A phylogenetic study of the genus *Dianthus* L. (Caryophyllaceae) in South Africa

By

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DECLARATION

Student Number: 48025933

I, Edward Sifiso Mnxati, declare that this thesis titled " A phylogenetic relationship of the genus *Dianthus* (Caryophyllaceae) in South Africa" is my own work. This work is original and has not been submitted in whole or in part for any other degree or qualification at this or any other university. I have properly acknowledged and cited all sources and references used in this thesis in accordance with the Harvard Style guidelines. I have conducted this research in accordance with ethical principles and have obtained all necessary approvals for research involving sensitive data. This thesis is free from plagiarism, and all borrowed ideas, text, or intellectual property have been duly acknowledged and referenced. I acknowledge any assistance I received during this research, including guidance from my supervisor, discussions with peers, and support from individuals or organizations.

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ABSTRACT

This study addresses the phylogenetic relationships within the genus *Dianthus* in South Africa, employing molecular analysis approach and the distribution pattern of the genus. We extracted DNA and sequenced South African taxa which were then added into other *Dianthus* sequence data including *Petrorhagia* species (outgroup) from GenBank. A robust phylogenetic tree comprising of three plastids (trnH-psbA, trnK-matK and trnK-psbA) and ITS nuclear region was assembled based on 94 taxa of *Dianthus* species and *Petrorhagia* using Mr Bayes analysis. Furthermore, we generated the distribution map for each *Dianthus* species using QGIS, points extracted from BRAHMS database. Our results have revealed *Dianthus* as monophyletic when *Petrorhagia* was used as an outgroup (s). Four strongly supported clades were identified (Africa Lineage, Eurasian radiation, Section Verruculosi and Section Armerium), indicating geographical distinctions. We identified potential discrepancies, notably in the placement of *D. mooiensis* and *D. namaensis*. An unexpected relationship between *D. thunbergii* and *D. bolusii* was also highlighted, challenging current morphological classifications. Defined clades were further observed within African *Dianthus* (Ethiopia & Northern African species; BS/100), and Western & Eastern region of South Africa; BS/100), while two subclades (BS/100 & BS/72) were observed within the South African clade. Additionally, ITS generated unsupported and unresolved trees independently, as did the three plastids (trnH-psbA, trnK-matK, and trnK-psbA). The identified clades and unresolved species placements warrant further investigation, possibly through additional molecular gene markers or broader taxon sampling. This study sets the groundwork for future research aimed at resolving the taxonomic revision of *Dianthus* in South Africa.

Keywords: *Dianthus*, caryophyllaceae, phylogenetic relationship, molecular phylogeny, ITS sequences, trnH-psbA, trnK-matK, trnK-psbA, monophyly, evolutionary patterns.

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ACRONYMS AND ABBREVIATIONS

ACCTRAN	Accelerated Transformation
AIC	Akaike Information Criterion
BI	Bayesian Inference
BS	Bootstrap
CTAB	Hexadecyltrimethylammonium Bromide
DELTRAN	Delayed Transformation
DD	Decimal Degrees
DMS	Degrees Minutes Seconds
DNA	Deoxyribonucleic Acid
IUCN	International Union for Conservation of Nature
ITS	Internal Transcribed Spacer
KZN	KwaZulu Natal
MCMC	Markov Chain Monte Carlo
MP	Maximum Parsimony
MulTrees	Multiple Equal Parsimonious Trees
NCBI	National Center for Biotechnology Information
NH	KwaZulu Natal Herbarium
NU	Bews Herbarium
PCR	Polymerase Chain Reaction

PP	Posterior Probability
PRE	National Herbarium
PVP	Polyvinyl Pyrolidone
QGIS	Quantum Geographic Information System
RSA	South Africa
SANBI	South African National Biodiversity Institute
SRAP	Sequence-Related Amplified Polymorphism
SW	South West
TBR	Tree Bisection Reconnection
UNISA	University of South Africa

Chapter One

1.1 General Introduction

Dianthus L., commonly known as wild pink dianthus or carnation is a large genus of ca. 384 accepted species, 150 subspecies, 12 heterotypic varieties and two forms distributed worldwide (Fassou, *et al*., 2022). The genus *Dianthus belongs* to the family Caryophyllaceae known as the Carnation family (Pooley, 1998). It is distributed in Europe, Asia, Africa and extending to South and North America (Pooley, 1998).

South Africa is home to 15 species, 11 subspecies, 7 varieties and two forms. Most of these species are concentrated in the eastern regions of the country (Germshuizen *et al*., 2006). These species share similar physical features (Hooper, 1961). They are herbaceous, and mostly with woody stems (Hooper, 1959 & 1961). Leaves are opposite and simple, linear in shape with smooth margins (Pooley, 1998). *Dianthus* flowers vary in colour, ranging from pink, purple, lilac, violet, red and white with 5 serrated petals at the edges (Hooper, 1959 & 1961; Figure 1.1).

South African *Dianthus* species, each is adapted to specific ecological niches and recognized for their distinctive floral characteristics. *Dianthus albens* Aiton is commonly found in the Western Cape, it grows in fynbos and renosterveld habitats. It is characterized by its white flowers and ability to thrive in nutrient-poor, acidic soils. *Dianthus basuticus* Burtt Davy is found in the high-altitude regions of the Drakensberg and Lesotho. It is well-suited to montane grasslands. It typically has narrow, linear leaves and produces small, pink to purple flowers. *Dianthus basuticus* consist of three lower ranks, subsp. *basuticus*, subsp. *fourcadei* and subsp. *grandiflorus*. *Dianthus bolusii* Burt Davy is known for its delicate, fringed pink flowers and is typically found in the fynbos regions of the Western Cape. It grows in nutrient-poor, acidic soils, often in rocky or sandy environments. *Dianthus burchellii* Ser. is found in grasslands and rocky outcrops across South Africa (Pooley, 1998; Goldblatt and Manning, 2000). It has narrow leaves and pink flowers with fringed edges (Pooley, 1998; Goldblatt and Manning, 2000).

Dianthus caespitosus Thunb. has two subspecies, subsp. *caespitosus* and subsp. *pectinatus*. It is found in rocky areas and grasslands, this species forms dense clumps and produces small, pink flowers. It is adapted to well-drained, sandy soils. *Dianthus crenatus* Thunb. is widespread across

South Africa. It grows in various habitats, including grasslands and rocky outcrops. It features erect stems and white to pale pink flowers with fringed petals (Pooley, 1998; Goldblatt and Manning, 2000). *Dianthus holopetalus* Turcz. is notable for its entire (non-fringed) petals, which are uncommon in the genus. It is typically found in grasslands and rocky areas in the higher altitudes of South Africa (Pooley, 1998; Goldblatt and Manning, 2000).

Dianthus kamisbergensis Sond. is endemic to the Kamiesberg Mountains in the Northern Cape, this species grows in rocky outcrops and produces bright pink flowers with deeply fringed petals. *Dianthus laingsburgensis* S.S. Hooper is endemic to the Laingsburg area in the Western Cape. This species is adapted to the dry, rocky conditions of the Karoo and produces pink to white flowers. *Dianthus micropetalus* Ser. is a species with very small flowers. It is adapted to arid conditions and is often found in sandy or rocky soils in the Northern Cape. *Dianthus mooiensis* F.N. Williams is native to the Mpumalanga province, this species thrives in rocky, well-drained soils. This species has three subspecies, subsp. *dentatus*, subsp. *kirkii*, and subsp. *mooiensis*. This species is characterized by its tufted growth form and pinkish-white flowers with fringed petals (Pooley, 1998; Goldblatt and Manning, 2000).

Dianthus namaensis Schinz is found in the Namaqualand region, this species is adapted to the arid conditions of the succulent Karoo biome. It has three subspecies, subsp. *dinteri*, subsp. *junceus* and subsp. *namaensis*. It has small, white to pale pink flowers. *Dianthus thunbergii* S.S. Hooper, consist of two subspecies, subsp. *maritimus* and subsp. *thunbergii*. This species is widespread across various habitats in South Africa. It features pink to purple flowers and is often found in rocky or grassy areas. *Dianthus transvaalensis* Burtt Davy is primarily found in the Transvaal region (now part of Gauteng, Limpopo, and Mpumalanga). It grows in grasslands and has pink to white flowers with fringed petals. *Dianthus zeyheri* Sond. has two subspecies, subsp. *natalensis* and subsp. *zeyheri*. It is distributed mainly in the Eastern Cape and KwaZulu-Natal but extending to the northern provinces of South Africa. *Dianthus zeyheri* prefers sandy, well-drained soils. This species is noted for its white or pale pink flowers with a slightly aromatic scent (Pooley, 1998; Goldblatt and Manning, 2000; Govaerts, 2000).

Furthermore, *Dianthus zeyheri* Sonder has been found to be of taxonomic importance, because of a distribution that extend from the Eastern Cape, throughout the coastal region of KwaZulu-Natal to the northern border of Zululand and into Mozambique (Hooper, 1961). This compares with other species which are only distributed in one or two regions of the country. *D. zeyheri* grows in grasslands and forest margins, in well-drained, loamy soil, particularly on sandstone (Pooley, 1998). *D. zeyheri* is characterized by palmate petals with fimbriate margins and a very long 5-lobed green calyx which is fused at the base (Hooper, 1959). Even though Raimondo *et al.* (2009) reported the conservation status (IUCN) of *D. zeyheri* as of least concern, population declining in the wild is a worry.

Fassou *et al*. (2022) reported that *Dianthus* is a genus with many taxonomically complex species groups, and they believed that maybe this is the reason why there is no complete treatment of the whole genus. Figure 1.1 shows the inflorescences and floral patterns of some of *Dianthus* species studied by Fassou, *et al*. (2022).

Figure 1.1 The inflorescence and floral pattern of the genus *Dianthus*. **A)** *D. sphacioticus* **B)** *D. stenopetalus* **C)** *D. critinus* **D)** *D. haematocalyx* **E)** *D. gabrielianae* **F)** *D. juniperinus subsp juniperinus* **G)** *D. juniperinus*. (Fassou *et al*., 2022).

1.2 Economical use of *Dianthus*

The woody root stalk of *D. basuticus* is used in Basotho traditional medicine to cleanse the blood (Moteetee and Van Wyk, 2011), and to treat constipation and flatulence (Kose *et al*., 2015). Plants are harvested from the wild, but at present medicinal use is not considered to be a severe threat to this taxon as it is still widespread and common (Moteetee and Van Wyk, 2011). *Dianthus plumarius* L. is an important environmental protection and greening plant, due to its strong reproductive capacity and fast-growing speed which can efficiently avoid soil erosion (Sun, 2006; Wang *et al*., 2018).

Dianthus chinensis L. and *Dianthus superbus* L. are used in traditional medicine for reducing fever and diarrhea, and as insecticidal pesticides (Zhang *et al*., 2019). *Dianthus barbatus* L. isalso known to be an important source of cut flowers and a greening plant and it is known for the purification of the air through absorption of harmful gases such as sulfur dioxide and chlorine (Zhang, 2011). Hence, *Dianthus* is said to be a useful genetic resource for flower breeding (Wang *et al*., 2021).

1.3 Conservation status

The population of *Dianthus,* specifically *D. zeyheri*, *D. basuticus* and *D. crenatus* (Thunb.) seem to be declining in the wild even though there are no reports on the conservation status (Raimondo *et al*., 2009). However, monitoring of wild resources is needed to gain a better understanding of the impact of harvesting on this taxon's risk of extinction (Kose *et al*., 2015).

1.4 Geographical distribution of *Dianthus*

Dianthus is widespread in Europe, Asia, and Africa, but the Mediterranean area acts as a diversity center for the whole genus (Constantinidis, 1999; Valente *et al*., 2010; Figure 1.2). Many of these species have native range, some are endemic in the Mediterranean area, South West Asia, and the Caucasus region, and with some species occurring in the eastern and southern Africa (Bittrich 1993). The distribution of this genus in Europe and Central Asia is common and native in these regions but found to be extending in North America, North Africa, and South Africa (Pooley, 1998; Valente *et al*., 2010; Fassou *et al*., 2022). South Africa inhabits about 15 species, which are distributed mostly in the eastern part of the country (Hooper, 1959; Pooley, 1998; Germshuizen *et al*., 2006).

The *Dianthus* genus, renowned for its diverse and striking floral species, expanded into South Africa by migrating through the high mountain ranges of eastern tropical Africa, eventually reaching regions of Ethiopia and Kenya (Hooper, 1959; Fassou et al., 2022). However, some African species of *Dianthus* are now at risk of extinction due to their use in traditional medicine within these areas (Balao et al., 2009). South African species of *Dianthus* are distributed in all provinces of South Africa, across various regions. It is more concentration in the Western Cape, Eastern Cape, and KwaZulu Natal especially in the Drakensberg mountains (Pooley, 1998; Goldblatt and Manning, 2000). These regions offer distinct ecological environments that cater to the genus's growth requirements. In the Western Cape, the Mediterranean climate with wet winters and dry summers supports several *Dianthus* species, particularly within the fynbos biome. This biome's acidic, nutrient-poor soils and frequent fires create a unique habitat where these species thrive (Goldblatt and Manning, 2000).

The Eastern Cape, with its more varied topography and climate, also harbors several *Dianthus* species, particularly in grasslands and rocky outcrops, where they benefit from well-drained soils and high levels of sunlight. The Drakensberg mountains, with their cooler temperatures and higher rainfall, provide another important habitat. Here, *Dianthus* species are often found in montane grasslands and alpine regions, adapting to the cooler and more humid conditions. This genus's ability to thrive in such varied environments across South Africa highlights its ecological versatility and contributes to its widespread distribution across these key regions (Goldblatt and Manning, 2000). All the species in Figure 2.1 occur in the eastern region of South Africa except *Dianthus albens* which is native to Western and Northern Cape Provinces of South Africa (Germshuizen, 2006).

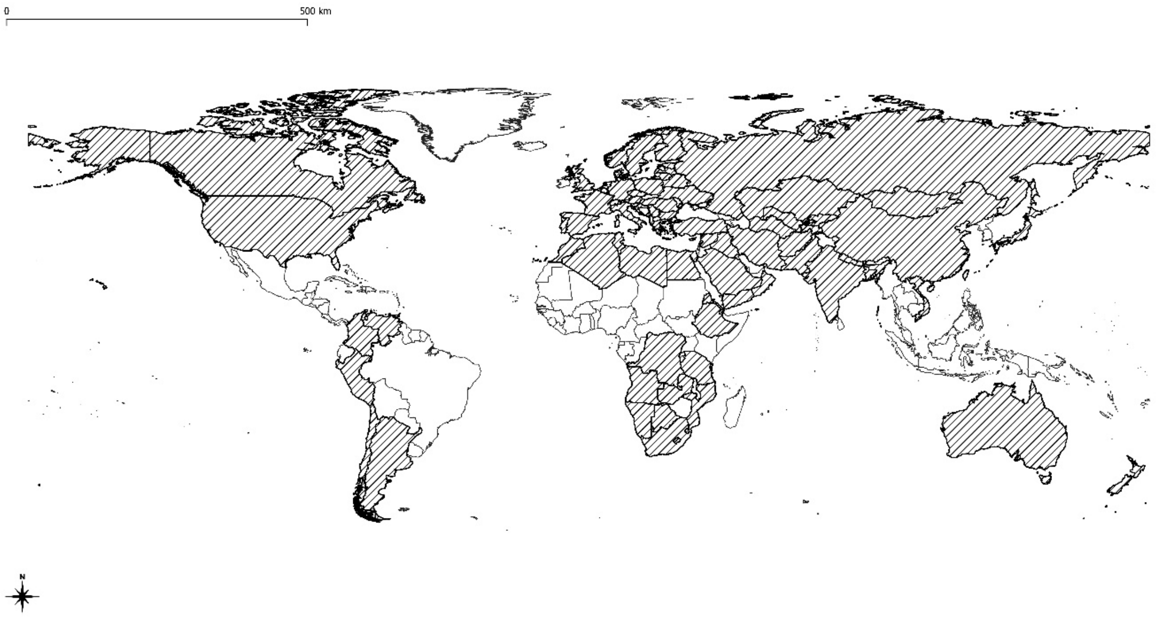


Figure 1.2 The species distribution within the genus *Dianthus* globally

1.5 Classification of *Dianthus*

Caryophyllaceae comprised of a clade of nearly 2200 species, consisting of herbs and subshrubs, with about 86 genera (Bittrich, 1993). Molecular studies showed that Caryophylles are monophyletic (Downie *et al*., 1997). Boissier (1867) came up with the first infrageneric classification of *Dianthus* which recognised six sections, however Rabeler (1993) considered the names used by Boissier (1867) unranked, namely, Carthusiani Boiss., Dentati Boiss., Fimbriati Boiss., Leiopetali Boiss. and Verruculosi Boiss., but this classification was based only on species occurring in the area of his Flora Orientalis. In 1893, Williams tried to improve the infrageneric classification system for *Dianthus* but failed to consider Boissier's (1867) work. Williams (1893) went further and using morphological characters proposed three subgenera, eight sections and 22 subsections. Pax and Hoffmann (1934) came up with the most currently used classification of seven sections.

Usually, caryophyllaceae is subdivided into three subfamilies (Table 1): Alsinoideae, Caryophylloideae, and Paronychioideae (Bittrich, 1993). Alsinoideae are distinguished by nectar glands located at the abaxial base of the episepalous stamens (Bittrich, 1993).

Caryophylloideae (*Dianthus* and 16 other genera under the tribe Caryophylleae) are distinguished by a tubular calyx, and fimbricated petals (Bittrich, 1993; Downie *et al*., 1997). Bittrich (1993) considered Alsinoideae and Caryophylloideae to be monophyletic due to its caryophyllad embryology and noted solanad embryology in Paronychioide.

The genus *Dianthus* is within the family Caryophyllaceae in major angiosperm groupings with about 384 accepted species, 150 subspecies, 12 heterotypic varieties and two forms globally distributed (Fassou *et al*., 2022). It is the second largest genus in the family surpassed only by *Silene* (Hooper, 1959). The genus is divided into two subgenera: *Dianthus* and *Carthusianastrum* F. Williams (Rabeler and Hartman, 2005). However, some researchers such as Kuzmina (2002, 2003) and Rabeler and Hartman (2005), have considered this classification to be artificial because it does not show any evolutionary relationships.

Table 1: Classification of the Caryophyllaceae (from Bittrich, 1993).

Subfamily	**Tribes**	**Total genera**	**Number of genera sampled in this study**
Caryophylloideae	Caryophylleae	17	1
	Sileneae	6	1
Alsinoideae	Alsineae Sclerantheae	28	4
	Geocarpeae Pycnophylleae	2	1
	Habrosieae	1	0
		1	0
		1	0
Paronychioideae	Paranychieae	15	4
	Polycarpeae Corrigiolleae	16	4
		2	0

1.6.1 Taxonomic confusion within the genus

Dianthus is considered as one of the most intricate taxa and is still not properly studied. Despite the popularity of *Dianthus* in horticulture, the genus needs a thorough investigation using modern technology. Various taxa belonging to this genus have been described at species or subspecies level, although their real taxonomical status is often uncertain (Farsi *et al.,* 2013). Many researchers have already stressed that this genus needs an in-depth taxonomic review (Lacaita, 1911; Grande, 1912; Pignatti, 1973; Arrigoni, 1984; Germshuizen *et al*., 2006; Fassou *et al*., 2022).

Another taxonomic concern is noted by Farsi *et al.* (2013) in the *Dianthus polylepis* (Bien. Ex Boiss.) complex which consists of two well-known endemic species, *Dianthus polylepis* and *Dianthus binaludensis* (Rech.f.), found in Khorassan-Kopetdagh floristic region. There has been debate about the taxonomic placement of the two species in the past (Assadi, 1985; Farsi *et al*., 2013). It has been indicated previously that the differentiation of *Dianthus polylepis* from *Dianthus binaludensis* through morphological characters is complex (Farsi *et al.,* 2013). Also, the correlation between the morphological and molecular results showed that *Dianthus polylepis* and *Dianthus binaludensis* species within the genus are undistinguishable either morphologically or throughmolecular studies (Assadi, 1985; Farsi *et al*., 2013). Therefore, a new combination of *Dianthus polylepis* subsp. *binaludensis* was proposed (Farsi *et al.,* 2013).

The taxonomic confusion within the species of *Dianthus* genus in South Africa, stems from several factors:

Morphological Similarity: Many species within the *Dianthus* genus exhibit very similar morphological traits, making it difficult to distinguish between species based solely on physical characteristics. This is especially true in regions with high species diversity, such as South Africa (Retief and Van Wyk, 1998).

Hybridization: The *Dianthus* genus is prone to hybridization, which further complicates species identification and classification. Hybrids may display intermediate characteristics that make them hard to classify under existing species definitions (McNeill, 1994).

Incomplete Taxonomic Revisions: The genus has not been comprehensively revised in South Africa, leading to uncertainties and inconsistencies in species identification. As a result, some species may be misidentified or classified under outdated taxonomic frameworks (Retief and Van Wyk, 1998; Goldblatt and Manning, 2000).

Lack of Comprehensive Floristic Studies: The flora of South Africa is diverse and not fully explored. Incomplete floristic surveys can lead to underestimations of species diversity or misidentification of species within the genus (Goldblatt and Manning, 2000).

Geographical Variability: The geographical distribution of *Dianthus* species across different habitats in South Africa introduces variability in traits such as flower color, size, and growth form, which can lead to taxonomic confusion (McNeill, 1994; Retief and Van Wyk, 1998).

1.6.2 Morphological confusion within *Dianthus*

Burtt Davy (1922) noticed that there is a specific limit amongst the South African *Dianthus* which are often ill-defined. The characters used for specific delimitation are less amenable to precise definition compared to the rest of other genera. In addition, this misconception of the species characteristics has led botanists to assign more than one name to similar species due towrong description of morphological characters (Burtt Davy, 1922).

Hooper (1959) indicated that the species that are occurring in South Africa possess certain characteristics which coexist across all native species. These characters are perennial hemicryptophytic habit with woody taproot and well-developed base (Hooper, 1959). They have setaceous to narrow-elliptic leaves and consist of solitary pedicellate flowers with outer-lower pairs of calyx-bracts smaller than the upper pairs, and a calyx is uniformly striate-nervose with hairs and globose petal-lamina (Hooper, 1959). However, Burtt Davy (1922) argued that the description employed by Sond. (1860) to categorize South African species based on the flowering stem is unreliable. This is because most *Dianthus* species in this region exhibit both simple and branched flowering stems within a single plant.

Some of the species that are of taxonomic concern, and which also shows blurring of morphological boundaries between species and their subspecies are *Dianthus zeyheri* which has been considered either as one coherent species (Sonder, 1860; Burtt Davy, 1922; Pooley, 1998) or consisting of two subsp.: zeyheri & *natalensis* Hooper (Hooper, 1959; Germshuizen *et al*., 2006). These two subspecies are distinguished only by a petal structure (Hooper, 1959). Hooper (1959) recognized *Dianthus zeyheri* Sond. subsp. *zeyheri* having 8-24 mm long petals, fringedwith deep incisions and petals of subsp. *natalensis* with 8-17 mm long petals with fine incisions. *Dianthus. zeyheri* also shares similar characters with *Dianthus crenatus* (Hooper, 1959). In addition, *Dianthus mooiensis* (F.N. Williams) and *Dianthus basuticus* also have a resemblance, sharing diagnostic characters, such as linear basal leaves and apical fimbriates petals (Burtt Davy, 1922; Pooley, 1998).

1.6.3. Problem statement

Balao *et al.* (2010) discovered that the taxonomy of *Dianthus* is infamously problematic because of the species being extremely morphologically diversified and noted that this resulted in taxonomic uncertainty. When a species is highly morphologically diverse, it becomes challenging to define clear diagnostic traits that consistently distinguish one species from another. Balao *et al.* (2010) further mentioned that this resulted in scientists assigning more than one name to similar species. This may cause wrong species to be conserved or misused in substitute of the correct species (Balao *et al.*, 2010). The identified low molecular variation, combined with morphological diversity, can result in inaccurate DNA barcoding of members of this genus (Valente *et al*., 2010). This variation is indicative of rapid species diversification, which poses a significant challenge in accurately identifying species within this genus in herbarium collections (Valente *et al*., 2010).

Balao *et al.* (2010) and Valente *et al.* (2010) further stressed that evolutionary radiation common in *Dianthus* is a major source of species diversification, therefore, acorrect identification to species level could prevent over exploitation of vulnerable species. Moreover, some researchers showed that polyploidy is a common phenomenon in *Dianthus,* but this could lead to misapplication of environmental laws if *Dianthus* concept is misapplied (Balao *et al*., 2009). Polyploidy is a condition in which an organism has more than two complete sets of chromosomes (Comai, 2005). In most organisms, cells are diploid, containing two sets of chromosomes (Comai, 2005). The biggest disadvantage of polyploidy in plants is often related to genetic instability (Madlung, 2013). Thus, this evolutionary force along with speciation often takes place via hybridization and genome duplication (Weiss *et al.,* 2002; Balao *et al.,* 2010). thus it is vital to get the concept correct to prevent hybridization and gene duplication of incorrect species for ornamental uses.

The polyploidy form of *Dianthus* causes gene redundancy in certain species of this genus which results in many varieties within members of the same species, a major source of speciation, which is a problem in the selection of correct species in traditional medicine (Weiss *et al.,* 2002; Balao *et al.,* 2010; Valente *et al.,* 2010).

Sonder (1860) and Burtt Davy (1922) used morphological characters to recognize 15 species of *Dianthus* including *Dianthus zeyheri* Sond. that were classified to species level. However, Hooper (1959) later identified two subspecies within *D. zeyheri*: *Dianthus zeyheri* subsp. *zeyheri* and *Dianthus zeyheri* subsp. *natalensis*. The distinction between these subspecies is complex and challenging due to the overlap in morphological features used to differentiate them. The two subspecies described by Hooper (1959) are *Dianthus zeyheri* subsp. *zeyheri* and *Dianthus zeyheri* subsp. *natalensis* has been having a complicated taxonomic interpretation and nomenclature. These are signified in Hooper (1959) where the author diagnosed *Dianthus zeyheri* subsp. *zeyheri* with cylindrical calyx length of 2.8-3.8 cm and petal lamina of 1.0-2.4 cm long, while diagnosing *Dianthus zeyheri* subsp. *natalensis* with a cylindrical calyx length of 2.8-4.5 cm and petal lamina of 1.5-1.2 cm long. These two interpretations are very confusing in the herbarium for taxonomists, especially when the genus is identified for environmental impact assessments.

This is due to the overlapping of the characters used to describe this taxon. Also, this is signified in the overlapping of the distribution of *Dianthus zeyheri* complex with other similar or closelyrelated species like *Dianthus crenatus*, which could be wrongly used commercially or medicinally. Additionally, the confusion is contributed by Sonder's (1860) description of *Dianthus zeyheri* Sond. Sonder (1860) described *Dianthus zeyheri* as a species with calyx lobe shorter than petal lamina, a species closely similar to *Dianthus crenatus* (Sonder, 1860) description in that both taxa have broadly obovate petals which are fimbricated, but the two species are separated by bracts which are about 6, ovate-lanceolate on *Dianthus zeyheri* and 4-6, ovate on *Dianthus crenatus*.

This conflict could easily result in wrong scientific findings and mislead anyone who has interest in the commercial value of the genus.

Dianthus is an important hereditary resource for flower cultivating and is an important part ofthe cut flower industry. Nevertheless, research on the genetic resemblance of *Dianthus* plants is scarce or limited (Wang *et al.,* 2021). However, few molecular studies have been conductedonthis genus and the molecular data have proven to be of importance in assessing phylogenetic relationships of species, plant diversity, identification, and species conservation (Farsi *et al*., 2013). Therefore, DNA analysis is vital in the study of conservation or protection of endangered *Dianthus* species (Farsi *et al*., 2013). For example, Wang *et al.* (2021) found *Dianthus* species to have a high detection efficiency and accuracy using molecular techniques.

They examined 20 primer pairs of SRAP markers which exhibited a high resolution and precise identification in 44 *Dianthus* samples, and they found a considerable difference of genetic distance between 5 group of *Dianthus* species namely, *Dianthus chinensis* L., *Dianthus superbus* L., *Dianthus barbatus* L., *Dianthus plumarius* L. and *Dianthus caryophyllus* L. *Dianthus chinensis* and *Dianthus superbus* displayed the highest genetic similarity followed by *Dianthus barbatus. Dianthus plumarius* showed the lowest genetic similarity compared to the other four species (Wang *et al.* 2021). In terms of phenotype, *Dianthus plumarius* displays the dense clump stem and the other four species show a sparsely clump stem. With the exception of *Dianthus plumarius*, *Dianthus caryophyllus* showed a higher plantheight than the rest of the other three species studied (Wang *et al.,* 2021).

The taxonomic identification of the genus *Dianthus* using morphology has been a complex issue in the past. This led to incorrect identification of certain species which in turn resulted in the inappropriate use of species in traditional medicine and conservation (Pooley, 1998). Hooper (1959) suggested that *Dianthus zeyheri* could be separated into two subspecies while Sonder (1860) and Burtt Davy (1922) suggested that it should remain at the species level based on overlapping morphological characters.

1.7 Aims and objectives of the study

Recent research has significantly advanced our understanding of the overall phylogenetic relationships within the genus *Dianthus*. However, there remains a lack of detailed knowledge about how South African *Dianthus* species are specifically related to each other within this context. This study was aimed at addressing this gap by addressing the phylogenetic relationship of the species in the genus *Dianthus* within the family Caryophyllaceae in South Africa. This study was aimed at addressing the geographical distribution patterns of South African species of *Dianthus* to clarify their phylogenetic relationships.

This was addressed by focusing on the following objectives:

- Investigating the phylogenetic relationship of *Dianthus* using DNA sequences from plastids (trnK-matK, trnK-psbA & trnH-psbA) and nuclear (ITS) datasets.
- To investigate and clarify the evolutionary relationships among species within the *Dianthus* genus in South Africa using phylogenetic analysis.
- Analyzing the geographical distribution patterns of these species to clarify their evolutionary relationships.

This, in turn, will contribute valuable insights to broader phylogenetic research and have practical implications for conservation and biodiversity management in the region.

It is hypothesized that molecular analysis of the *Dianthus* genus in South Africa will clarify the phylogenetic relationships among its species, resulting in more precise classification and identification within the group.

Chapter Two

Literature Review

2.1 Introduction

The genus *Dianthus* L. has been recognized by Hooper (1959) as the second largest genus in the family Caryophyllaceae after Silene L. *Dianthus*. One of the major centres of diversity in the genus *Dianthus* is in the Eurasian region (Valente *et al*., 2010; Fassou *et al*., 2022). Estimates for this area, encompassing Europe and Southeast Asia, indicate the presence of over 200 species, excluding those species with widespread distribution also found in Africa (Valente *et al*., 2010).

Another significant region for this genus is in the tropical and subtropical zones of North and South America (Valente *et al*., 2010; and Fassou *et al*., 2022). Considering every region globally, *Dianthus* comprises more than 84 additional species, bringing the worldwide estimate to approximately 384 species, , 150 subspecies, 12 heterotypic varieties and two forms occurring mostly in the northern hemisphere (Fassou *et al*., 2022). Africa stands out as another center of diversity, hosting predominantly endemic species of *Dianthus*, particularly in South Africa, with an estimated 15 endemic species distributed across all nine provinces in South Africa, and an addition of 11 subspecies, 7 varieties and two forms (Germshuizen *et al*., 2006; Fassou *et al*., 2022). Seven of these species are found in the eastern region of South Africa (Eastern Capeto KwaZulu-Natal Provinces). The South African species of *Dianthus* migrated and extended through high mountain ranges of eastern tropical Africa and have been recorded certain regions of Ethiopia and Kenya (Hooper, 1961; Valente *et al*., 2010).

Dianthus is mostly perennial, rarely annual or biennial, herbs, or shrubs with oblong toovate or linear and grass-like leaves (Hooper, 1959; Fassou, *et al*., 2022). The genus is characterized by hermaphroditic flowers which are solitary or in dense terminal cymes (Pax and Hoffmann, 1934). The flowers are protected by 2 to many epicalyx scales, with 5-toothed, tubular calyx, and have 20–60 well-marked parallel nerves (Fassou, *et al*., 2022).

2.2 Molecular studies of the genus *Dianthus*

The first molecular phylogenetic study of the Caryophyllaceae was done by Fior *et al*.(2006) which included *Dianthus furcatus* Balb. and *Dianthus seguieri* Vill. The study showed *Velezia rigida* L. to be closely related to *Dianthus* clade. Furthermore, Harbaugh *et al*. (2010) added *D. armeria* L. and *Dianthus* sp. (unidentified species) in Fior *et al*. (2006) datasets. This put *Velezia* in the same clade as *Dianthus* clade, the same clade which was found to be the sister clade to *Petrorhagia saxifraga* (L.) Link.

Valente *et al*. (2010) conducted a significant study on the molecular analysis of the *Dianthus* genus, utilizing sequences from *matK*, *psbA-trnK*, *trnH-psbA*, and *nrITS* extracted from 104 species. Their research proposed that the evolutionary history of the genus was influenced by a recent migration from the Mediterranean region, leading to its expansion into Africa. This hypothesis was later supported by Greenberg and Donoghue (2011), who conducted a phylogenetic analysis using five chloroplast regions (*matK*, *ndhF*, *trnL-trnF*, *trnQ-rps16*, *trnS-trnfM*) and nuclear ribosomal ITS (nrITS) sequences. Their findings reinforced Valente *et al*.'s conclusions, suggesting that the *Dianthus* species in Africa arose from relatively recent colonization events.

However, Fassou *et al*. (2022) challenged these conclusions by conducting a more comprehensive analysis using an updated DNA dataset and a broader representation of species within the genus *Dianthus*. Specifically, they found that Valente *et al*. (2010)'s conclusions were inappropriate when considering the genus *Petrorhagia*, a close relative of *Dianthus*. In their analysis, Fassou *et al*. (2022) included more than 30 species of *Petrorhagia*, including two species, *Petrorhagia thessala* and *Petrorhagia prolifera*, as outgroups. Crucially, this analysis excluded the type species *Petrorhagia saxifraga (L.) Link*, which is central to accurately understanding the phylogenetic relationships within the genus.

Fassou *et al*. (2022) noted that the omission of *Petrorhagia saxifraga* could have led to misinterpretations in the earlier studies by Valente *et al*. (2010) and Greenberg and Donoghue (2011). Furthermore, Fassou *et al*. (2022) observed that the phylogenetic tree constructed by Greenberg and Donoghue (2011) lacked several important lineages within *Dianthus*. This lack of comprehensive lineage representation weakened the conclusions drawn by these earlier studies, leading Fassou *et al*. (2022) to propose a revised evolutionary history for the *Dianthus* genus that suggests multiple, older migration events into Africa, rather than a single recent one.

Overall, Fassou *et al*. (2022) provided a more detailed understanding of the *Dianthus* genus's biogeographical history, highlighting the importance of including comprehensive species representation and considering the phylogenetic significance of key outgroups in evolutionary studies.

Wang *et al*. (2021) conducted a molecular study using 20 pairs of Sequence-Related Amplified Polymorphism (SRAP) markers to assess genetic diversity in 44 *Dianthus* species, including 13 lines of wild *Dianthus chinensis L.*, 7 lines of wild *Dianthus superbus L.*, and 24 commercial varieties such as *Dianthus caryophyllus L.*, *Dianthus plumarius L.*, and *Dianthus barbatus L.* The study found significant interspecific genetic diversity, particularly between different geographical populations, and highlighted the potential for these genetic resources in flower breeding. The genetic analysis showed high similarity between *Dianthus chinensis* and *Dianthus superbus*, while *Dianthus barbatus* and *Dianthus plumarius* had the lowest genetic similarity.

The study focused primarily on species from Europe and Asia and did not include any African or South African species in their analysis. Therefore, the placement of African or South African species within the *Dianthus* phylogeny was not addressed in this research. The results highlighted significant genetic diversity among the species analyzed, providing valuable insights into the phylogenetic relationships within the genus, particularly among the commercially important varieties.

But the lack of inclusion of African species suggests that further research would be necessary to fully understand the genetic diversity and evolutionary history of *Dianthus* species in Africa, including South Africa. However, molecular markers have become a powerful instrument for plant variation and identification (Chen *et al*., 2018; Wang *et al*., 2021). In Wang *et al.* (2021), 20 primer pairs of SRAP markers were found to have a high resolution and precise identification in 44 *Dianthus samples*. Therefore, in Wang *et al.* (2021), the phylogenetic classification was used to determine the species placement within the genus *Dianthus.*

2.3 South African species of *Dianthus*

Several species, including *Dianthus caryophyllus*, *Dianthus barbatus*, *Dianthus chinensis*, *Dianthus plumarius, D*ianthus *superbus*, are widely used as horticultural cultivars (Tanase *et al.*, 2012). Many flowers of *Dianthus* are divided into three groups (standards, sprays, and pot carnations) based on plant form, flower size, and flower shape (Valente *et al.*, 2010; Figure 2.1). Standards have a single large flower per stem, whereas sprays have a larger number of smaller flowers; both types are used for cutting flowers (Valente *et al.*, 2010). Pot carnation is a dwarf with many small flowers (Figure 2.1) that is used as a potted plant (Valente *et al.*, 2010).

D. albens *D. basuticus* *D. micropetulus* *D. mooiensis*

D. bolusii *D. thunbergii* *D. namaensis* *D. zeyheri*

Figure 2.1: The different species of the genus *Dianthus* in South Africa (Mnxati, 2008; World Checklist of Vascular Plants, 2021).

The recognition of a new species in *Dianthus* still relies on overall similarities of certain morphological traits and molecular data (Vaezi *et al.*, 2014) and molecular data does not only support delimiting species apart from the other sampled species, but it also associates it to its closest relative or ancestor (Johnson *et al*., 2012). South Africa is home to 15 species of *Dianthus,* each adapted to various habitats within the region, contributing to its botanical diversity. These species include *Dianthus albens*, *Dianthus basuticus*, *Dianthus bolusii*, *Dianthus burchellii*, *Dianthus caespitosus*, *Dianthus crenatus*, *Dianthus holopetalus*, *Dianthus kamisbergensis*, *Dianthus laingsburgensis*, *Dianthus micropetalus*, *Dianthus namaensis*, *Dianthus mooiensis*, *Dianthus thunbergii*, *Dianthus transvaalensis*, and *Dianthus zeyheri* (Germshuizen, 2006).

2.3.1 *Dianthus albens* Aiton

It is native to and common in the coastal areas along the Northern Cape (NC) and Western Cape (WC) coastline, growing in rocky areas and even in sand dunes. It is a subshrub and grows predominantly in semitropic biome (Govaerts, 2000). The plants of *Dianthus albens* are usually recognised by blossomed flowers as their leaves and stems are easily overlooked in the veld (Govaerts, 2000). *Dianthus albens* is closely related to other species in the *Dianthus* genus that share similar ecological adaptations and morphological traits. Species such as *Dianthus thunbergii* and *Dianthus micropetalus* may be among its closest relatives, given their similar adaptations to rocky and dry environments (Goldblatt and Manning, 2000).

2.3.2 *Dianthus basuticus* Burtt Davy

This species is native to Lesotho, Eastern Cape, Free State, KwaZulu-Natal, and Mpumalanga. It is typically found in the high-altitude regions of the eastern Highveld of South Africa and Lesotho, from Belfast to Barkly East (Burtt Davy, 1922; Govaerts, 2000). The species is known for its distinctive characteristics that adapt it to these mountainous environments (Govaerts, 2000). *Dianthus basuticus* thrives in rocky, well-drained soils in montane and alpine regions, often in grassland or shrubland areas. It is a perennial herb with a tufted growth habit, often forming dense mats, with grass-like leaves (Goldblatt and Manning, 2000).

2.3.3 *Dianthus bolusii* Burtt Davy

It occurs in Northern and Western Cape Provinces of South Africa. *Dianthus bolusii* is characterized by the basal leaves tufted at the crown of a woody slender, underground stem, which is roughly 10-17cm long (Govaerts, 2000). The woody stem is branched above the ground, hardly simple, have 4-8 flowers, bracts in pairs of 3 and 5. Petals are exserted, fimbriate and dark purple

(Burtt Davy, 1922). *Dianthus bolusii* thrives in rocky, well-drained soils and is commonly found in high-altitude grasslands or mountainous regions. It is adapted to areas with high sunlight and can withstand periods of drought. It is closely related to other species within the *Dianthus* genus that share similar ecological niches and morphological traits. (Goldblatt and Manning, 2000).

2.3.4 *Dianthus burchellii* Ser.

Dianthus burchellii is a perennial herb known for its slender, grass-like leaves and small, fragrant flowers, which are typically pink or white. This species is well-adapted to a variety of habitats, thriving in rocky and sandy soils. *Dianthus burchellii* is indigenous to South Africa, predominantly found in the Cape Provinces. It is commonly seen in fynbos, grasslands, and mountainous regions, contributing to the area's rich botanical diversity (Pooley, 1998; Goldblatt and Manning, 2000). *Dianthus burchellii* is closely related to other *Dianthus* species that share similar ecological adaptations and morphological traits (Goldblatt and Manning, 2000).

2.3.5 *Dianthus caespitosus* Thunb.

Dianthus caespitosus is a low-growing, perennial herb that forms dense, cushion-like mats. It has narrow, linear leaves and produces small, delicate flowers, typically pink or white, with a sweet fragrance. The species is known for its compact growth habit and resilience in harsh environments. *Dianthus caespitosus* is endemic to South Africa, where it is primarily found in the Drakensberg Mountain range. It thrives in high-altitude grasslands and rocky outcrops, often growing in well-drained, sandy soils. This species is adapted to the cool, moist conditions of the montane regions (Pooley, 1998; Goldblatt and Manning, 2000).

2.3.6 *Dianthus crenatus* Thunb.

It is native to Western and Eastern Cape Provinces, and KwaZulu-Natal (Govaerts, 2000). This species is more like *Dianthus basuticus* and *Dianthus mooiensis* in terms of morphological characters (Burtt Davy, 1922). Its distinctive feature is white or pale pink flowers with fringed petals, it often grows in high-altitude grasslands. *Dianthus crenatus* is closely related to other *Dianthus* species that share similar morphological traits and habitat preferences. Species such as *Dianthus basuticus* and *Dianthus mooiensis* may be among its closest relatives due to their shared growth habits and floral characteristics (Goldblatt and Manning, 2000).

2.3.7 *Dianthus holopetalus* Turz.

Dianthus holopetalus is a perennial herbaceous plant characterized by its slender stems and narrow, grass-like leaves. The plant produces small, star-shaped flowers that are usually pink or white, with petals that are notched or slightly fringed. It is adapted to thrive in various environmental conditions. *Dianthus holopetalus* is native to South Africa and is predominantly found in the eastern regions, including the Eastern Cape and KwaZulu-Natal. It typically grows in grasslands, rocky slopes, and other well-drained habitats. This species contributes to the region's diverse montane flora (Pooley, 1998; Goldblatt and Manning, 2000).

2.3.8 *Dianthus kamisbergensis* Sond.

Dianthus kamisbergensis is a perennial herb that forms low, dense tufts of narrow, linear leaves. The plant produces small, vibrant pink or white flowers with fringed petals, typically blooming in the spring and summer months. It is well-adapted to arid environments and rocky terrains. *Dianthus kamisbergensis* is endemic to South Africa, specifically found in the Kamiesberg region of the Northern Cape. This species thrives in high-altitude, rocky outcrops and mountainous areas, where it endures the harsh, dry conditions typical of this region (Pooley, 1998; Goldblatt and Manning, 2000).

2.3.9 *Dianthus laingsburgensis* S.S.Hooper

Dianthus laingsburgensis is a small, perennial herb known for its compact, tufted growth habit. The plant has narrow, grass-like leaves and produces small, fragrant flowers, typically pink or white, with finely fringed petals. It is well-adapted to the arid conditions of its native habitat. *Dianthus laingsburgensis* is endemic to South Africa, specifically in the Laingsburg region of the Western Cape. It grows in semi-arid environments, often found in rocky or sandy soils, and is well-suited to the harsh climatic conditions of this region (Pooley, 1998; Goldblatt and Manning, 2000).

2.3.10 *Dianthus micropetalus* Ser.

Native to Botswana, Cape Provinces, Free State, Northern Provinces of South Africa (Govaerts, 2000). This species is distinguishable from the rest of the species by short exserted petals but has the same resemblance with *D. thunbergii* and it has been previously confused with this species (Burtt Davy, 1922). It is known for its small, delicate flowers and its adaptation to specific ecological niches. This species is adapted to rocky, well-drained soils, often found in mountainous or high-altitude grasslands. It is well-suited to surviving in harsh, dry conditions with high sunlight exposure (Goldblatt and Manning, 2000).

2.3.11 *Dianthus mooiensis* F.N. Williams

It is native to Western Cape, Eastern Cape, Gauteng, Mpumalanga, Northwest, and KwaZulu-Natal (Govaerts, 2000). It is mostly found in high veld areas. *D. mooiensis* is closely related to *D. zeyheri* and *D. crenatus* sharing fimbriated flowers (Hooper, 1959;1961). This species is adapted to rocky, well-drained soils, often found in high-altitude grasslands or mountainous regions. It thrives in areas with good sunlight exposure and can withstand dry conditions. *Dianthus mooiensis* is closely related to other species within the genus *Dianthus* that inhabit similar environments in southern Africa. Species like *Dianthus basuticus* and *Dianthus micropetalus* share similar ecological niches and morphological traits, making them potential close relatives (Goldblatt and Manning, 2000).

2.3.12 *Dianthus namaensis* Schinz.

This species is native to southern Africa, particularly found in the arid and semi-arid regions of Namibia and South Africa. Ranges from Eastern Cape, through Western Cape up to Northern Cape and stretches to Namibia. The plant produces small, delicate flowers that are usually pink or white. The leaves are narrow, linear, and often grayish green, reflecting their adaptation to dry conditions. (Govaerts, 2000; Goldblatt and Manning, 2000). It is well-suited to withstand prolonged drought and intense sunlight, thriving in areas with limited water availability. (Goldblatt and Manning, 2000).

2.3.13 *Dianthus thunbergii* S.S. Hooper

The indigenous distribution of *Dianthus thunbergii* spans from the Eastern Cape to the Western Cape Provinces, (Govaerts, 2000). A distinguishing feature of *Dianthus thunbergii* is its petal-lamina, which is devoid of hairs (Hooper, 1959). It is a perennial herb, typically growing in a tufted or clump-forming manner. It tends to form dense mats, which is a common feature among *Dianthus* species adapted to rocky or mountainous habitats. *Dianthus thunbergii* is closely related to other *Dianthus* species that share similar habitats and morphological traits (Goldblatt and Manning, 2000).

2.3.14 *Dianthus transvaalensis Burtt Davy*

This species is native to the Free State and Mpumalanga Provinces (Govaerts, 2000), and it is mostly found in the high veld areas. It is known for its distinctive features and adaptations to its local environment. *Dianthus transvaalensis* is characterized by shortly branched rhizome above the ground, bearing several crowns. The leaves of this species form basal tufts which is 5-10 cm long (Burtt Davy, 1922). *Dianthus transvaalensis* is closely related to other *Dianthus* species that share similar morphological traits and ecological adaptation. Potential close relatives include *Dianthus thunbergii*, which shares similarities in growth form and floral characteristics, such as tufted growth and small, fringed flowers, and also occurs in rocky, high-altitude environments. Additionally, *Dianthus burchellii* displays comparable adaptations to dry, rocky soils, with similar growth habits and flower structures (Goldblatt and Manning, 2000).

2.3.15 *Dianthus zeyheri* Sond.

Dianthus zeyheri is a perennial, slender herb with a carrot-like root and a short, woodystem. Stems are branching, each branch with a robust flowering stalk, 200-750 mm tall. Leafy shoots appear close to the base of the plants, up to half the height of the flowering stalks. Leaves are lance-shaped, 20-90 mm long, grey green at least on thelower parts, and hairless. Flowering shoots form terminal clusters of 4-7 flowers (Pooley, 1998). *Dianthus zeyheri* occurs in all provinces of South Africa except the Western Cape, Northern Cape, and Free State (Govaerts, 2000). It is found in the EasternCape, extending throughout the coastal region of KwaZulu-Natal, to the northernmostparts of Zululand, close to the border of Mozambique (Germshuizen, 2006). *Dianthus zeyheri* is closely related to other species within the *Dianthus* genus that share similar ecological adaptations and morphological traits (Goldblatt and Manning, 2000).

Chapter Three

Materials and Methods

3.1 Taxon sampling and collection of plant material

Approximately 100 specimens per species of South African *Dianthus* were examined, sourced from the Bews Herbarium (NU), KZN Herbarium (NH), and National Herbarium (PRE). However, samples earmarked for DNA extraction were specifically acquired from only three species (*D. basuticus*, *D. kamisbergensis* and *D. transvaalensis*), and these samples were meticulously prepared for subsequent analysis. Details of these species can be found in Table 3.1. The DNA extraction and Polymerase Chain Reaction (PCR) procedures were conducted at the University of South Africa, Eureka Building, while the sequencing reactions took place at the Inqaba laboratory Biotechnology.

Moreover, among the 15 South African *Dianthus* species, *D. burchellii*, *D. crenatus*, and *D. holopetalus* were excluded from the DNA extraction and molecular analyses due to a lack of plant materials from the field and herbarium collection. To supplement the dataset, we obtained an additional 10 South African sequences and 85 non-South African sequences from GenBank/NCBI, based on Valente *et al*. (2010) and Fassou *et al*. (2022) studies. This aggregation resulted in a total of 98 taxa consisting of 94 *Dianthus*, and 4 *Petrorhagia* as outgroups. Detailed voucher information and GenBank accession numbers can be found in Table 3.1.

3.1.1 Choosing outgroups

Outgroups were chosen based on the molecular studies of Valente *et al*. (2010) and Fassou *et al*. (2022). Previous studies have indicated *Velezia* and some species of *Petrorhagia* being more closely related to *Dianthus* (Fior *et al*., 2006). Therefore, *Petrorhagia* species; *P. prolifera* (L.) P.W. Ball & Heywood; *Petrorhagia* spp. and *P. thessala* (Boiss.) P.W. Ball& Heywood were chosen as outgroups, following Fassou *et al*. (2022) and Valente *et al*. (2010).

Table 3.1: Voucher and GenBank accession/ID numbers for each DNA sequence used formolecular analyses.

Taxon	**Voucher Name**	**Gene Markers and Accession Numbers**			
		ITS	**trnK-matK**	**trnH-psbA**	**psbA-trnK**
Dianthus albens Sol.	Manning & Valente 171LV07 (MA)	GU440775	GU441115	GU441001	GU440891
Dianthus anatolicus Boiss.	Aldasoro *et al.* 2536 (MA690057)	GU440777	GU441117	GU441003	GU440893
Dianthus armeria L.	Vargas, P. 222PV99	GU440779	GU441119	GU441005	GU440895
Dianthus armeria L.	s.n.	GU440780	GU441120	GU441006	GU440896
Dianthus arrosti C. Presl	Certa & Di Martino 17869 (Reading 19 2006 29)	GU440781	GU441121	GU441007	GU440897
Dianthus barbatus L.	Aedo, Aizpuru & Pedrol	GU440782	GU441122	GU441008	GU440898
Dianthus basuticus Burtt Davy	Mnxati, E.S. 224 (NH)	NA	NA	NA	NA
Dianthus biflorus Sm.	Vargas, P. 80PV08	GU440785	GU441125	GU441010	GU440900
Dianthus bolusii Burtt Davy	Manning, J. 360	GU440786	GU441126	GU441011	GU440901
Dianthus caespitosus Thunb. subsp. *caespitosus* Thunb.	Manning, J. 361	GU440789	GU441129	GU441014	GU440904
Dianthus caespitosus Thunb. subsp. *pectinatus* Hooper	Manning & Valente 178LV07	GU440788	GU441128	GU441013	GU440903
Dianthus calocephalus Boiss.	Medina *et al*. 2569 (MA 742233)	GU440791	GU441131	GU441016	GU440906
Dianthus capitatus Balbis ex DC	Vargas, P. *et al*. 147PV06	GU440792	GU441132	GU441017	GU440907
Dianthus carmelitarum Reut. ex Boiss	Herrero & al AH1183 (MA 687243)	GU440793	GU441133	GU441018	GU440908
Dianthus carthusianorum L.	Aldasoro 8729 (MA727515)	GU440794	GU441134	GU441019	GU440909
Dianthus charidemi Pau	Vargas, P. 71PV06	GU440795	GU441135	GU441020	GU440910
Dianthus chinensis L.	Kim, 2005-0815	GU440796	GU441136	GU441021	GU440911
Dianthus cibrarius Clem.	Nieto G 1571GN (MA 644333)	GU440797	GU441137	GU441022	GU440912
Dianthus ciliatus Guss.	Aldasoro J 3193 (MA 699818)	GU440798	GU441138	GU441023	GU440913
Dianthus cintranus Boiss. & Reuter	Monjardino J 45145 (MA509698)	GU440799	GU441139	GU441024	GU440914
Dianthus corymbosus Sibth. & Sm.	Vargas, P. 119PV08	GU440801	GU441141	GU441026	GU440916
Dianthus crassipes R. de Roemer	Devesa JA & Vazquez FM QD31 (MA 628878)	GU440803	GU441143	GU441028	GU440918
Dianthus cretaceus Adam	Aedo & al 11669 (MA 743655)	GU440804	GU441144	GU441029	GU440919
Dianthus crinitus Sm.	Balao *et al*. (SEV 249775)	GU440805	GU441145	GU441030	GU440920
Dianthus crinitus Sm.	Muñoz Garmendia & al 4661 (MA 688938)	GU440806	GU441146	GU441031	GU440921
Dianthus cruentus Griseb.	Vargas, P. 127PV08	GU440807	GU441147	GU441032	GU440922
Dianthus cyri Fisch. & Mey.	Medina *et al*. 2462 (MA742743)	GU440808	GU441148	GU441033	GU440923
Dianthus deltoides L.	Christenhusz MJM 4326 TUR	GU440809	GU441149	GU441034	GU440924
Dianthus deltoides L.	Rico *et al.* SALA 111141	GU440810	GU441150	GU441035	GU440925
Dianthus diffusus Sm.	Wilkinson, S. SW65	GU440811	GU441151	GU441036	GU440926
Dianthus diffusus Sm.	Wilkinson, S. SW72	GU440812	GU441152	GU441037	GU440927
Dianthus eretmopetalus Stapf.	RBG Edinburgh, Living Coll., FBI 23	GU440813	GU441153	NA	NA

Dianthus erinaceus Boiss.	RBG Edinburgh, Living Coll., FBI 19	GU440814	GU441154	GU441038	GU440928
Dianthus excelsus Hooper	Bidgood, S., Mbago F. & Vollesen, K. 2562	GU440815	GU441155	GU441039	NA
Dianthus fischeri Sprengel	K Skvortsov 17871 (MA625551)	GU440816	GU441156	GU441040	GU440929
Dianthus gallicus Pers.	Vargas, P. 274PV06	GU440817	GU441157	GU441041	GU440930
Dianthus giganteus D'Urv.	Castroviejo & Nisa 15741 SC (MA 689440)	GU440819	GU441159	GU441043	GU440931
Dianthus giganteus D'Urv.	Wilkinson, S. SW88	GU440820	GU441160	GU441044	GU440932
Dianthus glacialis Haenke	Tribsch, Solstad & Jörgensen 11095	GU440821	GU441161	GU441045	GU440933
Dianthus gracilis Sm.	Wilkinson, S. SW83	GU440822	GU441162	GU441046	GU440934
Dianthus gredensis Pau ex Caballero	Vargas, P. 278PV06	GU440823	GU441163	GU441047	GU440935
Dianthus haematocalyx Boiss. & Heldr.	Vargas, P. 87PV08	GU440824	GU441164	GU441048	GU440936
Dianthus hyssopifolius L.	Aedo, Aizpuru & Pedrol CA9506 (MA 707266)	GU440826	GU441166	GU441049	GU440937
Dianthus integer Vis.	Vargas, P. 83PV08	GU440827	GU441167	GU441050	GU440938
Dianthus kamisbergensis Sond.	Pretorius, W.A.J. 236. (NU)	NA	NA	NA	NA
Dianthus knappii (Pant.) Ascherson & Kanitz ex Borbás	Christenhusz 4316 TUR	GU440828	GU441168	GU441051	GU440939
Dianthus laingsburgensis Hooper	Valente, LM 136LV07	GU440829	GU441169	GU441052	GU440940
Dianthus langeanus Willk.	Martin-Blandco 3126 (MA615484)	GU440830	GU441170	GU441053	GU440941
Dianthus laricifolius Boiss. & Reuter	Molina C & Montamart G 30TWM7346 (MA730782)	GU440831	GU441171	GU441054	GU440942
Dianthus legionensis (Willk.) F.N. Williams	Wilkinson, S. SW4	GU440832	GU441172	GU441055	GU440943
Dianthus leptoloma Steud. Ex A. Rich.	Friis, I. 4531 K	GU440833	GU441173	GU441056	GU440944
Dianthus leucophaeus Sibth. & Sm.	Vargas, P. 161PV06	GU440834	GU441174	GU441057	GU440945
Dianthus libanotis Lab.	Gonzalo *et al.* 56 (MA743059)	GU440835	GU441175	GU441058	GU440946
Dianthus longiglumis Del.	Polunin, O. 11634 K	GU440836	GU441176	GU441059	NA
Dianthus micranthus Boiss. & Heldr.	Aldasoro *et al.* 2619 (MA690359)	GU440839	GU441179	GU441062	GU440949
Dianthus microlepis Boiss.	Bowen, 7730 (Reading 19 2006 21)	GU440840	GU441180	GU441063	GU440950
Dianthus micropetalus Ser.	Retief en Germishuizen 231 K	GU440841	GU441181	GU441064	NA
Dianthus mooiensis F.N. Williams	Valente, LM 313LV07	GU440842	GU441182	GU441065	GU440951
Dianthus multiaffinis Pau	Riera J *et al.* 30SYK2818 (MA 692259)	GU440843	GU441183	GU441066	GU440952
Dianthus multiceps Costa ex. Willk.	Pedrol J 5848JP (MA 614702)	GU440844	GU441184	GU441067	GU440953
Dianthus myrtinervius Griseb.	Wilkinson, S. SW91	GU440845	GU441185	GU441068	GU440954
Dianthus namaensis Schinz	Vargas, P. 435PV00	GU440846	GU441186	GU441069	GU440955
Dianthus orientalis Adams	Nisa *et al.* 1023(MA 689355)	GU440847	GU441187	GU441070	GU440956
Dianthus pallens Sibth. & Sm.	Aldasoro & Alarcón A9764	NA	GU441188	GU441071	GU440957
Dianthus pavonius Tausch.	Tribsch & Bendiksby 11112	GU440848	GU441189	GU441072	GU440958
Dianthus plumarius L.	Christenhusz 75-1201 TUR	GU440851	GU441192	GU441075	GU440961
Dianthus pungens L.	Güemes J *et al.* 2679 (MA628967)	GU440852	GU441193	GU441076	GU440962

Dianthus pygmaeus Hayata	RBG Edinburgh, Living Coll., FBI 36	GU440853	GU441194	GU441077	GU440963
Dianthus pyrenaicus Pourret	Aedo, Aizpuru & Pedrol 12198 (MA 732210)	GU440854	GU441195	GU441078	GU440964
Dianthus rupicola Biv.	Garcia-Verdugo C., 19CG06	GU440855	GU441196	GU441079	GU440965
Dianthus serotinus Waldst. & Kit.	Christenhusz 4324 TUR	GU440857	GU441198	GU441081	GU440967
Dianthus serratifolius Sm.	Wilkinson, S. SW74	GU440858	GU441199	GU441082	GU440968
Dianthus serrulatus Desf.	Balao *et al*. (SEV 218923)	GU440859	GU441200	GU441083	GU440969
Dianthus serrulatus Desf.	Lafkih *et al.* 695 (Reading 19 2006 60)	GU440860	GU441201	GU441084	GU440970
Dianthus stenopetalus Griseb.	Wilkinson, S. SW89	GU440861	GU441202	GU441085	GU440971
Dianthus strictus Banks & Solander	Dagher & Dardas 1676. P00 05257 Reading	GU440862	GU441203	GU441086	GU440972
Dianthus strymonis Rech.	Wilkinson, S. SW82	GU440863	GU441204	GU441087	GU440973
Dianthus subulosus Freyn et Conr.	Tribsch, 11172	GU440865	GU441206	GU441089	GU440975
Dianthus superbus L.	Filimonova T.93-03 (MA722668)	GU440866	GU441207	GU441090	GU440976
Dianthus superbus L.	RBG Edinburgh, Living Coll., FBI 34	GU440867	GU441208	GU441091	GU440977
Dianthus sylvestris Wulfen	Aedo C *et al*. 7993 (MA698149)	GU440868	GU441209	GU441092	GU440978
Dianthus tenuiflorus Griseb.	Vargas, P. 123PV08	GU440871	GU441212	GU441095	GU440981
Dianthus thunbergii Hooper	Manning, J. 359	GU440872	GU441213	GU441096	GU440982
Dianthus toletanus Boiss. & Reuter	Wilkinson, S. SW43	GU440873	GU441214	GU441097	GU440983
Dianthus transvaalensis Burtt Davy	Abbott, A.7888. (NU)	NA	NA	NA	NA
Dianthus trifasciculatus Kit.	Cernoch 47357 Reading	GU440874	GU441215	GU441098	GU440984
Dianthus tripunctatus Sibth. & Sm.	Aldasoro & Alarcón A9792	GU440875	GU441216	GU441099	GU440985
Dianthus turkestanicus Preobr.	RBG Edinburgh, Living Coll., FBI 22	GU440876	GU441217	GU441100	GU440986
Dianthus tymphresteus (Boiss. & Spruner) Boiss.	Wilkinson, S. SW75	GU440877	GU441218	GU441101	GU440987
Dianthus versicolor Fischer ex Link	Castroviejo & Valdés Bermejo 14266 SC (MA 614400)	GU440878	GU441219	GU441102	GU440988
Dianthus viscidus Bory & Chaub.	Wilkinson, S. SW67	GU440879	GU441220	GU441103	GU440989
Dianthus zederbaueri Vierh.	Aldasoro *et al*. 2645 (MA689787)	GU440880	GU441221	GU441104	GU440990
Dianthus zeyheri Sond.	Valente, LM 312LV07	GU440881	GU441222	GU441105	GU440991
Dianthus zonatus Fenzl	Aldasoro & Alarcón A9822	GU440882	GU441223	GU441106	GU440992
Petrorhagia prolifera (L.) P.W. Ball & Heywood	Valente, LM 124LV07	GU440883	GU441224	GU441107	GU440993
Petrorhagia spp.	Vargas, P. 77PV08	GU440884	GU441225	GU441108	GU440994
Petrorhagia spp.	Wilkinson, S. SW40	GU440886	GU441227	GU441110	GU440996
Petrorhagia thessala (Boiss.) P.W. Ball & Heywood	Vargas, P. 109PV08	GU440885	GU441226	GU441109	GU440995

3.2 DNA extraction, amplification and sequence alignment

Genomic DNA was isolated from 0.01 – 0.3g of herbarium-dried leaf materials using DNA extraction kit (i.e., Zymo Research, USA). The DNA extraction was done following the manufacturer protocol. Purification of samples was done using Zymo research kit and all the DNA extraction and PCR amplification reactions were carried out at the University of South Africa, Eureka building, Johannesburg. The UNISA laboratory was used for DNA extraction and the sequencing reactions took place at the Inqaba laboratory Biotechnology.

All PCRs was performed using ReadyMix Master (Advanced Biotechnologies, Epson,Surrey, UK). The primers used for PCR reactions are listed in Table 3.2. Bovine serumalbumin (3.2% BSA) was added to both plastid reactions. This additive serves as stabilizer for enzymes, reduces problems with secondary structure, and improves annealing (Palumbi, 1996). The PCR amplification was performed using the Fast Thermal Cycler machine.

Programs used for PCR amplification was as follows:

a) for ITS the protocol used consisted of pre-melt at 94°C for 60 sec, denaturation at 94°C for 60 sec, annealing at 48°C for 60 sec, extension at 72°C for 3 min (for 28 cycles), followed by a final extension at 72°C for 7 min and

b) for *matK* the protocol used consisted of pre-melt at 94°C for 3 min, denaturation at 94°C for 60 sec, annealing at 52°C for 60 sec, extension at 72°C for 2 min (for 30 cycles), final extension was at 72°C for 7 min.

c) for *trnH-psbA* the protocol used consisted of a pre-melt at 94°C for 3min, denaturationat 94°C for 60 sec, annealing at 48°C for 68 sec, extension at 72°C for 1 min (for 28 cycles), final extension at 72°C for 7 min.

d) for *trnK-psbA* the protocol used consistedof a pre-melt at 96°C for 1m 30 sec, denaturation 95°C for 30 sec, annealing 50°C for1 min, extension 72°C for 1 min 30 sec (for 35 cycles) and final extension at 72°C for20 min.

e) For *trnK-matK* the protocol is similar to *trnK-psbA* except that the extension step was only 1 min.

The PCR products were verified by electrophoresis in 1% agarose gels stained with ethidium bromide. The PCR products were sent to the Inqaba laboratory Biotechnology for cycle sequencing. Cycle sequencing reactions werecarried out at Inqaba Laboratory Biotechnology.

3.3 Data analyses

Complementary strands were assembled, and edited using Sequencer 3.1 (Gene Codes, Ann Arbor, Michigan, USA). ITS, trnH-psbA; trnK-matK and trnK-psbA were aligned manually in PAUP* (version 4.0b.10; Swofford, 2003). All sequences were aligned using the MUSCLE program, and any necessary alignment adjustments or exclusions of ambiguously aligned sequences were also performed using MUSCLE.

3.4 Molecular phylogenetic analyses

The maximum parsimony (MP) tree was constructed based on plastids trnK-matK, trnK-psbA, trnH-psbA, and nuclear (ITS) sequences. The tree searches were analyzed using Heuristic search with 1000 replicates of random taxon addition, holding 10 trees at each step during stepwise addition with tree bisection reconnection (TBR) branch swapping algorithm and saving multiple equal parsimonious trees (MulTrees). All character transformations were treated as likely thus Fitch parsimony (Fitch, 1971) and all the analyses were conducted using PAUP version.4.0b 10 program (Swofford, 2003). Branch lengths were calculated using DELTRAN (Delayed transformation) character optimisation instead of ACCTRAN (accelerated transformation) due to report errors with PAUP version.4.0b 10.

Bootstrap analysis was used to estimate the support for each clade (Felsenstein, 1985) using TBR swapping with Fitch weights and retaining 10 trees per replicates. Bootstrap support was categorized as high (85-100), moderate (75-84) and low (50-74).

Bayesian Inference (BI) was conducted with MrBayes v.3.2.7 (Ronquist and Huelsenbeck 2003), using four parallel Markov Chain Monte Carlo (MCMC) runs for a total of 10 million generations and MODELTEST version.3.06 (Posada and Crandall, 1998). A model test was done to determine the most appropriate model for each genebased on Akaike information criterion (AIC; Sugiura, 1978; Table 4.1). All tree file outputs were burnt using tree annotator v. 2.7.4 and the trees were visualized on FigTree, a graphical viewer of phylogenetic trees.

3.5 Distribution maps analyses

Occurrence data, encompassing latitude and longitude geographic coordinates of 14 *Dianthus* species was extracted from the SANBI online BRAHMS database. These coordinates underwent conversion from degrees, minutes, seconds (DMS) to decimal degrees (DD) points. Georeferencing was done and quality control was also done before these points were incorporated and visualized in Quantum Geographic Information System (QGIS version 3.16) software as a shapefile. To ensure accuracy, outliers were reviewed in relation to the South Africa Province border shapefile (Opendatasoft, 2024) and removed as needed.

In the layout manager, distribution maps were generated for each species. Two types of maps were created: one illustrating the combined distribution of all 14 *Dianthus* species, and individual maps for each species. A map for *Dianthus burchellii* could not be generated due to insufficient locality data. The final map layouts were exported in PDF format to facilitate a clear and comprehensive analysis of the species distribution maps.

Table 3.2: Details of primers used for PCR amplification.

Locus	**Primer**	**Reference**
ITS		
ITS5	GGA AGT AAA AGT CGT AAC AAG G	White *et al.* 1990
ITS4	TCCTCCGCT TAT TGATAT GC	White *et al.* 1990
trnH-psbA		
trnH	TGA TCC ACT TGG CTA CCG CC	Sang *et al.* 1997
psbA	GCT AAC CTT GGT ATG GAA GT	Sang *et al.* 1997
trnK-matK		
trnK-F	GGG TTG CTA ACT CAA TGGTAG AG –	Wicke and Quandt 2009
CARYmatK1440R	AKC GTA AAT GAG AGG ATT G	Schäferhoff *et al.* 2009
trnK-psbA		
trnK	GGG TTG CTA ACT CAA TGGTAG AG –	Sang *et al.* 1997
psbA	GCT AAC CTT GGT ATG GAA GT	Sang *et al.* 1997

Chapter Four

Results

4.1. Statistics of molecular data

A robust phylogenetic tree of three plastids (trnH-psbA, trnK-matK and trnK-psbA) and ITS nuclear region was assembled using 98 taxa of *Dianthus* and *Petrorhagia* species. Among the gene regions, trnH-psbA produced the shortest basepair characters, while trnK-matK exhibited the highest basepair characters and with greater stability. The summary of DNA matrix and maximum Parsimony statistics for individual gene regions is presented inTable 4.1, along with the best JMODELTEST analyses results.

The ITS dataset encompasses 95 taxa with 659 characters, 181 constant characters and 117 potentially parsimony informative characters. The F81+I+G was chosen as the best JMODELTEST (Table 4.1). The trnH-psbA dataset contained 258 characters from 93 taxa with183 constant characters and 36 potentially parsimony informative characters. For trnH-psbA, TN93+I+G model was selected as the best by JMODELTEST (Table 4.1). The trnK-matK dataset had the highest number of total characters (1376) from 95 taxa, with 1252 constant characters and 52 potentially parsimony informative characters. The GTR+I+G model was selected as the best by JMODELTEST (Table 4.1). The trnK-psbA dataset comprised of 772 characters from 91 taxa, 668 constant characters and the lowest number (21) of potentially parsimony informative characters. The HKY85+I+G model was chosen as the best JMODELTEST (Table 4.1).

However, the combined dataset including ITS, trnH-psbA, trnK-matK, and trnK-psbA was comprised of 98 taxa that resulted in 3065 characters. The dataset had 2284 constant characters, 555 uninformative characters, and 226 parsimony-informative characters (Table 4.1).

Table 4.1. Summary of DNA matrix and maximum Parsimony statistics for the aligned, analyzed and number of informative for the individual gene regions. The best JMODELTEST analyses results were also indicated.

Gene regions	**No. of characters**	**No. of Taxa**	**Characters constant**	**Parsimony uninformative**	**Parsimony informative**	**Missing**	**jModeltest AIC**	**Number of Trees per run**
ITS	659	95	181	361	117	>5%	F81+I+G	
trnH- psbA	258	93	183	39	36	>5%	TN93+I+G	
trnK-matK	1376	95	1252	72	52	>5%	GTR+I+G	
trnK-psbA	772	91	668	83	21	>5%	HKY85+I+G	
Combined regions	3065	98	2284	555	226	>5%	-	20001

4.2 Phylogenetic Analyses of all the *Diànthus* species in this study

The results of the separate analysis of plastid sequences did not support the use of plastid gene markers independently to assess the phylogenetic relationships within the genus *Dianthus*. However, the combination of both nuclear (ITS) and plastid (trnH-psbA, trnK-matK, and trnK-psbA) datasets was well-supported with 0.92 PP (Figure 4.1) and 100/BS (Figure 4.2). On the other hand, the separate analyses of ITS sequences (Appendix A) revealed unsupported topological conflicts in resolving the two *D. caespitosus* as a sister group to the subclade of *D.namaensis*. According to the ITS phylogenetic tree, South African species are monophyletic, with the exception of one species, *D. laingsburgensis*. *Dianthus laingsburgensis* is nested with Ethiopian species clade, forming a sister group/species to *D. leptoloma*.

The phylogenetic analyses of the combined datasets (Figure 4.1 and Figure 4.2) revealed *Dianthus* as monophyletic when *Petrorhagia* was used as an outgroup (s). Four strongly supported clades were identified (Africa Lineage, Eurasian radiation, Section Verruculosi and Section Armerium), indicating geographical distinctions (Figure 4.1). Africa Lineage is primarily found in southern and eastern Africa, including countries like South Africa and Ethiopia. Eurasian Radiation is widespread across Europe and Asia, particularly in regions extending from the Mediterranean to Central Asia. Section Verruculosi is concentrated in the eastern Mediterranean, with significant representation in countries like Greece and Turkey. Section Armerium is also mainly found in the Mediterranean region, particularly in southwestern Europe, including Spain and France. These distributions highlight the diverse habitats and evolutionary adaptations of the *Dianthus* genus across different continents (Fassou *et al*., 2022).

The Eurasian Radiation is comprised of five subclades, while sections Verruculosi and Armerium each formed well-supported distinct clades (Figure 4.1). The African lineage (Figure 4.1) included two subclades, one with South African species. However, the specific placement of certain species (*D. laingsburgensis*, *D. serrulatus* B, *D. crinitus* A, *D. crinitus* B, and *D. excelsus*) remains unresolved within the African Lineage.

To gain a deeper understanding of the relationships within the African clades, particularly focusing on the South African species, it is crucial to consider the results from the Bayesian analyses. In Figure 4.2, the outgroup *Petrorhagia* supported the monophyletic state of *Dianthus*. *Petrorhagia thessala* was resolved as sister to the African *Dianthus* species, while *Petrorhagia* sp. B was resolved (BS/100) possibly as *Petrorhagia prolifera*, but a physical specimen was not checked to confirm this assumption due to logistics in getting the material from Turkey. According to the

Bayesian Analyses of the African lineage or species (Figure 4.2), two geographically distinct clades can be identified, the Ethiopia & Northern African species (BS/100), and Western & Eastern region of South Africa (BS/100). Within the South African species, two more subclades with strong bootstrap support can be identified. One consists of *D. laingsburgensis*, *D. transvaalensis*, *D. basuticus*, *D. caespitosus*, *D. namaensis*, *D. mooiensis*, *D. micropetalus* and *D. kamisbergensis* while the other consists of *D. thunbergii*, *D. bolusii*, *D. albens* and *D. zeyheri*.

4.3 Phylogenetic analysis and relationships among South African *Dianthus*

In both Figures 4.1 and 4.2, all African species were grouped within the African Lineage group, with South African species forming one cluster and Ethiopian and Northern African species forming another. However, *D. excelsus*, *D. serrulatus*, and *D. longiglumis* showed unresolved phylogenetic positions.

The unresolved status of these three Northern African species could be attributed to several factors. First, incomplete lineage sorting may have occurred if these species diverged rapidly during a period of high speciation, leading to indistinct phylogenetic signals. Second, historical hybridization events may have complicated their exact placement within the phylogeny. Finally, the genetic markers used in the analysis might lack sufficient resolution to differentiate these species adequately, particularly if they share a significant portion of their genetic material due to common ancestry or recent divergence.

The positioning of these Northern African species at the base or as sister taxa to the South African clade suggests they represent an older lineage that diverged before the more recent speciation events that produced the diverse South African species. This implies that the ancestors of these species were part of an early wave of *Dianthus* migration into Africa, diverging before the genus expanded and diversified in Southern Africa.

Moreover, the presence of unresolved Northern African species at the base of the South African clade suggests that *Dianthus* may have originated or initially diversified in Northern Africa or nearby regions, with subsequent dispersal events leading to the colonization of Southern Africa. The high species diversity and recent divergence within the South African clade indicate that once *Dianthus* species reached Southern Africa, they encountered an environment highly conducive to rapid speciation. The unique climatic and ecological conditions of Southern Africa, particularly in the Cape Floristic Region, likely provided numerous niches that facilitated this diversification.

The rooted Bayesian Inference (BI) tree (Figure 4.2) derived from the analysis of combined genes revealed that South African species of *Dianthus* are nested together in two distinct clades, each consisting of smaller subclades. *Dianthus zeyheri* was identified as the closest species to *D. albens*. This relationship is also evident in Figure 4.1, with *D. zeyheri* identified as the closest species to *D. albens* (BS/88; Figure 4.1). *Dianthus zeyheri* and *D. albens* both have fimbriated petal margins, a character used to identify these species (Figure 4.3 **A).**

Surprisingly, *D. thunbergii* and *D. bolusii*, which belong to different regions (Eastern and Western, respectively), were discovered to be closely associated. Both species share similar floral characteristics, particularly in the shape and size of their petals, which are typical of the *Dianthus* genus. They frequently feature fringed petals, a common trait among many *Dianthus* species. Additionally, *Dianthus thunbergii and Dianthus bolusii form* a sister subclade alongside *D. zeyheri* and *D. albens*, representing species from the eastern and western regions, respectively.

In the Western and Eastern South Africa clade (Figure 4.2), a well-supported group with 100% bootstrap support had three subclades. Specifically, *D. caespitosus* is the sister taxon to *D. mooiensis*, while *D. namaensis* is the sister taxon to *D. micropetalus*, and both are sisters to the *D. kamisbergensis* species. Notably, *D. namaensis* and *D. micropetalus* are consistently supported as sister species in both phylogenetic trees (Figure 4.1 and Figure 4.2). Additionally, *D. laingsburgensis* was observed to form a group with *D. basuticus*, both of which are closely related to *D. transvaalensis*. However, results depicted in Figure 4.1 introduced some ambiguity regarding *D. transvaalensis*, which appears nested within Eurasian radiation species, but weakly supported (BS/64), falling below the accepted clade credibility value (BS/75). The connection between *D. basuticus* and *D. transvaalensis* may arise from the shared presence of basal leaves in both species. This characteristic serves as a distinguishing feature for identifying these species (Figure 4.3 **H**).

The Bayesian analysis of the plastid dataset (Appendix B) provided estimates of phylogeny that placed *D. s albens* weakly supported with *D. leptoloma* (the Ethiopian species). The remaining southern African taxa exhibited scattered distribution and lacked clear definition, with low posterior probability values in their respective clades. The monophyly of the ITS BI tree (Appendix A) resulted in improved resolution of relationships compared to the plastids BI tree (Appendix B). Despite these improvements, there still existed topological incongruence and insufficient support between the ITS and plastid datasets. This inconsistency was notably highlighted in the placement of *D. zeyheri*, depicted as a closest related species to *D. albens* in the ITS tree (see Appendix A), while its status remained unresolved in the plastids tree (Appendix B).

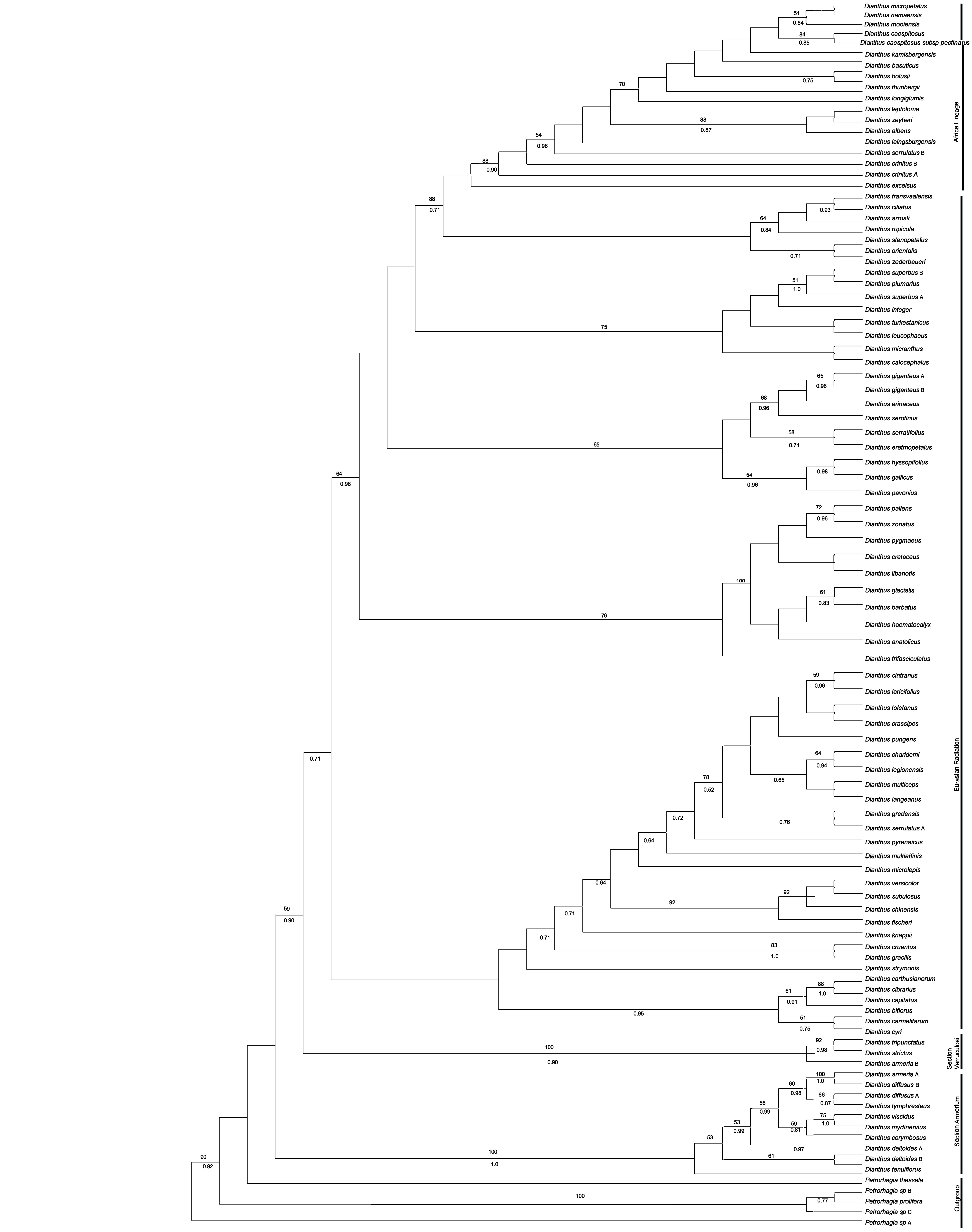
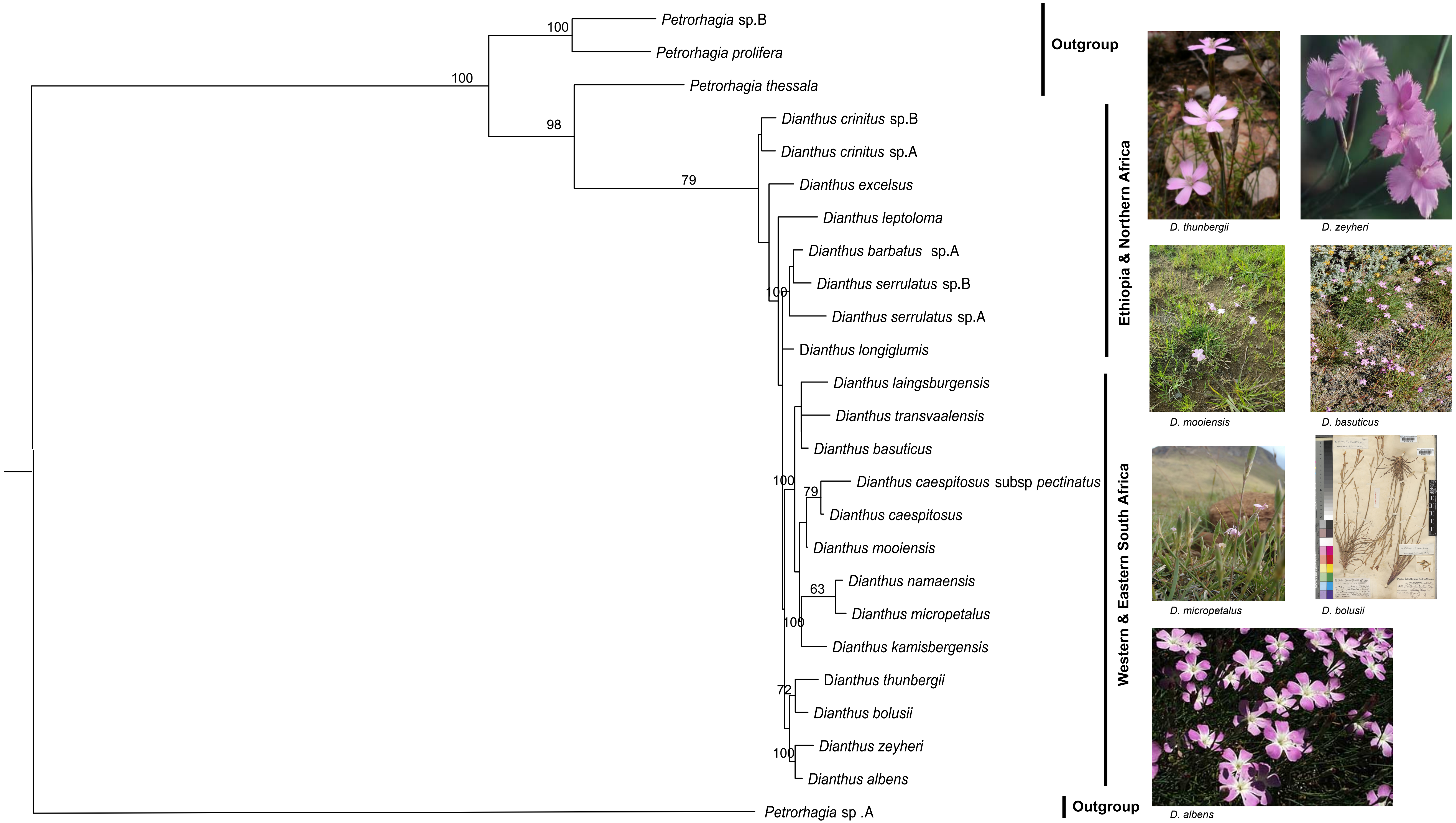

Dianthus micropetalus
Dianthus namaensis
Dianthus mooiensis
Dianthus caespitosus
Dianthus caespitosus subsp pectinatus
Dianthus kamisbergensis
Dianthus basuticus
Dianthus bolusii
Dianthus thunbergii
Dianthus longiglumis
Dianthus leptoloma
Dianthus zeyheri
Dianthus albens
Dianthus laingsburgensis
Dianthus serrulatus B
Dianthus crinitus B
Dianthus crinitus A
Dianthus excelsus
Dianthus transvaalensis
Dianthus ciliatus
Dianthus arrosti
Dianthus rupicola
Dianthus stenopetalus
Dianthus orientalis
Dianthus zederbaueri
Dianthus superbus B
Dianthus plumarius
Dianthus superbus A
Dianthus integer
Dianthus turkestanicus
Dianthus leucophaeus
Dianthus micranthus
Dianthus calocephalus
Dianthus giganteus A
Dianthus giganteus B
Dianthus erinaceus
Dianthus serotinus
Dianthus serratifolius
Dianthus eretmopetalus
Dianthus hyssopifolius
Dianthus gallicus
Dianthus pavonius
Dianthus pallens
Dianthus zonatus
Dianthus pygmaeus
Dianthus cretaceus
Dianthus libanotis
Dianthus glacialis
Dianthus barbatus
Dianthus haematocalyx
Dianthus anatolicus
Dianthus trifasciculatus
Dianthus cintranus
Dianthus laricifolius
Dianthus toletanus
Dianthus crassipes
Dianthus pungens
Dianthus charidemi
Dianthus legionensis
Dianthus multiceps
Dianthus langeanus
Dianthus gredensis
Dianthus serrulatus A
Dianthus pyrenaicus
Dianthus multiaffinis
Dianthus microlepis
Dianthus versicolor
Dianthus subulosus
Dianthus chinensis
Dianthus fischeri
Dianthus knappii
Dianthus cruentus
Dianthus gracilis
Dianthus strymonis
Dianthus carthusianorum
Dianthus cibrarius
Dianthus capitatus
Dianthus biflorus
Dianthus carmelitarum
Dianthus cyri
Dianthus tripunctatus
Dianthus strictus
Dianthus armeria B
Dianthus armeria A
Dianthus diffusus B
Dianthus diffusus A
Dianthus tymphresteus
Dianthus viscidus
Dianthus myrtinervius
Dianthus corymbosus
Dianthus deltoides A
Dianthus deltoides B
Dianthus tenuiflorus
Petrorhagia thessala
Petrorhagia sp B
Petrorhagia prolifera
Petrorhagia sp C
Petrorhagia sp A
Africa Lineage
Eurasian Radiation
Section Verruculosi
Section Armerium
Outgroup
51
0.84
84
0.85
0.75
70
88
0.87
54
0.96
88
0.90
88
0.71
64
0.84
0.93
0.71
51
1.0
75
65
0.96
68
0.96
58
0.71
65
54
0.96
0.98
64
0.98
72
0.96
100
61
0.83
76
59
0.96
64
0.94
0.65
78
0.52
0.76
0.72
0.64
0.71
0.64
92
92
0.71
0.71
83
1.0
88
1.0
61
0.91
0.95
51
0.75
59
0.90
100
0.90
92
0.98
100
1.0
60
0.98
66
0.87
56
0.99
53
0.99
59
0.81
75
1.0
0.97
53
61
100
1.0
90
0.92
100
0.77

Figure 4.1: Bayesian inference consensus tree showing sectional groupingsamong *Dianthus* species based on analysis of Combined dataset. Posterior probability (PP) values are presented below branches and Bootstrap support(BI) values are presented above branches.



D. thunbergii *D. zeyheri*

D. mooiensis *D. basuticus*

D. micropetalus *D. bolusii*

D. albens

Figure 4.2: Bayesian Analyses (BI) tree from analysis of combined dataset for African *Dianthus.* Bootstrap support (BS)values presented above the relevant branches.

Figure 4.3: Representative forms in South African *Dianthus*. **A** solitary branched flowers *Dianthus* species form**; B** white form *Dianthus* with fimbriated petal margins**; C** pink form *Dianthus* with fimbriated petal margins; **D** pink form *Dianthus* with deep incision petal lobes; **E** solitary white form *Dianthus* species with deep incision petal lobes; **F** cauline leaves form *Dianthus*; **G** combination of basal & cauline leaves form *Dianthus* species; **H** basal leaves form *Dianthus* species in rocky area habitat (Photo: Mnxati, E. S.).

4.4 *Dianthus* distribution in South Africa

Locality data collected from herbarium specimens and the SANBI online BRAHMS database indicated that *Dianthus* species are distributed across all nine provinces of South Africa (Figure 4.4; species distribution map). Individual species distribution maps are included in the Appendices (C-P). Some species are exclusively found in coastal areas, along the Western Cape coastline (e.g., *D. albens*, *D. bolusii*, *D. holopetalus*, and *D. caespitosus*, extending to Eastern Cape province). *Dianthus basuticus* is distributed across seven provinces, spanning Western Cape, Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga, certain areas of Gauteng, and part of Northern Cape. Based on available data, *D. crenatus* is confined to the eastern region of South Africa.

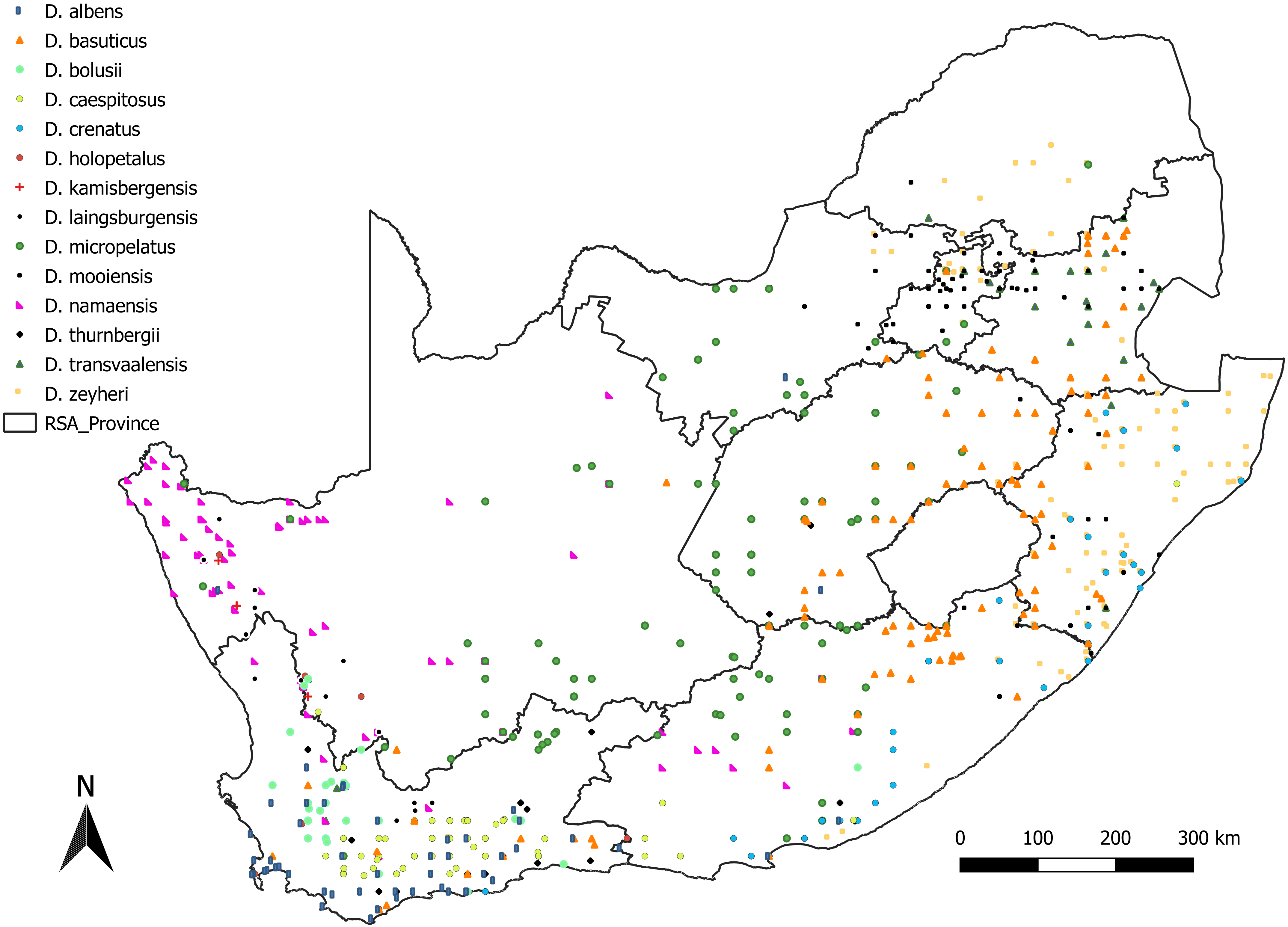

D. albens
D. basuticus
D. bolusii
D. caespitosus
D. crenatus
D. holopetalus
D. kamisbergensis
D. laingsburgensis
D. micropelatus
D. mooiensis
D. namaensis
D. thurnbergii
D. transvaalensis
D. zeyheri
RSA_Province
N
0
100
200
300 km

Figure 4.4: Distribution of South African species of *Dianthus* across all provinces of South Africa

Chapter Five

Discussion

The primary objective of this study was to investigate the relationship among South African species of *Dianthus* using molecular gene markers namely, ITS, trnH-psbA, trnK-matK, and trnK-psbA. The study aims to explore the monophyly of *Dianthus* and examine interspecific relationships between and within its various species. Additionally, the study seeks to elucidatethe influence of geography within the South African *Dianthus* genus, as inferred from our results.

5.1 Phylogenetic incongruence and topological conflicts

This study chooses to utilize the combined datasets of nuclear ITS and plastid markers trnH- psbA, trnK-matK, and trnK-psbA, employing Bayesian Analyses to generate phylogenetic trees. This decision is based on the consistent observation that various analyses of combined datasets, regardless of the methods applied, yield comparable topologies to those found in previous studies (Hershkovitz and Zimmer, 1999; Soltis *et al*., 2001; Valente *et al*., 2010; Fassou *et al*., 2022).

The incongruence observed in the separate analyses of ITS, trnH-psbA, trnK-matK, and trnK-psbA datasets, as well as topological conflicts in the placement of certain species, prompted the combination of datasets. This combination sought to address conflicting signals and establish a more reliable framework for understanding the phylogenetic relationships among *Dianthus* species in South Africa. The observed results might also be influenced by the absence of two South African species, *D. burchellii* and *D. crenatus*, from the dataset. Including these missing species in future studies could significantly alter the observed relationships or strengthen the bootstrap support for some of them.

Although the combination of the nuclear marker ITS and plastid markers trnH-psbA, trnK- matK, and trnK-psbA was supported by a statistical test of homogeneity, the individual parsimony tree topologies (trees not shown) estimated by each of these markers exhibited poorly supported conflicts. While the statistical test indicates congruence between ITS and plastid markers, the tree topologies from the two datasets differ in some respects. According to Wendel and Doyle (1998), the causes of these conflicts and poorly supported trees may include inappropriate model choice, homoplasy, insufficient signals, data paucity, horizontal gene transfer, and incomplete lineage sorting, among other possibilities.

The current study on South African *Dianthus* examined the effectiveness of ITS sequences in resolving phylogenetic relationships among species. The findings indicated that ITS sequences were not useful at the species level, showing limited utility. In contrast, Álvarez and Wendel (2003) found ITS sequences highly effective in resolving relationships at both species and genus levels within Caryophyllaceae, including *Dianthus*. Hershkovitz and Zimmer (1999) and Soltis *et al.* (2001) successfully used ITS to resolve phylogenetic relationships at broader taxonomic levels, such as sub-families, suggesting its utility beyond species-level analysis. These earlier studies imply that while ITS is beneficial for broader taxonomic categories, it may not be as effective for closely related species like those in South African *Dianthus*.

This study challenges the assertions made by Hershkovitz and Zimmer (1999); Soltis *et al*. (2001); Álvarez and Wendel (2003), which suggest that well-supported taxa remain unaffected by adjusting the alignment or discarding uncertainly aligned sequences. In the current study, this was also done during sequence alignment. Despite the success of ITS in generating reliable phylogenetic trees, Madhani *et al.* (2018) conducted a study of *Dianthus* in Turkey focusing on the genetic diversity and population structure. The study utilized ITS molecular marker to assess genetic variation and population structure. They found substantial genetic diversity within and between *Dianthus* populations. This diversity is essential for the species' adaptation and survival. Madhali *et al*. (2018) further clarified the evolutionary relationships among different *Dianthus* species in Turkey, identifying species complexes and ancestral lineages.

Of the *Petrorhagia* species used as the outgroup in this study, *P. thesalla* is the closest relative to the *Dianthus* species, while the other *Petrorhagia* species are more distantly related to *Dianthus*. Consequently, the genus *Petrorhagia* appears to be paraphyletic, raising several questions about its classification.

These findings align with the discoveries of Madhani *et al*. (2018), wherein *Petrorhagia* sect. Pseudotunica (Fenzl) Post & Kuntze and sect. Pseudogypsophila (A. Braun) P. Ball & Heywood were also linked with *Dianthus*.

Additionally, Madhani *et al*. (2018) noted that, considering *Dianthus* sect. Armeriastrum Ser. forms the most basally branching clade of the genus, and the clade composed of *Velezia rigida* and part of *Petrorhagia* sect. Pseudotunica that are sister to the *Dianthus* crown group, which seems predictable to extend the previously suggested grouping approach to place *Petrorhagia* sect. Pseudotunica in *Dianthus*.

5.2 Phylogenetic relationships within *Dianthus*

The findings of this study align with recent molecular phylogenetic studies on *Dianthus* (Valente *et al*., 2010; and Fassou *et al*., 2022), which have signaled the necessity for substantial changes in the classification of species and have accepted the inclusion of *Petrorhagia thessala* to *Dianthus*, as previously suggested (Soltis *et al*., 2001;Valente *et al*., 2010; and Fassou *et al*., 2022).

Our study has revealed that *Dianthus* is monophyletic with the inclusion of *Petrorhagia* as an outgroup. The branches in both phylogenetic trees (Figure 4.1 and Figure 4.2) are all synapogens, indicating a monophyletic group. The monophyly of *Dianthus* is consistent with the findings of Valente *et al*. (2010) and Fassou *et al*. (2022) when two genera, *Petrorhagia* and *Velezia*, which are sister to *Dianthus*, are both used as an outgroup. Furthermore, the current study has identified four strongly supported clades (Figure 4.1), with species from similar geographic regions grouped together. This clearly indicates that geography plays a crucial rolein the evolution of *Dianthus*, as our findings consistently assemble all *Dianthus* species according to their geographical locations.

The practice of classifying *Dianthus* species based on their geographical location is also evident in Valente *et al*. (2010), where species are segregated into lineages (Eurasian, African, Verruculosi, and Armerium) based on their country of occurrence. Notably, only one South African species, *D. transvaalensis*, is found nested within the Eurasian radiation (Figure 4.1). A possible hypothesis for the finding that *Dianthus transvaalensis* is the only South African species nested within the Eurasian radiation could be related to historical biogeographic events, such as long-distance dispersal or past connections between Africa and Eurasia. This suggests that *D. transvaalensis* might have a unique evolutionary history compared to other South African *Dianthus* species, possibly due to ancient migration or environmental shifts that allowed it to establish and persist in South Africa after originating or having ancestors in the Eurasian region. Another hypothesis could involve convergent evolution, where *D. transvaalensis* developed similar traits to Eurasian species due to similar environmental pressures, despite being geographically isolated. In contrast, Fassou *et al*. (2022)

present a differing perspective from Valente *et al*. (2010) by highlighting disagreement, particularly concerning South African species (*D. mooiensis*, *D. namaensis*, and *D. zeyheri*), which are observed to be nested within the Eurasian radiation clade.

The African lineage (Figure 4.1) is divided into two subclades and two clades in Figure 4.2. The first subclade includes species from Ethiopia and Northern Africa, while the second subclade encompasses species from South Africa. This suggests a classification based on the geographic origin within the African region. However, in Figure 4.1 the specific placement of species such as *D. laingsburgensis*, *D. serrulatus* B, *D. crinitus A*, *D. crinitus* B,and *D. excelsus* remains unresolved. This may be attributed to one or more of the following reasons: inappropriate model choice, homoplasy, insufficient signals, data paucity, horizontal gene transfer, and incomplete lineage sorting, among other factors. As noted by Wendel and Doyle (1998), these shortcomings can lead to poorly placed species within phylogenetic trees or unresolved species.

Furthermore, Figure 4.2 confirms that the outgroup *Petrorhagia* supports the monophyletic state of *Dianthus*. The results additionally affirm *Petrorhagia thessala* as sister to the African *Dianthus* species clade. Moreover, *Petrorhagia* sp. B was highly resolved (BS/100), possibly identified as *Petrorhagia prolifera* (Figure 4.2), indicating a potentially accurate classification of this species. This could be that *Petrorhagia sp. B* is indeed a distinct species that closely aligns with *Petrorhagia prolifera* based on its genetic and morphological characteristics. This strong resolution suggests that the species has clear, defining traits that differentiate it from other species within the genus, thereby supporting its accurate classification. Additionally, the robust support for this identification could imply that there is a low likelihood of misclassification, reinforcing the accuracy of the species determination within the phylogenetic framework presented. Geographically defined clades were observed within African *Dianthus* (Ethiopia & Northern African species; BS/100) and Western & Eastern regions of South Africa (BS/100), while two subclades (BS/100 & BS/72) were noted within the South African clade (Figure 4.2).

Within the clade consisting of species from Western and Eastern regions of South Africa, the Bayesian Inference tree highlighted four subclades among South African*Dianthus* species (Figure 4.2). The first subclade consists of *D. laingsburgensis-D. basuticus*, second subclade consists of *D. caespitosus–D. mooiensis*, third subclade consists of *D. namaensis–D. kamisbergensis*, and and fourth subclade consists of *D. thunbergii-D. albens*. In the third subclade , *D. namaensis* is resolved as the closest sister to *D. micropetalus* (Figure 4.2). However, Fassou *et al*. (2022) identified *D. mooiensis* as the closely related species to *D. namaensis*. This finding could be attributed to the smaller sample size of South African species in their study, as well as the fact that both species share white flowers with deeply incised petal lobes (Figure 4.3 **B** and **C**).

In the fourth subclade , *Dianthus zeyheri* consistently emerged as the closest species to *D. albens* (Figure 4.2) The relationship between *D. albens* and *D. zeyheri* was also evident in Figure 4.1 in our results with strong support (BS/88; PP/0.87). Nevertheless, Fassou *et al*. (2022) positioned *D. zeyheri* alone below the *D. namaensis-D. mooiensis* subclade. The isolation of *D. zeyheri* and the placement of *D. mooiensis* as the sister to *D. namaensis* in the study by Fassou *et al*. (2022) may be attributed to limited sampling of South African species. This limitation is also evident in Valente *et al*. (2010) study, where few representative members of South African species were included, providing no clear indication of the relationships among these species.

Dianthus thunbergii and *D. bolusii*, despite their distinct regional occurrences, were found nested together in the fourth subclade (Figure 4.2). According to Burtt Davy (1922) and Hooper (1959), these two species share similar morphological features, including fimbriated petals, 2-6 bract-pairs, and blue-grey leaves. Notably, the leaves in *D. bolusii* are densely clumped near the ground (Hooper, 1959; Vlok and Schutte-Vlok, 2010). Furthermore, the current findings identify *D. thunbergii* as the closely related species to *D. albens*, a species nested within the fourth subclade . This subclade functions as a sister subclade to both *D. thunbergii* and *D. bolusii* (see Figure 4.2). However, according to Hooper (1959), distinctions between *D. thunbergii* and *D. albens* can be made based on a combination of morphological characteristics. Specifically, *D. thunbergii* is characterized by narrower-ovate lobes at the tip of its calyx and lanceolate-elliptic bracts at the calyx base. In contrast, *D. albens* exhibits ovate-elliptic bracts.

Within the third subclade , *D. namaensis* and *D. micropetalus* emerge as the closest related species, supported by a moderate bootstrap (BS/63; Figure 4.2). This relationship is further confirmed by their adjacency in Figure 4.1, where they are depicted as closely related species but poorly supported bootstrap (BS/51). According to Burtt Davy (1922), these two species commonly share specific morphological characters, including basal leaves longer than stem-leaves, exserted petals, and fimbriated petals. Additionally, Burtt Davy (1922) provided some distinction between *D. micropetalus* and *D. namaensis*. *D. micropetalus* is characterized by very minute petals that are toothed or narrowly fimbriate, along with shorter basal leaves. In contrast, *D. namaensis* features longer basal leaves and lacerate fimbriated petals.

Furthermore, within third subclade , *D. kamisbergensis* is strongly supported (BS/100) as a sister species to both *D. namaensis* and *D. micropetalus* (Figure 4.2). *Dianthus kamisbergensis* share certain morphological characteristics with its closely related sister species, such as basal leaves longer than intermediate cauline leaves and exserted petals. Furthermore, Burtt Davy (1922) points out distinctive features of *D. kamisbergensis*, including a very short calyx and the presence of microscopic hairs on basal leaves, that segregate it from its sister species in third subclade .

The second subclade emerges as the sister subclade to the third subclade . Species within the second subclade are more closely related to those in the third subclade than to any other subclade in the combined datasets, as illustrated in Figure 4.2. The molecular grouping of these species may be attributed to shared morphological features, specifically fimbriate and exserted petals, as indicated by Burtt Davy (1922), Hooper (1959), and Vlok and Schutte-Vlok (2010). The strong molecular support for the close relationship between *D. caespitosus* and *D. mooiensis*, along with all species identified as related in the current study, aligns with evidence from previous research highlighting the presence of similar morphological characters (Burtt Davy, 1922; Hooper, 1959; and Vlok and Schutte-Vlok, 2010). However, the two species in the second subclade are still morphologically distinct. For instance, *D. caespitosus* exhibits basal leaves longerthan cauline leaves, while in *D. mooiensis,* leaves are predominantly cauline, with basal leavesbeing shorter than the intermediate cauline leaves. Additionally, cauline leaves in *D. caespitosus* are fewer and linear, whereas in *D. mooiensis* the leaves are broader and more abundant (Burtt Davy, 1922; Hooper, 1959).

It should be emphasized that the current findings deviate from those presented by Fassou *et al*.(2022). In their study, *D. mooiensis* was placed within the clade containing Euro-Asian species,specifically embedded within the *D. namaensis* species. Notably, Fassou *et al.* (2022) did not provide evidence of similar morphological features that would support the emergence of these two species as the closest related species in their phylogenetic tree.

In contrast, the phylogenetic results of the present study have led to the separation of these two species, likely attributed to the presence of distinctive characteristics in both *D. namaensis* and *D. mooiensis* when compared to the remaining *Dianthus* species. Burtt Davy (1922) and Hooper (1959) have specifically highlighted these uncommon features. They observed that *D.namaensis* exhibits leaves that are predominantly basal, long, linear, and form a tuft. Conversely, *D. mooiensis* displays leaves that are primarily cauline, with basal leaves being shorter than the cauline leaves, and the cauline leaves are broader.

In the first subclade (Figure 4.2), *D. laingsburgensis*, *D. transvaalensis*, and *D. basuticus* emerge as the species closest to each other in this subclade based on the phylogenetic tree from the combined datasets. A close relationship between *D. laingsburgensis*, *D. transvaalensis*, and *D.basuticus* could be inferred based on the characters of basal leaves, calyx length, and bract pairs, as postulated by Burtt Davy (1922) and Hooper (1959).

The close grouping of *Dianthus basuticus*, *Dianthus laingsburgensis*, and *Dianthus transvaalensis* in a phylogenetic tree suggests that these species share a common ancestor and have diverged relatively recently in evolutionary terms. This close relationship is likely due to their similar ecological niches, growth forms, and adaptations to the challenging environments in which they are found. Despite being distributed across different regions of southern Africa, these species have evolved similar traits to survive in rocky, high-altitude, or semi-arid habitats. However, this relationship will have to be examined with complete morphometric data. The three species exhibit notable autapomorphies, including exserted fimbriated petals, erect branches, narrowly elliptic or linear, long leaves (up to 20 cm), and paired bracts (Burtt Davy, 1922). These distinctive features could prove valuable in future taxonomic studies of *Dianthus* species.

5.3 *Dianthus* distribution in South Africa

The results from both phylogenetic trees (Figure 4.1 and 4.2) mutually support each other by grouping *Dianthus* species together based on geographical location. The result is consistent with previous studies (Valente *et al*., 2010), which also grouped South African species together, except for *D. transvaalensis*, which emerged within Eurasian species in Figure 4.2. This contrasts with the findings of Fassou *et al*. (2022), where South African species were grouped within the Eurasian radiation, creating some confusion regarding their classification. Clearly, this lends support to Madhani *et al*. (2018) 's theory that geography appears to have played a prominent role in theevolution of carnations in Eurasia and the rest of the lineages. Madhani *et al*. (2018) further asserted that *Dianthus* lacks major interspecific ecological differentiation but contains many narrow endemics, indicating a strong signal that the fundamental model of speciation, especially within carnations, has been driven by geographical factors.

In Africa, *Dianthus* species were found north of the Sahara but mostly widespread in the southern regions (Figure 1.2; Figure 4.4). African species of *Dianthus* are primarily restricted to Eastern Tropical Africa (Burtt Davy, 1922). According to Burtt Davy (1922), South African species of *Dianthus* appear to have reached South Africa through high mountain ranges in Eastern Tropical Africa, reflecting an earlier phase of diversity and evolution.

Dianthus laingsburgensis and *Dianthus kamisbergensis* are known from the literature to occur in the Northern Cape Province around the Namaqualand area (Burtt Davy, 1922; Hooper, 1961). However, current distribution records confirm that these two species are now found distributednot only in the Northern Cape but also in the Western Cape provinces of South Africa (Figure 4.4; Appendices I and J). The native range of these species is primarily in the subtropical biomeof the Cape provinces. Interestingly, in the phylogenetic tree (Figure 4.2), these speciesare grouped separately, likely based on certain morphological characteristics as mentioned earlier.

The distribution maps (Figure 4.4; Appendices C-P) of South African *Dianthus* species across provinces further highlight the diverse ecological niches occupied by different species. Coastal species, such as *D. albens* and *D. bolusii*, are confined to the Western Cape, while *D. basuticus* spans in multiple provinces.

Moreover, *D. albens* is predominantly found in the far Western Cape Province, extending its distribution range to the Eastern Cape where it is sparsely distributed. It also extends through the high mountain grasslands of the Free State Province up to the Northwest Province. *Dianthus albens* co-occurs with some related species within the same distribution range, namely *D. thunbergii*, *D. caespitosus,* and *D. bolusii*, with their distribution range extending only to the Eastern Cape Province (Hooper, 1959; Figure 4.4; Appendices C-P). The exclusive eastern distribution of *D. crenatus* emphasizes the importance of considering geographic factors in understanding the evolution and distribution of *Dianthus* species in South Africa, currently unexplainable.

One major trend is apparent within the winter rainfall season: most South African species, particularly those known to be endemic to the Western Cape province, are observed in the current results extending to the Eastern Cape, through the Free State to the North West Province, as well as the Northern Cape. These trends, however, are based on current locality distribution records, and they need to be analyzed more critically using biogeographical and phylogeography methods.

A further trend is evident in the well-known species in Lesotho, *D. basuticus*. According to Hooper (1961), this species was previously known to occur only in the high mountain areas of the eastern region grasslands of the Eastern Cape, Free State, KwaZulu-Natal, and Mpumalanga Province. However, the current results indicate that this species is now distributed in the fynbos vegetation of the winter rainfall Western Cape region of South Africa (Figure 4.4; Appendix D). The shift in distribution could be attributed to various factors, such as changes in climate, land use, or ecological dynamics. For instance, alterations in rainfall patterns, temperature variations, or habitat modifications might have facilitated the species' migration to new areas. Additionally, changes in the local ecosystem, such as disturbances or new ecological interactions, could influence the species' ability to thrive in the fynbos environment.

The only two species that seem to be adapted solely to the eastern regions up to the northern regions of South Africa are *D. zeyheri* and *D. mooiensis* (Figure 4.4; Appendix L and P). There are no records of these species in the Northern and Western Cape provinces of SouthAfrica (Figure 4.4; Appendix L and P). *Dianthus micropetalus* is the only species of *Dianthus* that occurs in seven provinces of South Africa, excluding KwaZulu-Natal and Mpumalanga provinces (Figure 4.4; Appendix K). In the literature, its range is known to extend from Cradock, through Kalahari, to the dry areas of Great Namaqualand (Burtt Davy, 1922).

An unusual aspect of the phytogeography of *Dianthus* is the extent of its development in the winter rainfall area of southern Africa, and the complete high diversity of similar radiations into dry summer rainfall climates elsewhere in the world (Fassou *et al*., 2022). This may reinforce the hypothesis that the genus has had a long association with Africa especially south of the Sahara (Madhani *et al*., 2018).

The current results reveal that only the most derived South African species, such as *Dianthus basuticus*, have adapted to a winter rainfall regime. In contrast, many other South African species, particularly those in the Western Cape Province like *Dianthus albens* and *Dianthus bolusii*, still exhibit traits indicative of their ancestral summer rainfall patterns, as evidenced by their flowering seasons (Hooper, 1959; Figure 4.4; Appendix C-P). This observation supports Deacon's (1983) hypothesis regarding the relatively recent emergence of the winter rainfall pattern in South Africa.

The evolutionary history of *Dianthus* in Africa is intertwined with the continent's geological past. The African continent, which is believed to have formed from the breakup of the supercontinent

Gondwana (Hallam, 1975), is home to several endemic species of *Dianthus* (Burtt Davy, 1922). This geological history plays a crucial role in understanding the biogeography of the genus (Hallam, 1975).

Long-distance dispersal across the high mountains of Eastern Tropical Africa is thought to be the primary factor shaping the distribution of *Dianthus* species in tropical and subtropical regions. The rugged topography and varying climates of these mountains likely provided corridors and refuges that facilitated the spread of *Dianthus* species across vast distances. This mode of dispersal is considered more significant than vicariance (the geographical separation of populations due to barriers) and extinction, which are believed to have had only marginal effects on the current distribution patterns of the genus (Burtt Davy, 1922; Hooper, 1959 and 1961; Valente *et al*., 2010).

These findings also align with the hypothesis that *Dianthus* species in South Africa are relatively recent arrivals from Northern Africa or nearby regions. The unresolved phylogenetic positions of *D. excelsus*, *D. serrulatus*, and *D. longiglumis*, coupled with the derived adaptation of *D. basuticus* to a winter rainfall regime, suggest that the genus's migration and subsequent diversification in Southern Africa occurred over an extended period. The presence of traits linked to ancestral summer rainfall patterns in many South African species further supports the idea that the shift to winter rainfall is a relatively recent phenomenon.

Overall, the biogeographic and evolutionary patterns of *Dianthus* in Africa reflect a complex history shaped by long-distance dispersal, recent climatic changes, and the continent's dynamic geological past. The adaptation of some species to the winter rainfall regime and the persistence of summer rainfall traits in others highlight the ongoing evolutionary processes that continue to shape the diversity and distribution of *Dianthus* in Southern Africa. Moreover, the distribution of South African *Dianthus* species across different but ecologically similar habitats suggests that they may have diverged from a common ancestor that was widely distributed across southern Africa. Over time, as populations became geographically isolated due to changing climates, topography, or other factors, they evolved into distinct species while retaining many of the adaptive traits of their common ancestor.

5.4 Implications for conservation

The recent discovery of *Dianthus basuticus* in the fynbos vegetation of the Western Cape, a region known for its Mediterranean climate with winter rainfall, suggests a significant ecological adaptation. This shift has important conservation implications.

Habitat Vulnerability: The fynbos biome, although highly diverse, is one of the most threatened ecosystems due to factors like urban expansion, agriculture, and invasive species. The establishment of *D. basuticus* in this region may heighten its susceptibility to habitat loss and environmental changes, highlighting the need for targeted conservation strategies to protect these newly established populations.

Climate Change Adaptation: The change in distribution might be an early sign of how climate change is impacting species' habitats. Conservation efforts should account for these dynamics, focusing on preserving both the current and potential future habitats of *D. basuticus* to ensure its survival amid changing climatic conditions.

The exclusive distribution of *D. kamisbergensis* in the Kamiesberg Mountains in the Northern Cape Province of South Africa underscores the importance of considering geographic factors in conservation planning. Since *D. kamisbergensis* is confined to a specific geographic area, conservation efforts must be tailored to protect its habitat in these regions. This includes safeguarding the high-altitude endemic mountains of Kamiesberg where it is found. This may involve collaborating with local communities and stakeholders to reduce habitat disturbance and manage land use effectively. Geographic exclusivity might also indicate a limited gene pool for *D. kamisbergensis*, making it more vulnerable to environmental changes and genetic bottlenecks. Conservation programs should explore strategies like habitat corridors or managed relocation to enhance genetic diversity and resilience.

Dianthus species with broader distributions across multiple provinces in South Africa, such as *D. zeyheri* and *D. crenatus* may encounter a variety of threats across different regions. Conservation strategies need to be adaptable and region-specific, addressing the unique threats in each area while also considering the overall connectivity of populations across provinces.

The study’s findings on species relationships and the frequent misidentification by botanists carry direct conservation implications. Accurate species identification is crucial for effective conservation, as misidentification can lead to insufficient or misdirected conservation efforts. Clarifying the taxonomy of closely related species will ensure that conservation resources are allocated appropriately, and that all species receive the necessary protection. Moreover, understanding the genetic relationships between closely related species can help determine conservation priorities. For example, if two species are closely related and share similar habitats, conserving one might also benefit the other. However, if they occupy distinct ecological niches, both may require separate conservation strategies.

Chapter Six

Conclusion and Future Directions

In conclusion, the integrated analysis of nuclear and plastid datasets has provided a comprehensive view of the phylogenetic relationships within *Dianthus*, emphasizing the need for a holistic approach in molecular studies. The identified clades and unresolved species placements warrant further investigation, possibly through additional molecular markers or broader taxon sampling. This study lays the foundation for future research aiming to unravel the intricate evolutionary history and ecology of *Dianthus* in South Africa. The presence of well-supported subclades within the South African clade indicates potential for further exploration of regional evolutionary patterns. In addition, the ITS, trnH-psbA, trnK-matK, and trnK-psbA gene sequences, when considered independently, have proven to be unreliable for assessing phylogeny in the genus *Dianthus*. However, the simultaneous analysis of all four markers (ITS, trnH-psbA, trnK-matK, trnK- psbA) has significantly enhanced reliability and the ability to differentiate closely related taxa. Despite unresolved controversial taxonomic issues, this study did not provide initial evidence for a clear phylogenetic divergence between *D. zeyheri* and *D. crenatus*, primarily due to the absence of *D. crenatus* DNA sequences during the investigation.

The inclusion of additional gene sequences is evidently essential and may contribute to a better understanding of the evolution of certain *Dianthus* species, particularly concerning the differentiation between two subspecies of *D. zeyheri*, namely *D. zeyheri* ssp. *zeyheri* and *D. zeyheri* ssp. *natalensis*, which appear to exhibit overlapping morphological characters. It is therefore recommended that, in the future, a comprehensive taxonomic treatment/revision of this genus in South Africa be conducted, comparing both morphometric and molecular phylogeny results. This approach aims to address and resolve the controversial taxonomic issues within this genus.

Furthermore, the results of this study propose a comprehensive examination of the taxonomy of *Dianthus* and *Petrorhagia* to arrive at a definitive conclusion regarding the classification ofthe genera. This echoes the sentiment of previous studies (Valente *et al*., 2010; Madhani *et al*., 2018; Fassou *et al*., 2022), which recommended further investigation incorporating more material from *Dianthus*, *Gypsophila*, *Petrorhagia*, and *Saponaria*, as these genera were inadequately represented in their analyses. In conclusion, this study highlights the need for continued research, stressing the importance of including all South African species, subspecies, and varieties for accurate conclusions, and emphasizing the need to better integrate morphological characteristics in future studies.

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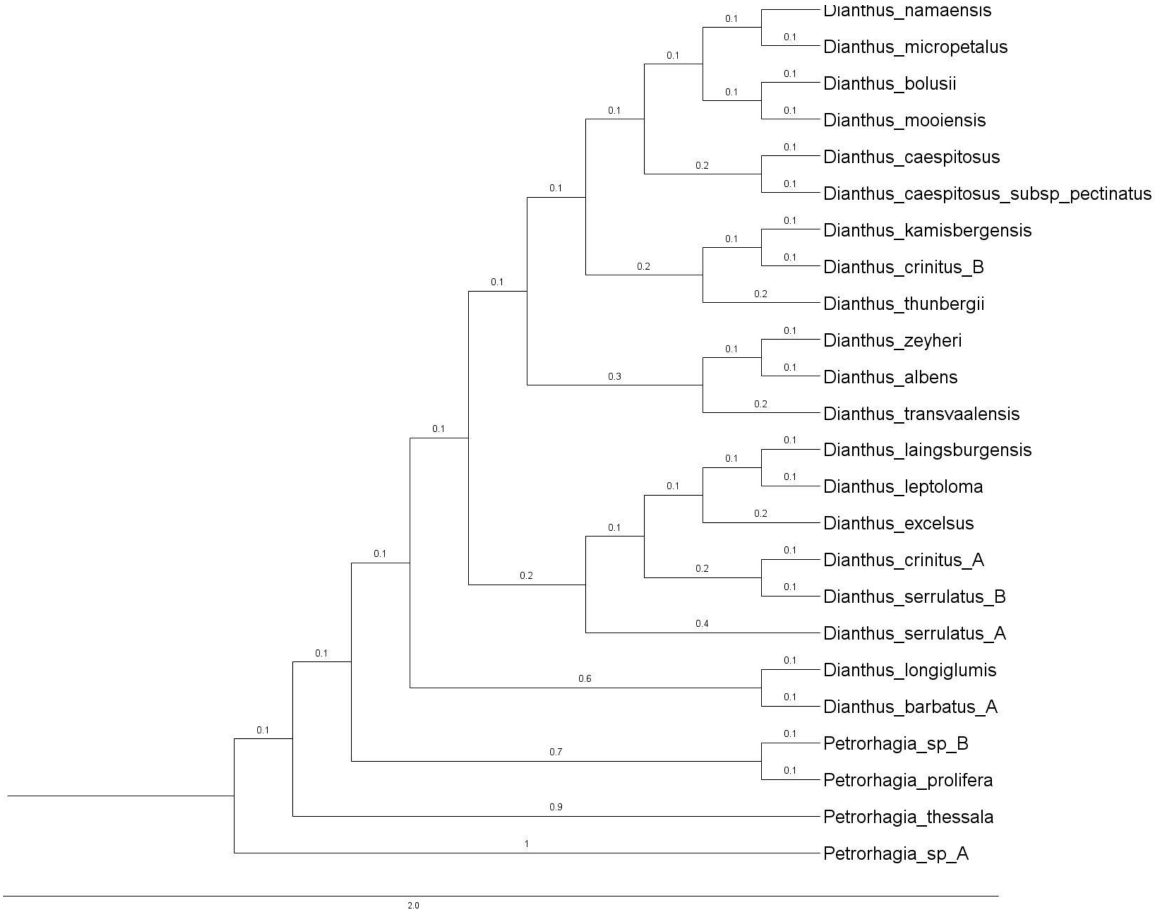
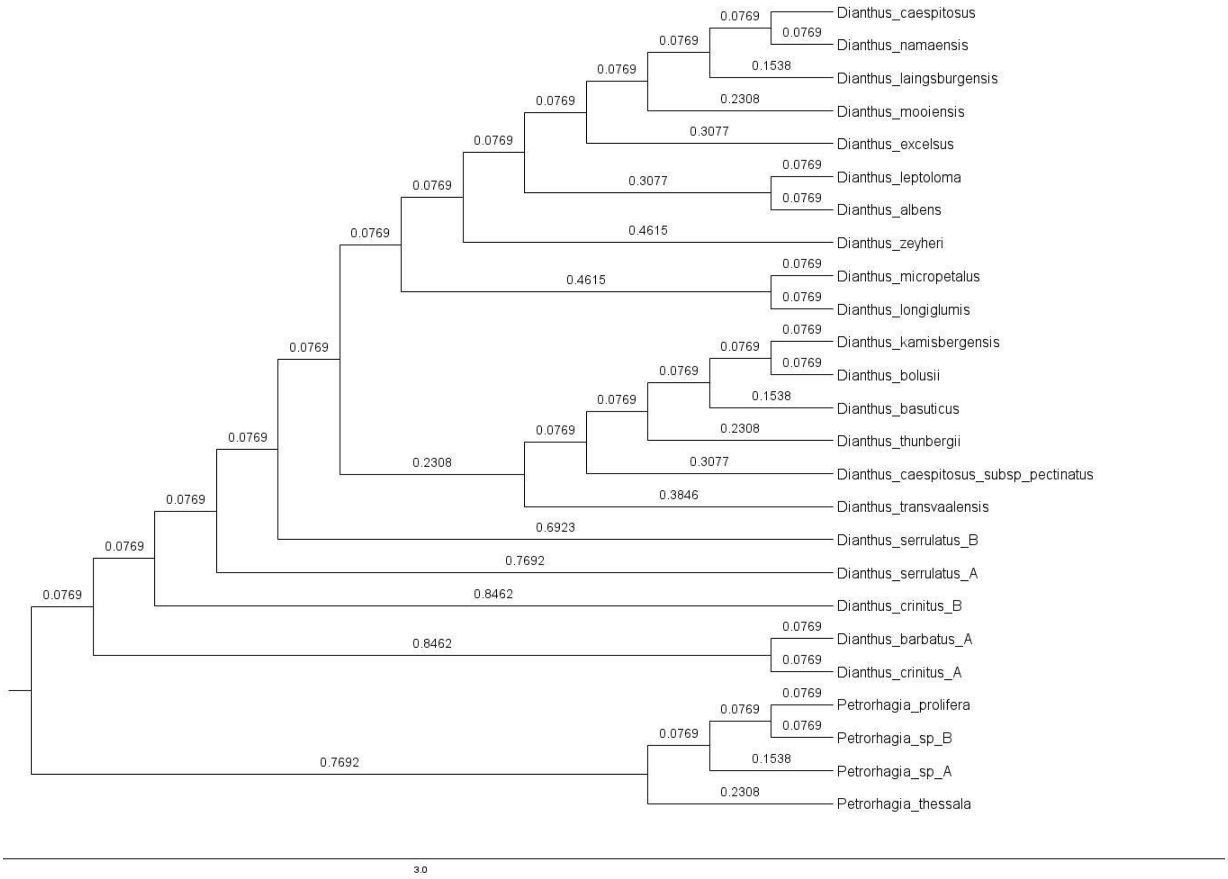
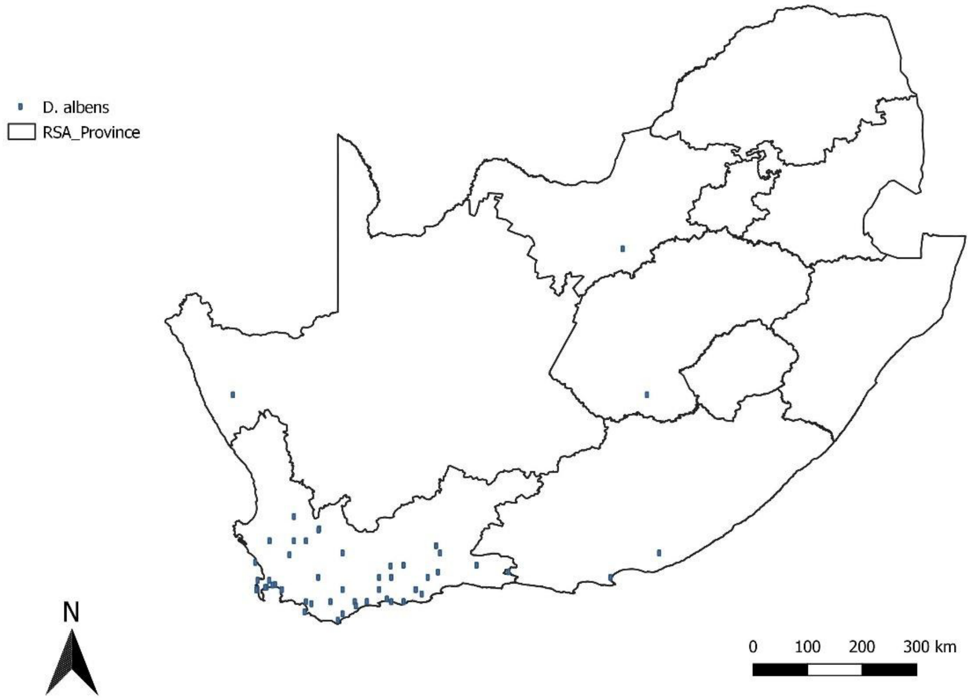
Appendices



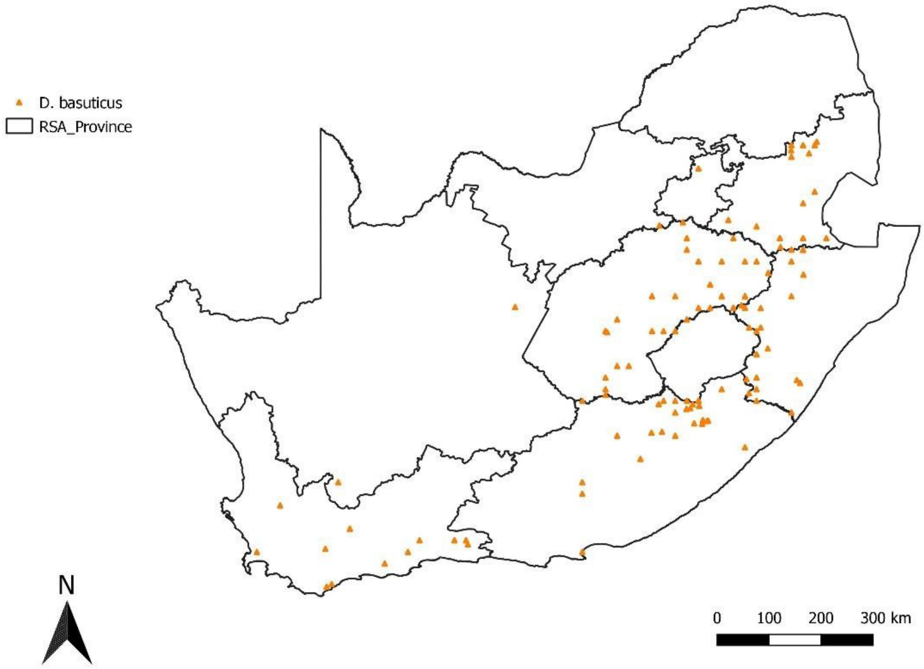
Appendix A: Bayesian Analysis (BI) tree resulting from the analysisof nuclear ribosomal DNA internal transcribed spacer (ITS) sequences for African *Dianthus*. Posterior Probability (PP) values are presented above branches. Independent analyses of the ITS sequences separately yielded trees with numerous polytomies, indicative of poor phylogenetic resolution.



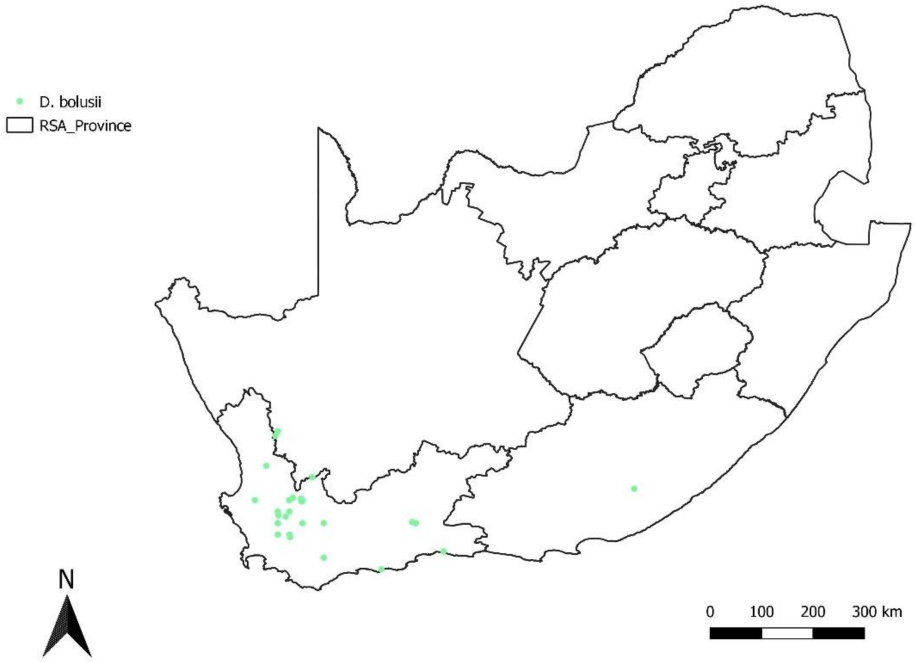
Appendix B: Bayesian Analysis (BI) tree resulting from the analysis of the combined plastids dataset, respectively, for African Dianthus. Posterior Probability (PP) values are presented above branches. Independent analyses of the plastid markers yielded trees with numerous polytomies, suggesting poor phylogenetic resolution.



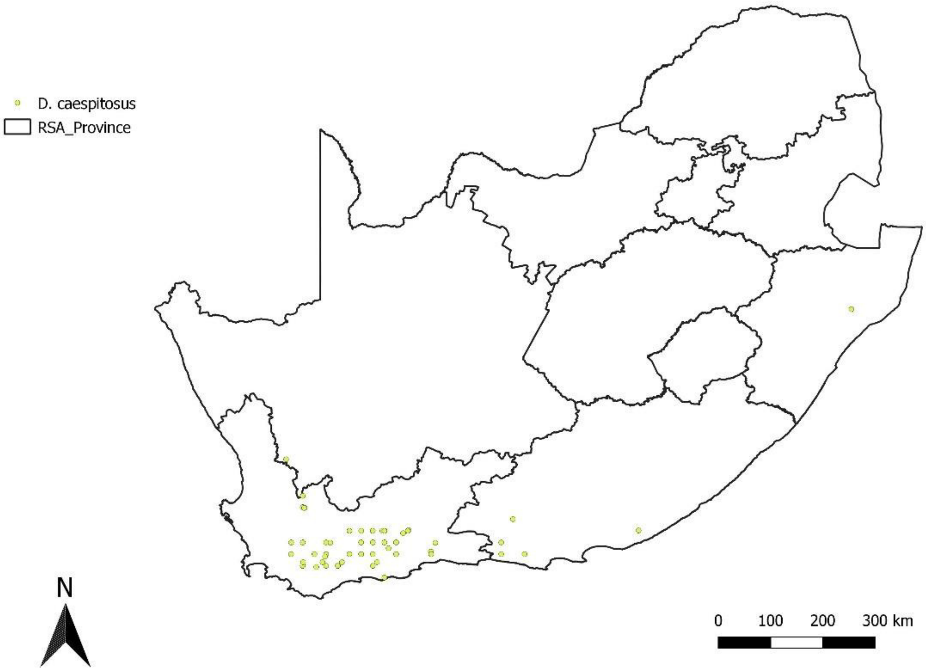
Appendix C: Distribution of *D. albens* in South Africa



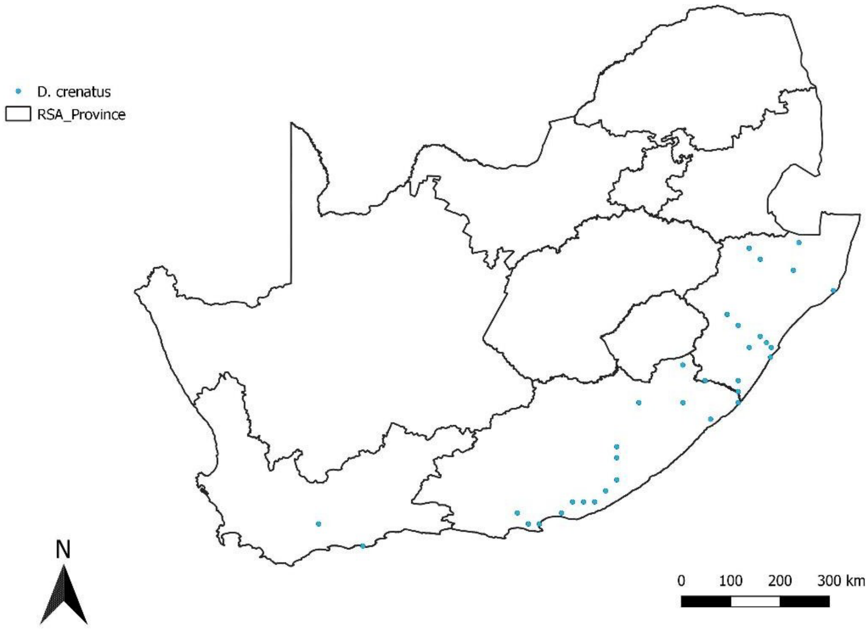
Appendix D: Distribution of *D. basuticus* in South Africa



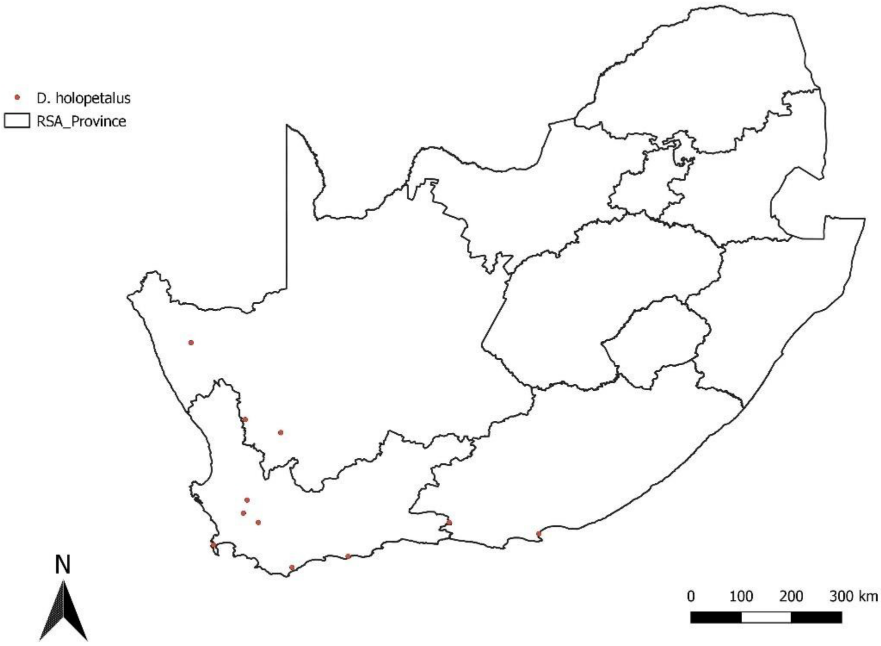
Appendix E: Distribution of *D. bolusii* in South Africa



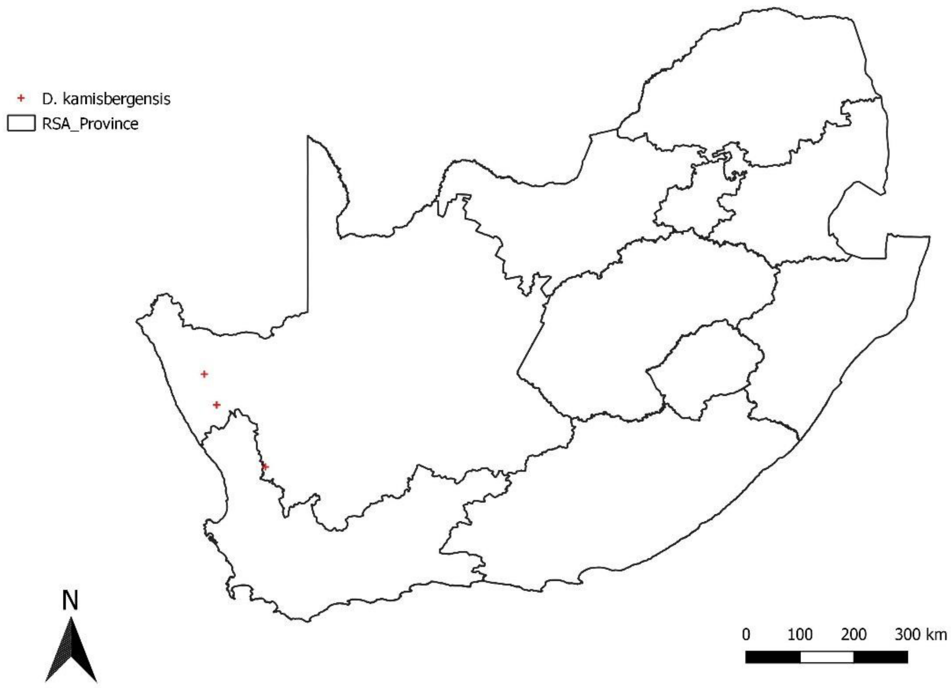
Appendix F: Distribution of *D. caespitosus* in South Africa



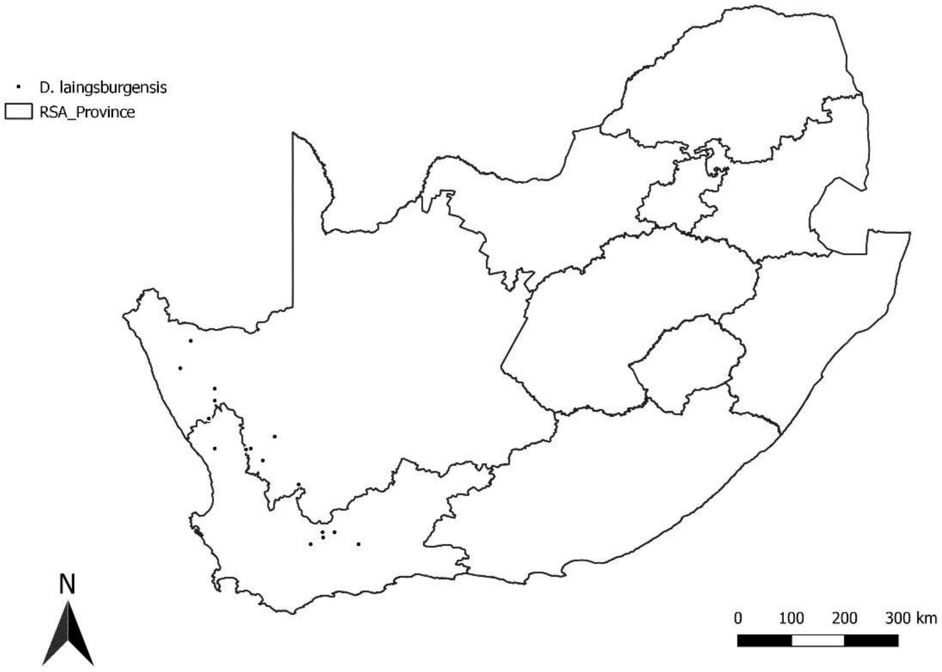
Appendix G: Distribution of *D. crenatus* in South Africa



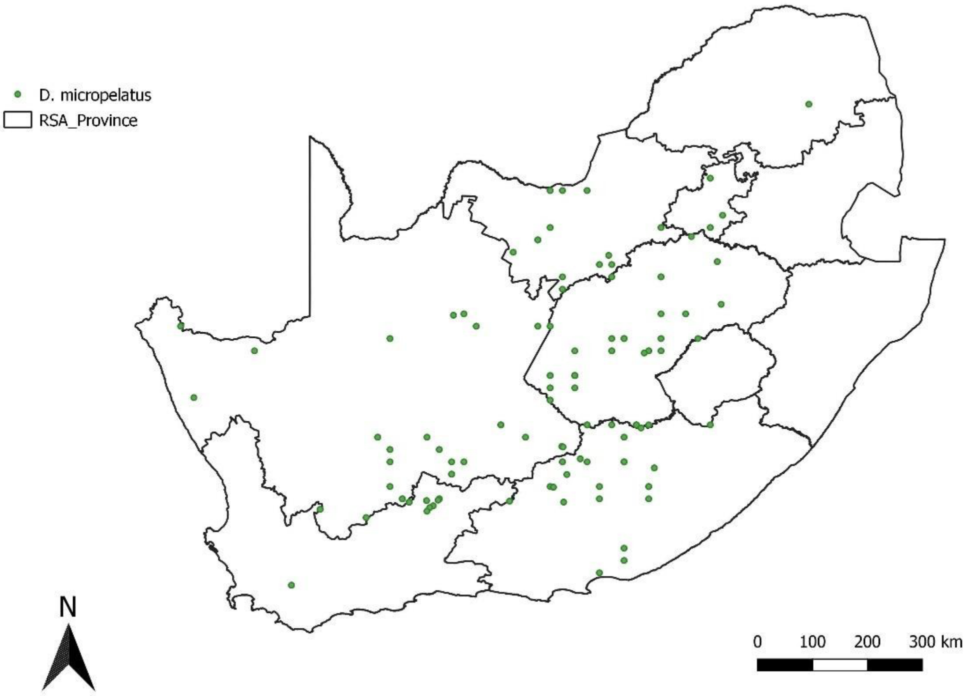
Appendix H: Distribution of *D. holopetalus* in South Africa



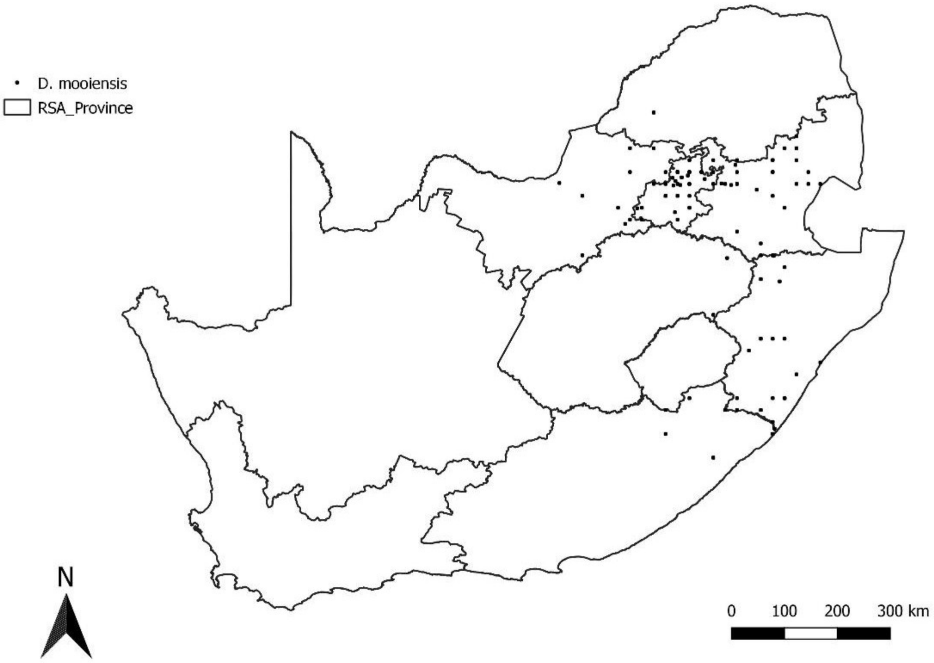
Appendix I: Distribution of *D. kamisbergensis* in South Africa



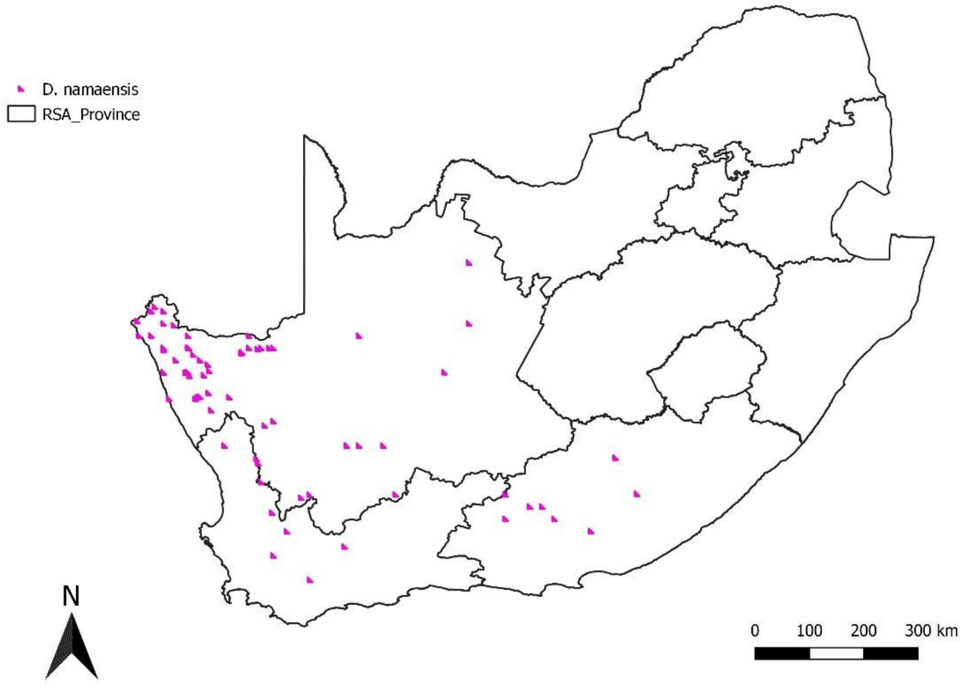
Appendix J: Distribution of *D. laingsburgensis* in South Africa



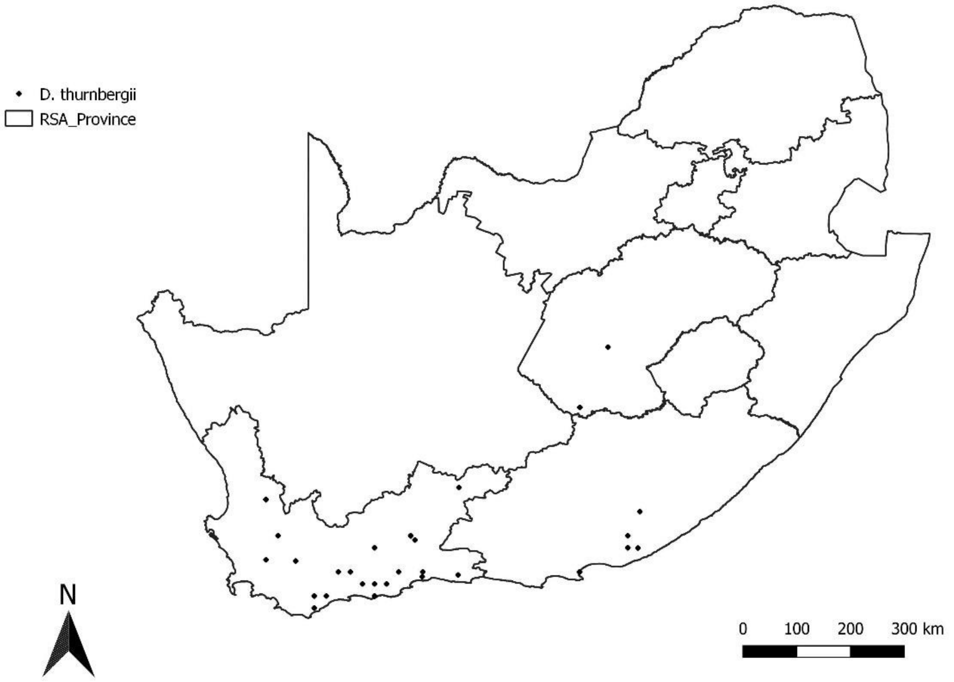
Appendix K: Distribution of *D. micropetalus* in South Africa



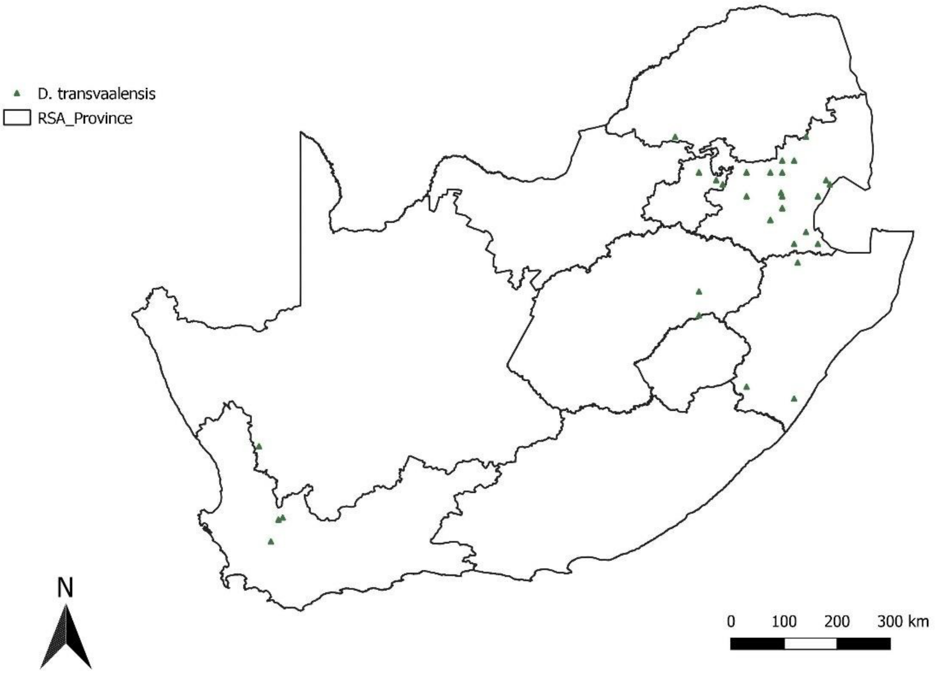
Appendix L: Distribution of *D. mooiensis* in South Africa



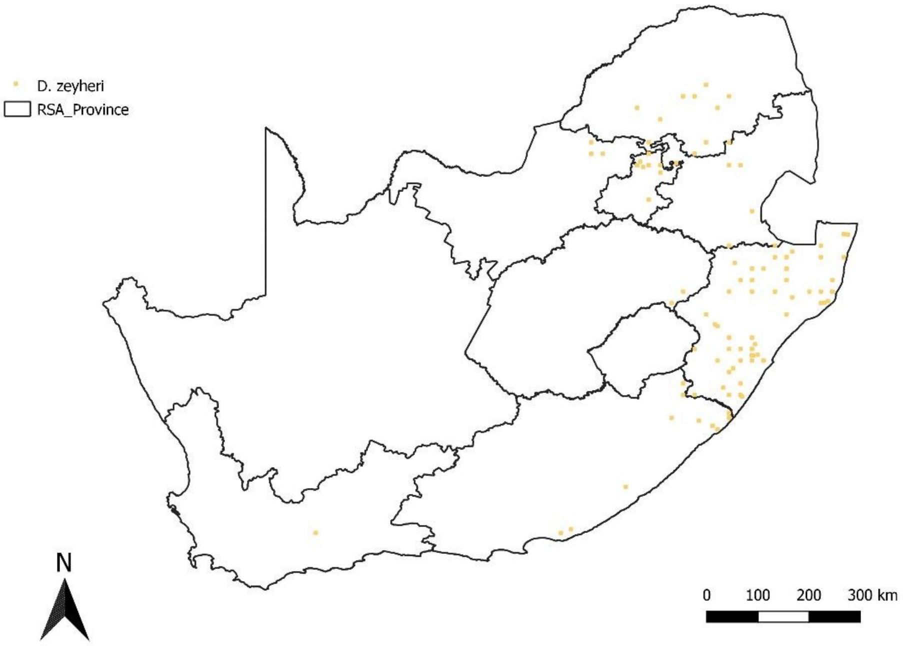
Appendix M: Distribution of *D. namaensis* in South Africa



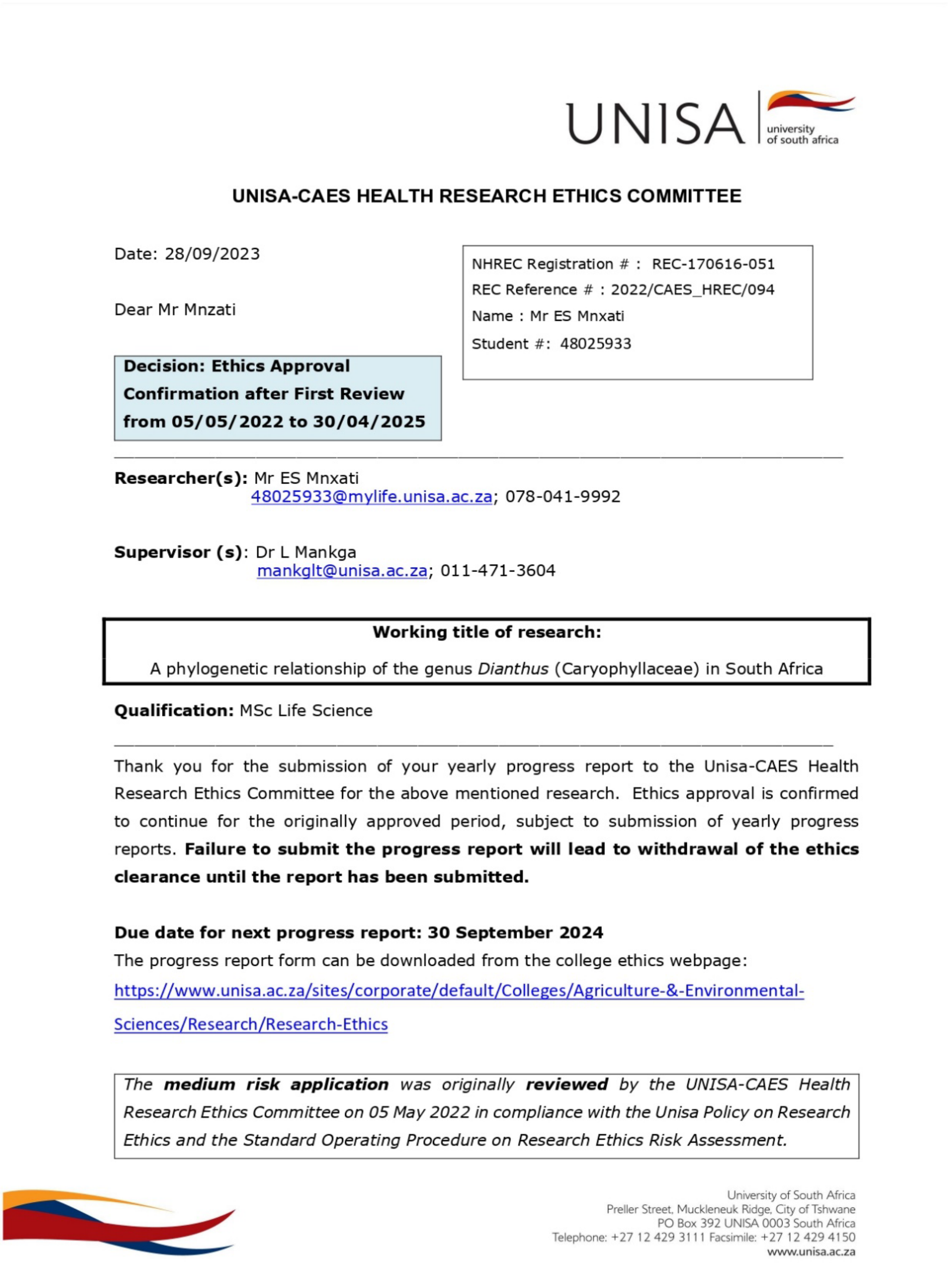
Appendix N: Distribution of *D. thunbergii* in South Africa



Appendix O: Distribution of *D. transvaalensis* in South Africa



Appendix P: Distribution of *D. zeyheri* in South Africa



UNISA-CAES HEALTH RESEARCH ETHICS COMMITTEE

Date: 28/09/2023

Dear Mr Mnzati

NHREC Registration # : REC-170616-051
REC Reference # : 2022/CAES_HREC/094
Name : Mr ES Mnxati
Student #: 48025933

Decision: Ethics Approval Confirmation after First Review from 05/05/2022 to 30/04/2025

Researcher(s): Mr ES Mnxati
48025933@mylife.unisa.ac.za; 078-041-9992

Supervisor (s): Dr L Mankga
mankglt@unisa.ac.za; 011-471-3604

Working title of research:

A phylogenetic relationship of the genus *Dianthus* (Caryophyllaceae) in South Africa

Qualification: MSc Life Science

Thank you for the submission of your yearly progress report to the Unisa-CAES Health Research Ethics Committee for the above mentioned research. Ethics approval is confirmed to continue for the originally approved period, subject to submission of yearly progress reports. **Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

Due date for next progress report: 30 September 2024
The progress report form can be downloaded from the college ethics webpage: https://www.unisa.ac.za/sites/corporate/default/Colleges/Agriculture-&-Environmental-Sciences/Research/Research-Ethics

*The **medium risk application** was originally **reviewed** by the UNISA-CAES Health Research Ethics Committee on 05 May 2022 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

University of South Africa
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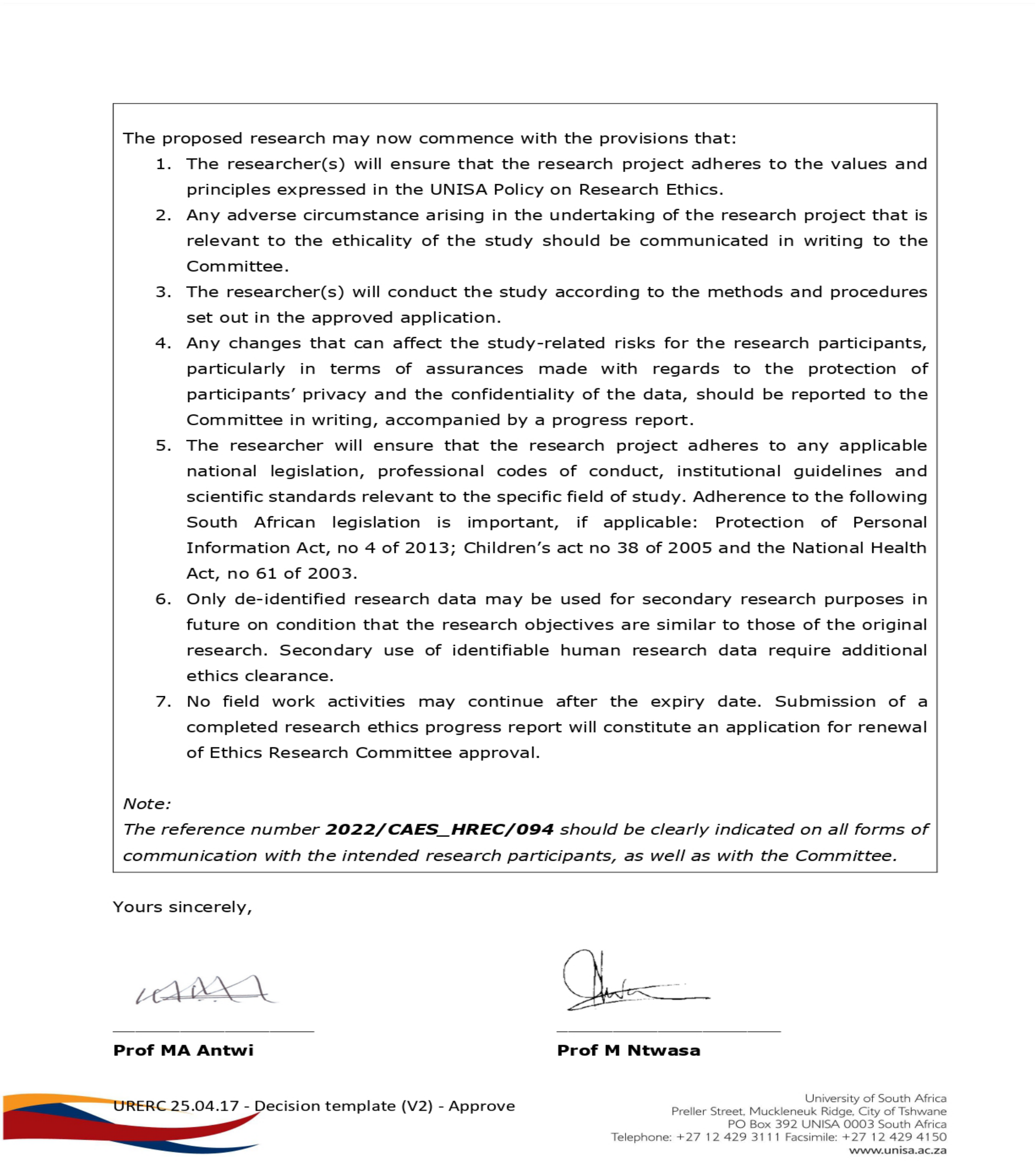
Appendix A1: Ethics Approval Certificate to Conduct Research page 1



The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:
The reference number ***2022/CAES_HREC/094*** *should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

Prof MA Antwi

Prof M Ntwasa

URERC 25.04.17 - Decision template (V2) - Approve

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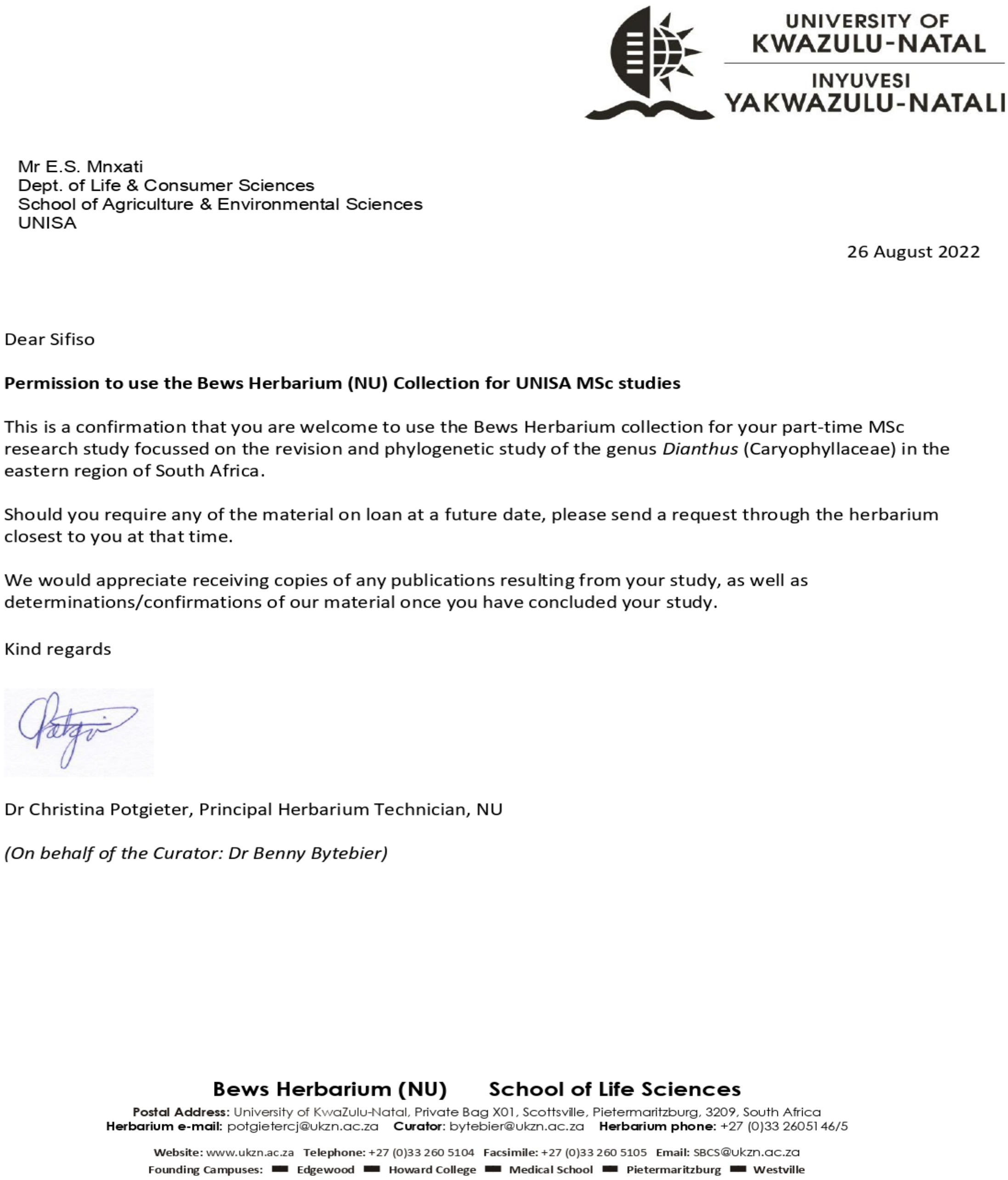
Appendix A1: Ethics Approval Certificate to Conduct Research page 2



UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

Mr E.S. Mnxati
Dept. of Life & Consumer Sciences
School of Agriculture & Environmental Sciences
UNISA

26 August 2022

Dear Sifiso

Permission to use the Bews Herbarium (NU) Collection for UNISA MSc studies

This is a confirmation that you are welcome to use the Bews Herbarium collection for your part-time MSc research study focussed on the revision and phylogenetic study of the genus *Dianthus* (Caryophyllaceae) in the eastern region of South Africa.

Should you require any of the material on loan at a future date, please send a request through the herbarium closest to you at that time.

We would appreciate receiving copies of any publications resulting from your study, as well as determinations/confirmations of our material once you have concluded your study.

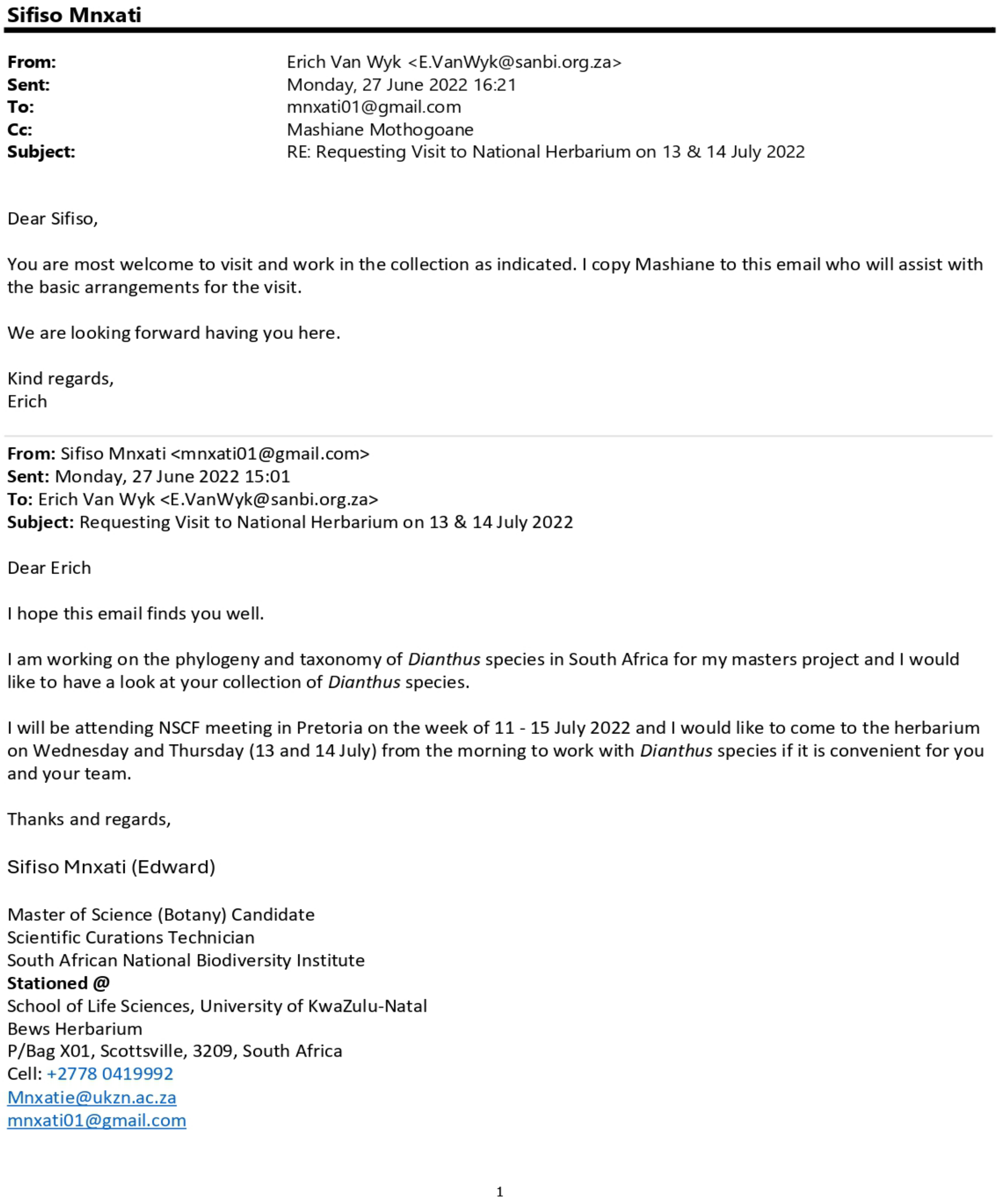
Kind regards

Dr Christina Potgieter, Principal Herbarium Technician, NU

(On behalf of the Curator: Dr Benny Bytebier)

Bews Herbarium (NU) **School of Life Sciences**
Postal Address: University of KwaZulu-Natal, Private Bag X01, Scottsville, Pietermaritzburg, 3209, South Africa
Herbarium e-mail: potgietercj@ukzn.ac.za **Curator:** bytebier@ukzn.ac.za **Herbarium phone:** +27 (0)33 2605146/5
Website: www.ukzn.ac.za **Telephone:** +27 (0)33 260 5104 **Facsimile:** +27 (0)33 260 5105 **Email:** SBCS@ukzn.ac.za
Founding Campuses: ▬ Edgewood ▬ Howard College ▬ Medical School ▬ Pietermaritzburg ▬ Westville

Appendix A2: Permission to use Bews Herbarium Collection



Sifiso Mnxati

From:	Erich Van Wyk <E.VanWyk@sanbi.org.za>
Sent:	Monday, 27 June 2022 16:21
To:	mnxati01@gmail.com
Cc:	Mashiane Mothogoane
Subject:	RE: Requesting Visit to National Herbarium on 13 & 14 July 2022

Dear Sifiso,

You are most welcome to visit and work in the collection as indicated. I copy Mashiane to this email who will assist with the basic arrangements for the visit.

We are looking forward having you here.

Kind regards,
Erich

From: Sifiso Mnxati <mnxati01@gmail.com>
Sent: Monday, 27 June 2022 15:01
To: Erich Van Wyk <E.VanWyk@sanbi.org.za>
Subject: Requesting Visit to National Herbarium on 13 & 14 July 2022

Dear Erich

I hope this email finds you well.

I am working on the phylogeny and taxonomy of *Dianthus* species in South Africa for my masters project and I would like to have a look at your collection of *Dianthus* species.

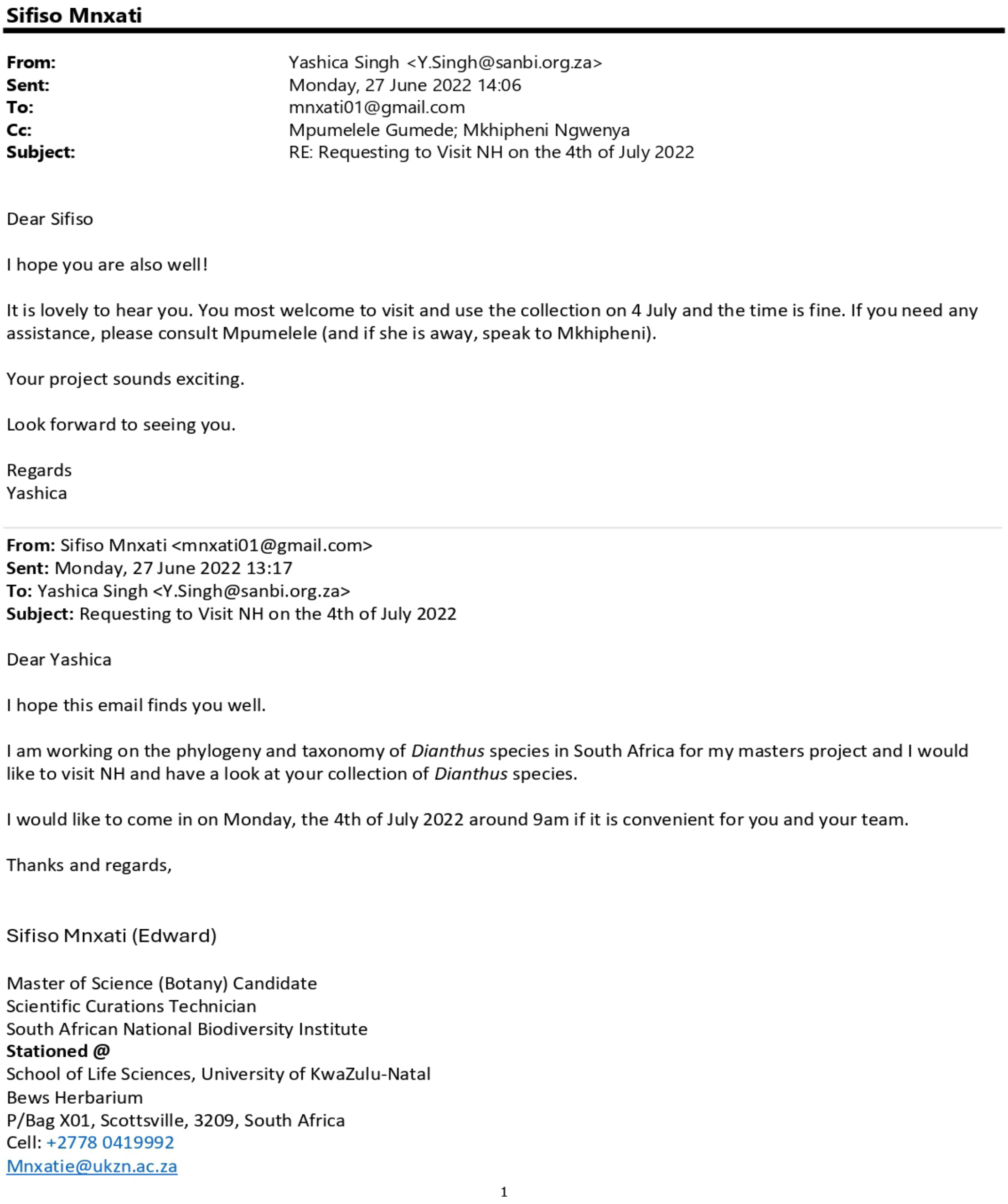
I will be attending NSCF meeting in Pretoria on the week of 11 - 15 July 2022 and I would like to come to the herbarium on Wednesday and Thursday (13 and 14 July) from the morning to work with *Dianthus* species if it is convenient for you and your team.

Thanks and regards,

Sifiso Mnxati (Edward)

Master of Science (Botany) Candidate
Scientific Curations Technician
South African National Biodiversity Institute
Stationed @
School of Life Sciences, University of KwaZulu-Natal
Bews Herbarium
P/Bag X01, Scottsville, 3209, South Africa
Cell: +2778 0419992
Mnxatie@ukzn.ac.za
mnxati01@gmail.com

Appendix A3: Permission to use SANBI National Herbarium Collection



Sifiso Mnxati

From: Yashica Singh <Y.Singh@sanbi.org.za>
Sent: Monday, 27 June 2022 14:06
To: mnxati01@gmail.com
Cc: Mpumelele Gumede; Mkhipheni Ngwenya
Subject: RE: Requesting to Visit NH on the 4th of July 2022

Dear Sifiso

I hope you are also well!

It is lovely to hear you. You most welcome to visit and use the collection on 4 July and the time is fine. If you need any assistance, please consult Mpumelele (and if she is away, speak to Mkhipheni).

Your project sounds exciting.

Look forward to seeing you.

Regards
Yashica

From: Sifiso Mnxati <mnxati01@gmail.com>
Sent: Monday, 27 June 2022 13:17
To: Yashica Singh <Y.Singh@sanbi.org.za>
Subject: Requesting to Visit NH on the 4th of July 2022

Dear Yashica

I hope this email finds you well.

I am working on the phylogeny and taxonomy of *Dianthus* species in South Africa for my masters project and I would like to visit NH and have a look at your collection of *Dianthus* species.

I would like to come in on Monday, the 4th of July 2022 around 9am if it is convenient for you and your team.

Thanks and regards,

Sifiso Mnxati (Edward)

Master of Science (Botany) Candidate
Scientific Curations Technician
South African National Biodiversity Institute
Stationed @
School of Life Sciences, University of KwaZulu-Natal
Bews Herbarium
P/Bag X01, Scottsville, 3209, South Africa
Cell: +2778 0419992
Mnxatie@ukzn.ac.za

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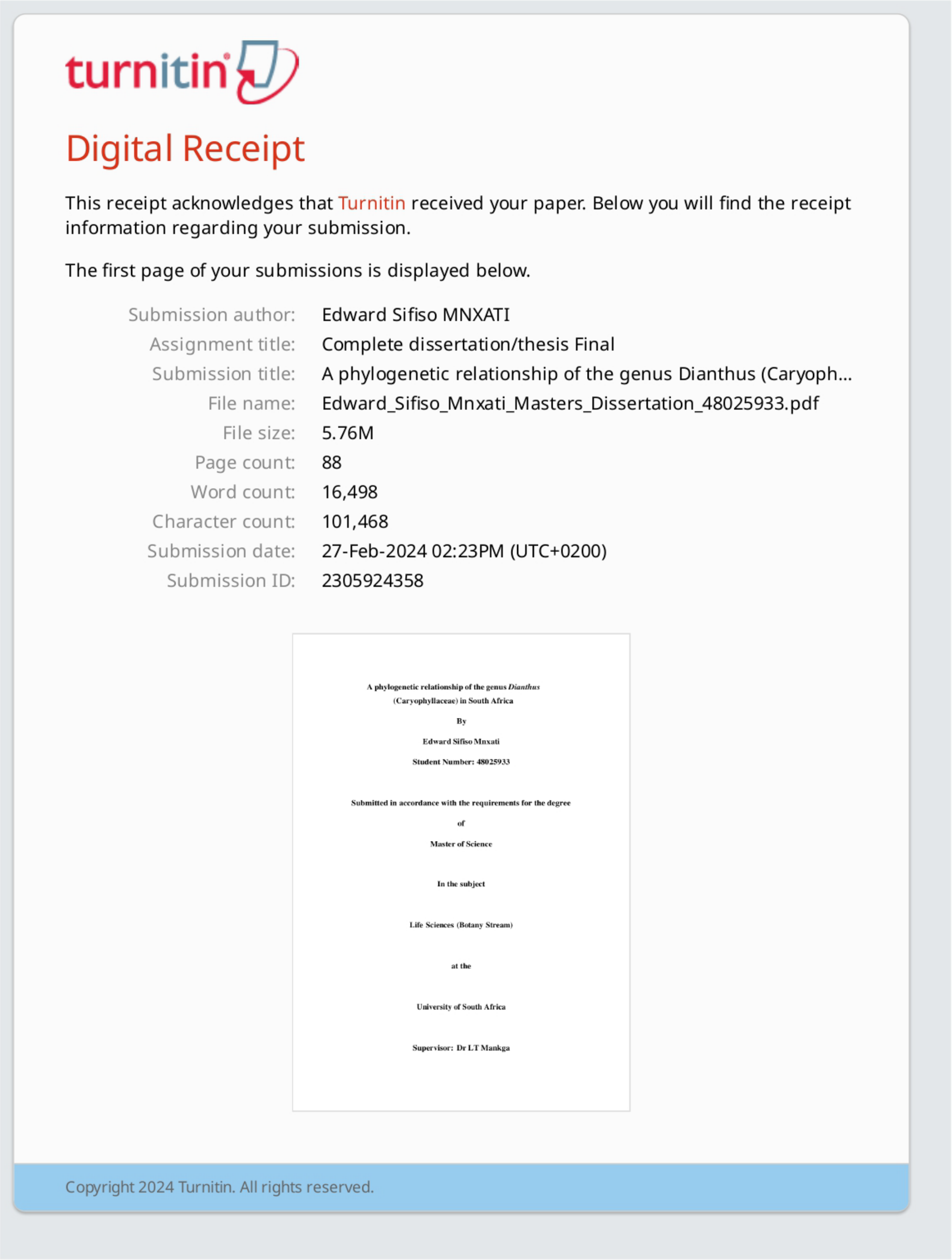
Appendix A4: Permission to use SANBI KZN Herbarium Collection



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Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author: Edward Sifiso MNXATI
Assignment title: Complete dissertation/thesis Final
Submission title: A phylogenetic relationship of the genus Dianthus (Caryoph...
File name: Edward_Sifiso_Mnxati_Masters_Dissertation_48025933.pdf
File size: 5.76M
Page count: 88
Word count: 16,498
Character count: 101,468
Submission date: 27-Feb-2024 02:23PM (UTC+0200)
Submission ID: 2305924358

A phylogenetic relationship of the genus *Dianthus*
(Caryophyllaceae) in South Africa

By

Edward Sifiso Mnxati

Student Number: 48025933

Submitted in accordance with the requirements for the degree

of

Master of Science

In the subject

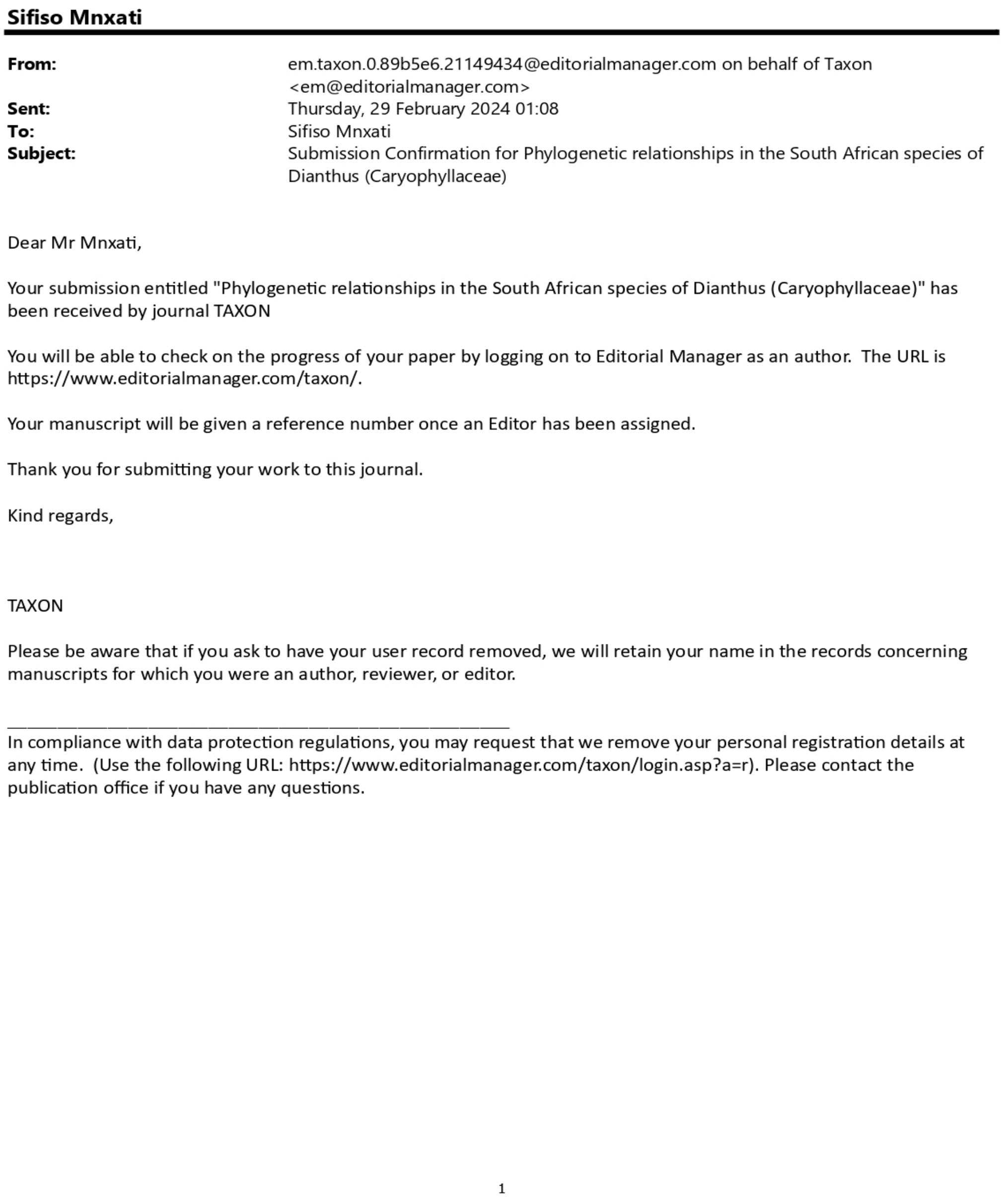
Life Sciences (Botany Stream)

at the

University of South Africa

Supervisor: Dr LT Mankga

Appendix A5: Turnitin Proof of Submission Digital Receipt



Sifiso Mnxati

From: em.taxon.0.89b5e6.21149434@editorialmanager.com on behalf of Taxon <em@editorialmanager.com>
Sent: Thursday, 29 February 2024 01:08
To: Sifiso Mnxati
Subject: Submission Confirmation for Phylogenetic relationships in the South African species of Dianthus (Caryophyllaceae)

Dear Mr Mnxati,

Your submission entitled "Phylogenetic relationships in the South African species of Dianthus (Caryophyllaceae)" has been received by journal TAXON

You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is https://www.editorialmanager.com/taxon/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

TAXON

Please be aware that if you ask to have your user record removed, we will retain your name in the records concerning manuscripts for which you were an author, reviewer, or editor.

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In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Use the following URL: https://www.editorialmanager.com/taxon/login.asp?a=r). Please contact the publication office if you have any questions.

Appendix A6: Manuscript Proof of Submission