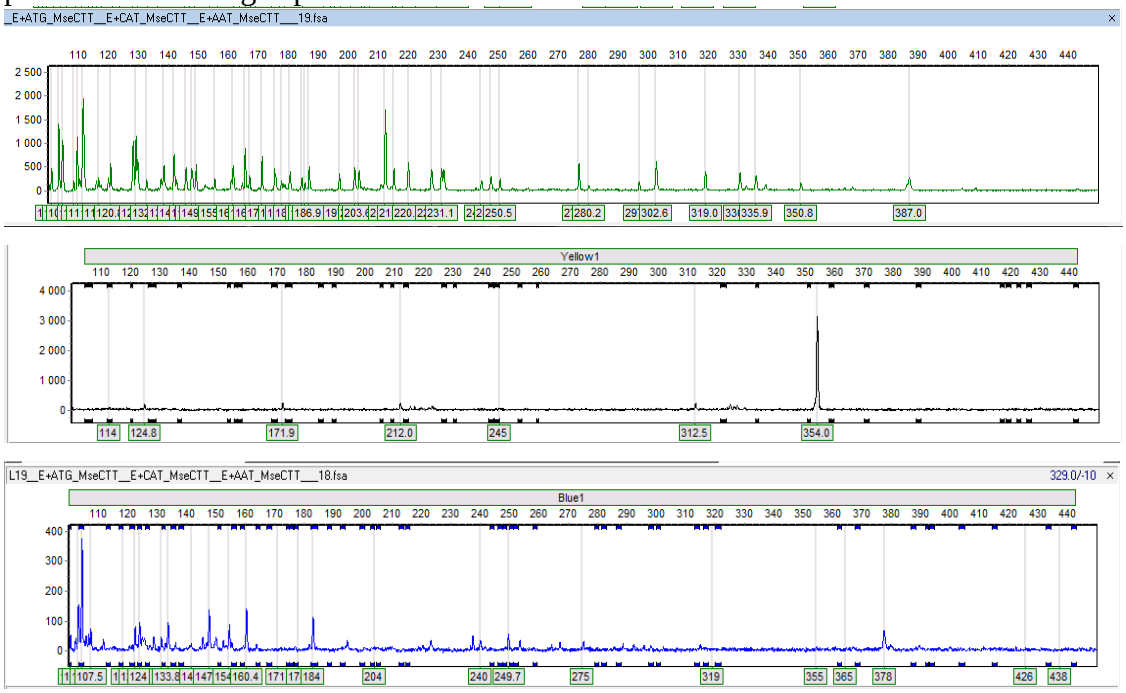

Figure IV: Successful chromatographs from Limpopo lineage of *Opuntia engelmannii*, *E+ATG/MseCTT* (Green dye), *E+CAT/MseCTT* (Black dye), *E+AAT/MseCTT* (Blue dye) primer combination group.

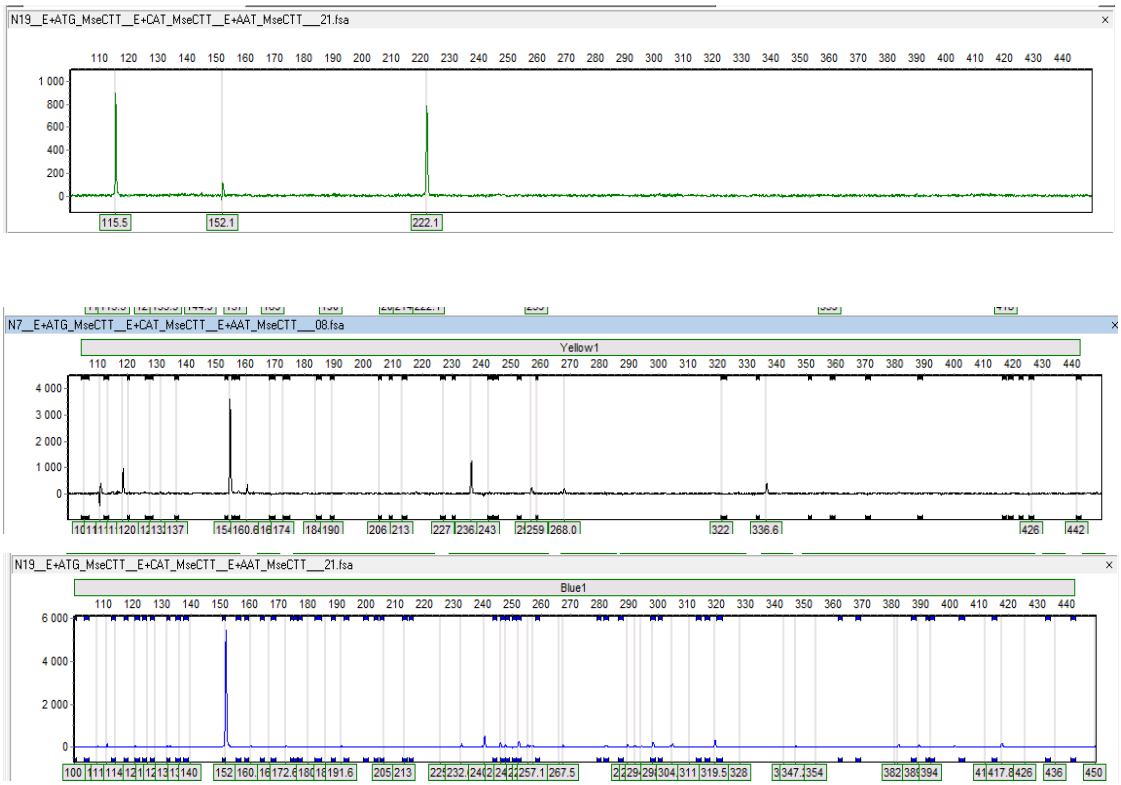

Figure V: Successful chromatographs from Northern Cape lineage of *Opuntia engelmannii*, *E+ATG/MseCTT* (Green dye), *E+CAT/MseCTT* (Black dye), *E+AAT/MseCTT* (Blue dye) primer combination group.

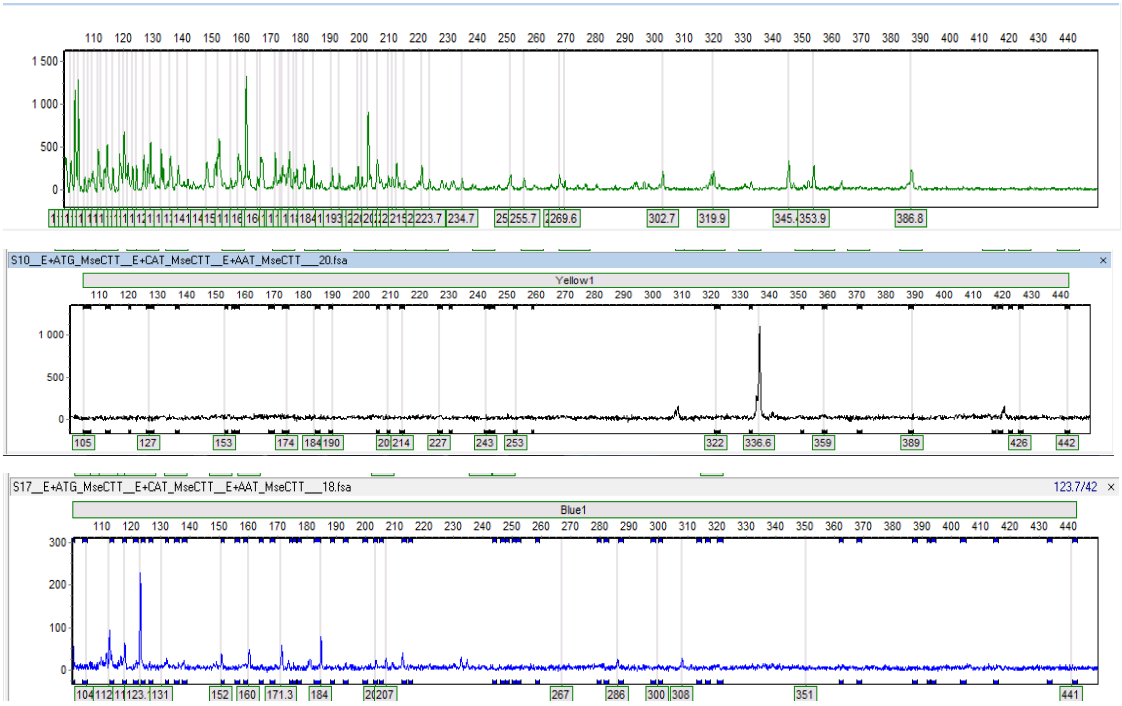

Figure VI: Successful chromatographs from *Opuntia stricta E+ATG/MseCTT* (Green dye), *E+CAT/MseCTT* (Black dye), *E+AAT/MseCTT* (Blue dye) primer combination group.

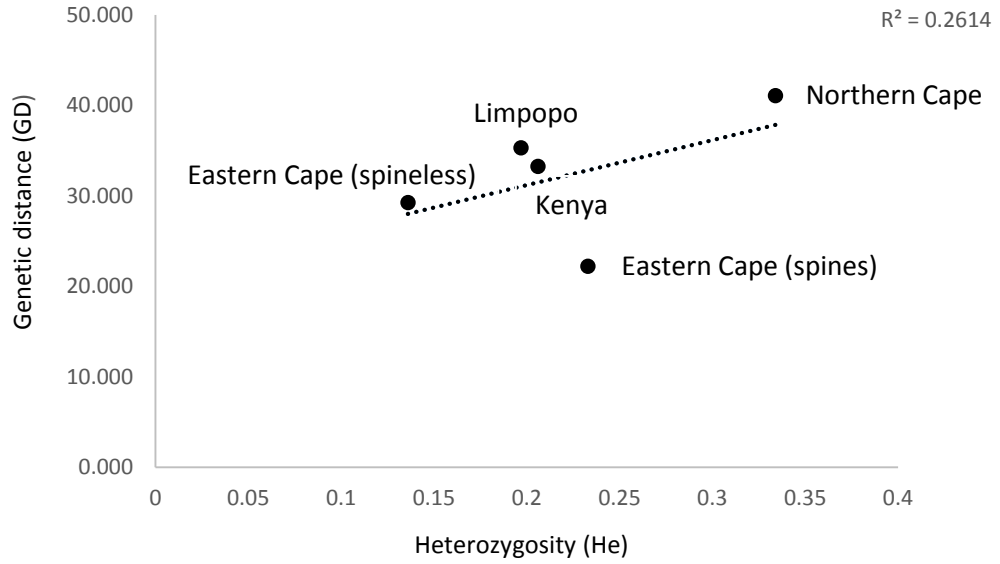

Figure VII: The weak positive relationship (26%) between the Genetic distance and Heterozygosity between *Opuntia engelmannii* lineages.

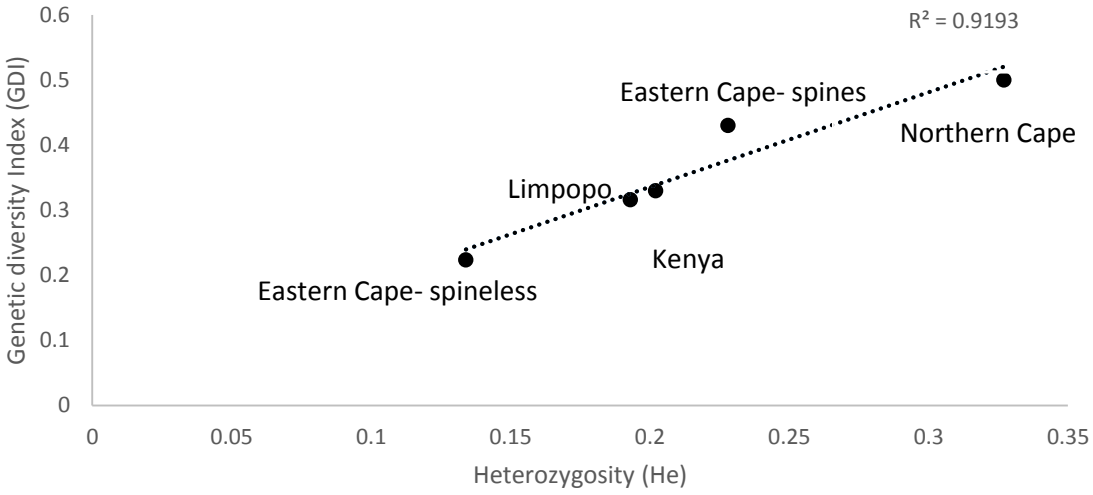

Figure VIII: The strong positive relationship (92%) between the Genetic diversity Index and Heterozygosity among *Opuntia engelmannii* lineages.

Appendix IV: Chromosome squashes and counts protocol for *Opuntia engelmannii* using root tips (from Majure *et al.*, 2011) - trials were done however, there are no useful observation made at this point with regards to the differences in ploidy and the work is currently on-going.

1. Place root tips in 2mM 8-hydroxyquinoline for 8 hours at 4˚C. Rinse with distilled water.
2. Fix the root tips in 3:1 absolute ethanol glacial acetic acid for 2-24 hours at room temperature. Rinse with distilled water.
3. Place the root tips in 70% ethanol for 2 hours. Rinse with distilled water
4. Digest the root tips in 70% HCl for 5-10 minutes at room temperature. Rinse with distilled water.
5. Place root tips back in 70% ethanol and store at 4˚C until use. Rinse with distilled water before squashing.
6. Squash root tips fixed in 60% acetic acid.
7. Stain with 1% aceto-orcein dye.
8. View on a photo-microscope III (Carl Zeiss).

Appendix V: Morphological key to *Opuntia Engelmannii* in South Africa using assumed diagnostic characters. Data from the study suggests that there are three distinct *Opuntia engelmannii* lineages.

Lineage 1
Lineage 2
Lineage 3

Lineage	Spines	Cladodes	Areoles	Fruits
Eastern Cape (spines) 	Spines approximately 30 mm long, white spines, 90˚spine angle*, spine thickness approximately 1.5 mm, atleast 50 spines per cladode	Fairly thick cladode (15-20mm), 15-40 areoles in each cladode	Brown oval shaped areoles, areole has +-10 glochids, 1-2 spines in each areole	No glochids on the base of the fruit (like that of *O. stricta*), 1-2 fruits per individual, approximately 30 mm long fruits, 20 mm thickwith presence of both bracts and spines on the fruit, Fruits have a V-shape
Eastern Cape (spineless) 	No spines	Very thick cladode (20-30 mm), 20 -40 areoles in each cladode	Small, round and dark brown areoles, areole has 2-3 glochids, No spines per areole	No fruits throughout the experiments

Kenya 	Spines approximately 40 mm long, thinner spine (+-1 mm), Spines are two coloured, a brown base and a yellow top, spine angle approximately 75˚, atleast 100 spines per cladode	Thin cladode (5-10 mm), variable number of areoles in each cladode (10-40)	Brown round shaped areoles, areole has +-15 glochids, 4-5 spines per areole, areoles fairly big to accommodate the number of spines	Glochids present at the base of the fruit, atleast 1 fruit per individual, 30 mm long fruit, 20 mm thick fruits with some spines and no bracts,fruits have a U-shape
Limpopo 	Significantly longer spines, approximately 50 mm long, Yellow spines, 90˚ spine angle *, atleast 200 spines per cladode	Fairly thick cladode (10 - 20 mm), 35-50 areoles in each cladode	Brown round shaped areoles, areole has +-20 glochids, 3-4 spines per areole, areoles fairly big to accommodate the number of spines	Glochids present at the base of the fruit, atleast 5 fruits per individual, 20-40 mm long fruits, approximately 25mm thick with some spines and no bracts, fruits have a U-shape

Northern Cape 	Shorter spines, approximately 20 mm long, very thin spines (+-0.5mm), Spines have a 3 –coloured, Red on the base, yellow in the middle and glossy yellow tips, 60-75˚ spine angle*, atleast 70 spines per cladode	Thick cladode (5-15 mm), 20-40 areoles on each cladode	20-40 areoles on each cladode	Glochids present at the base of the fruit, 1-2 fruits per individual, 30 mm long fruit, 15 -20 mm thick fruits with some spines and no bracts, fruits have a U-shape with a more rounder top

*Angle base measured from the ventricular base of the cladode to the spine.

Appendix VI: Summary conceptual diagram of key questions and key findings, conclusion and recommendations from the study