STUDIES ON SOUTH AFRICAN AND NEW ZEALAND SPECIES OF *BULBINELLA* USING NUCLEAR AND CHLOROPLAST SEQUENCE DATA

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ABSTRACT

The genus *Bulbinella* Kunth consists of geophytes occurring in South Africa and New Zealand and includes a number of beautiful, conspicuous, mostly threatened flowering species. The genus is composed of about 23 species and is taxonomically related to *Bulbine* Wolf and *Kniphofia* Moench. There are six species in New Zealand and 17 species in South Africa. The genus represents one of the most understudied genera in South Africa. The species relationships and complexes are poorly understood due to morphological homogeneity and it has been flagged as a priority to study due to its ethnomedicinal value. The aim of this thesis was to establish the first set of DNA sequence data for phylogenetic studies complimenting previous morphological and taxonomic studies because molecular techniques offers increased precision by permitting assessment of additional characters. This was done using a number of conventional phylogenetic genes for plants, as well as following a phylogenomic approach of the chloroplast. In the thesis the taxonomy, morphology and importance of species in *Bulbinella* were reviewed. The 94 specimens were sampled, of which 86 specimens were in-group and eight outgroup sequences, using either sequences obtained from GenBank or those generated in this study. DNA sequencing of four gene regions (*ITS*, *rbcL*, *matK* and *psbA-trnH*) was conducted to resolve some of the major questions in the phylogeny of *Bulbinella* in South Africa and New Zealand. Due to the fact that South African species relationships needed more definition, a subsequent phylogenetic analysis based on 34 protein-coding genes from 16 taxa was done in a phylogenomic approach to improve resolution and give a better understanding of the evolutionary process of *Bulbinella*. Phylogenies were constructed using Maximum Likelihood (ML) conducted in Garli v2 and Bayesian Inference (BI) using Mr Bayes v3.2, with consensus topologies generated using PHYLIP v3.695. For chloroplast draft genome assembly, the filter reads were processed in a bioinformatics pipeline, annotated and used in phylogenetic analyses. In each of the gene analyses (separate and combined) New Zealand species always grouped on their own but in the overall group of *Bulbinella*. New Zealand and South African species included distinct, polyphyletic or possible synonymous species. The standard DNA barcode region *matK* (but not *rbcL*), were able to distinguish most South African and New Zealand species, but not others. The *psbA-trnH* spacer and *ITS* could be used as a supplementary barcode. Based on the genome data, phylogenetic trees confirmed the gene tree results and conclusions but provided greater statistical support and could distinguish between previously indistinguishable species. The results suggested that the following genes can be used or recognized as barcode genes to distinguish *Bulbinella* species and these are *atpA*, *atpF, atpI, rbcL, ndhI, ndhH, ndhF, rpl2, rpoC, rpoC2, rps15, orf188, rps2, matK, ndhE, ndhG, ccsA, psaC, ycf2, psbA, rpoB* and *ndhD*. The study has established multigene phylogenies for the genus for the first time which will strengthen the taxonomy of the genus, aid identifications for users of the plants for medical applications, the ornamental industry, as well as facilitate biodiversity and conservation efforts to protect the diversity of this genus. However, our results showed that there is a great need for increased sampling and morphological supported studies for these species, while the genes identified in the whole genome sequencing approach will be helpful to support the phylogeny of this genus.

DECLARATION

I declare that this thesis has been composed by me for the Philosophiae Doctorate degree at the University of the Free State and the work contained within unless otherwise stated, is my own and has not previously been submitted by me at another university/faculty. I further more cede copy of the dissertation in favour of the University of the Free State".

C. Musara (December 2017).

IN VERITATE SAPIENTIAE LUX

UNIVERSITY OF THE **FREE STATE**

DEDICATION

To my parents Reverend Elisha and Nomsa for the “prayers and good genes”; you are the reason and cause for my journey to stardom!

FOREWORD

This study is a contribution to the taxonomic and phylogenetic understanding of the plant genus *Bulbinella* following on previous efforts (Moore, 1964; Moore and Edgar 1970; Perry 1987; 1999, Milicich, 1993, Boatwright and Manning, 2012). These taxonomic treatments combined as before presented previously published descriptive taxonomy with the newest genetic technology to provide a baseline for biosystematic evaluations presented in this study.

My thesis is presented in six chapters. The research chapters 2 to 4 are preceded by the introduction, motivation and general objectives of the study in **Chapter 1. Chapter 2** is the first research chapter in the form of a literature review dealing with the distribution, conservation status and economic importance of *Bulbinella* genus in South Africa and New Zealand. It represents an overview of the classification of *Bulbinella* based on morphology and emphasises the need for molecular systematics. The Chapter also describes most indispensable techniques which can be used for the characterisation and assessment of germplasm, genetic diversity and the phylogenetic history of organisms. These suggestions were formalised and **published** in the Botanical Science Journal of Mexico. The title of the paper is as follows: "A review of the genus *Bulbinella* (Asphodelaceae), its distribution, conservation status and economic importance". (Botanical Sciences 95 (2): 1-14, 2017. DOI: 10.17129/botsci.696). The review emphasises that an accurate *Bulbinella* classification is fundamental knowledge for breeders and taxonomists.

Chapter 3 deals with the materials and methods employed on constructing and elucidating the diversity and phylogenetic relationships of *Bulbinella* species from

South Africa and New Zealand using a combination of Illumina sequencing based on 34 chloroplast protein-coding genes (genome sequence analysis) and DNA sequencing of four gene regions (*ITS, rbcL, matK* and *psbA-trnH*). These approaches were aimed to resolve some of the major remaining questions in the current phylogeny of *Bulbinella* in South Africa and New Zealand.

Chapter 4 and **Chapter 5** are the last research chapters present results and general discussions on the phylogenetics of *Bulbinella* species both in South Africa and New Zealand. **Chapter 6** is the conclusions. Additional information and results are included in an Appendix.

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My heartfelt appreciation goes to my parents, Reverend Dr Elisha and Nomsa Musara for their spirited selfless support and undoubted moral and financial support and guidance during my studies. I also extend my gratitude to my sisters and brothers and all my nieces and nephews for their patience in missing their well-deserved time with me during the study.

Last, but certainly not least, I thank the ALMIGHTY LORD GOD for His eternal love, grace, mercy and power throughout this work; thank You LORD JESUS CHRIST.

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LIST OF ABBREVIATION

%	Percentage
˚C	Degrees Celsius
µl	Microliter
2n	Somatic chromosome number
ABI	Applied Biosystems
AIC	Akaike information criterion
ARC	Agriculture Research Council
B.	*Bulbinella*
Be.	*Bulbine*
BI	Bayesian Inference
BOLD	Barcode for Life Data Systems
Bp	Base pair
BP	Bootstrap Percentage (support)
CO_2	Carbon dioxide
CBOL PG	Consortium for the Barcode of the Life Plant Working Group
CTAB	Cetyl trimethylammonium bromide
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide
DOGMA	Dual Organellar GenoMe Annotator
EDTA	Ethylene Diaminetetra Acetic Acid
ESS	Estimated Sample Sizes
Ethanol	Ethyl alcohol
Fig.	Figure
g	Gram

GenBank	National Centre for Biotechnology Information
ITS	Internal Transcribed Spacer Region
IUCN	International Union for Conservation of Nature
JRAU	Herbarium of the University of Johannesburg, Johannesburg, South Africa
K	*Kniphofia*
Kunth	Kunth, Karl Sigismund (1788-1850)
L	Linnaeus (von Linn´), Carl (1707-1778)
M	Molar
MCMC	(Bayesian) Markov Chain Monte Carlo.
Min	Minute
Ml	Milliliter
ML	Maximum likelihood
mM	Millimolar
MEGA	Molecular Evolutionary Genetics Analysis
MUSCLE	Multiple Sequence Comparison by Log-Expectation
N	Gametic chromosome number
NADH	Nicotinamide adenine dinucleotide + hydrogen
NaCl	Sodium chloride
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
PP	Posterior Probabilities
PsbA-trnH	Intergenic spacer locus
RbcL	Ribulose-1, 5 biphosphate carboxylase large subunit
RNA	Ribonucleic acid
SANBI	South African National Biodiversity Institution
subsp.	Subspecies
TAE	Tris; Acetic acid;

x Basic chromosome

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CHAPTER 1: GENERAL INTRODUCTION AND OBJECTIVES

1.1: Background for the study

South Africa is renowned for its high species richness and endemism, and harbours approximately 10% of the world's plant taxa (Goldblatt and Manning, 2000). Of the more than 20 000 plant species that occur in South Africa, more or less 2 700 species, from 15 families can be classified as geophytes (Ferreira and Hancke, 1985). Geophytes are perennial plants with a life-form in which the perennating bud is borne on a subterranean storage organ (Halevy, 1990; Özhatay *et al.*, 2013). Geophytes form an integral part of the world floriculture industry because many species are worth jointly an estimated US$1 billion on the floriculture market (Kamenetski and Miller, 2010). They are not only desired for their ornamental value, but also for their usefulness in traditional medicine (Koetle *et al.*, 2015). Their ecological importance includes the ability to develop a myriad of adaptive features that help them survive environmental stresses in a wide array of ecological habitats (Khodorova, 2011; Kamenetsky *et al.*, 2013).

Despite the ecological and economical importance of indigenous geophytes from South Africa, not much scholarly attention has been given to them (Von Staden *et al.*, 2013). Furthermore, there is a major decrease in the number of active taxonomic revisions of these plants, which is a trend not only found in South Africa but also on a global scale (Von Staden *et al.*, 2013). This is problematic because taxonomic revisions are used as the basis for assessing the extinction risks of plants and aid in conservation. To address this problem, priority genera in South Africa that is in urgent need of revision have been identified (Von Staden *et al.*, 2013). One of these is *Bulbinella* Kunth, a plant genus known for its horticultural importance and uses for humans. Such uses, for instance, include livestock feed and herbal remedies for ailments caused by bacterial and fungal infections due to a range of produced phenylanthraquinones (Bringmann *et al.,* 2008; Richardson *et al*, 2017; Musara *et al.*, 2017). For these reasons, *Bulbinella* was chosen as the topic of a phylogenetic study in this thesis.

1.2: *Bulbinella*

The genus *Bulbinella* was first described in 1843 by Kunth (Kunth, 1843). *Bulbinella* is a member of the family Xanthorrhoeaceae, subfamily Asphodeloideae, Order Asparagales (Van Wyk *et al.,* 2006; Bringmann, 2008), consists of 23 species and is taxonomically related to *Bulbine* Wolf and *Kniphofia* Moench (Perry, 1999; Kuroda, 2003). In a systematic study of the Asphodelaceae based on plastid *trnL-F* and nrDNA Internal transcribed spacer (*ITS)* sequences, *Bulbinella* forms a monophyletic group with *Eremurus* M. Bieb., *Kniphofia* and *Trachyandra* Kunth, sister to a clade consisting of *Aloe* L., *Bulbine*, *Hawortia* Duval, and *Jodrellia* Baijnath (Devey *et al.,* 2006; Naderi Safar *et al.*, 2014).

Bulbinella is a summer-green perennial herb producing leaf rosettes and flowers during summer, but the bulbs remain dormant below the ground surface in winter (Moore, 1964; Milicich, 1993). While *Bulbinella* has disjunct outlier representatives in New Zealand (6 species), the greatest species diversity (17 species) is found in South Africa (Ramdhani *et al.,* 2006; Bringmann, 2008; Klopper *et al.,* 2010). In South Africa, species occur mostly in wet habitats and is confined to the winter rainfall area of the

Northern and Western Cape Provinces (Perry, 1999). In New Zealand, endemic species are found predominantly in winter rainfall areas with some in the central Otago region, which enjoys a similar climate to the Cape Floristic Region of South Africa (Perry, 1999). The high biodiversity in the South African group suggests the potential for further improvement of cultivar development (Perry, 1999).

An ecological important characteristic of *Bulbinella* is its ability to spread fast and survive even under marginal dry areas of South Africa (Perry, 1999). This has been evidenced also by Evans (1987) when he stated that *Bulbinella* was one of the few native plants that had spread because of its tuberous roots enabling plants to resist burning. In New Zealand numerous new roots are formed each season that act as storage organs and assist in perennation for the plant (Milicich, 1993). Additionally, for *Bulbinella nutans* (Thunb.) Spreng., *B. cauda-felis* (L. f.) T. Durand & Schinz and *B. triquetra* (L. f.) Kunth the thicket formation (sheaths) act as food reserves to enable the plant to survive unfavourable conditions (Perry, 1999). Furthermore, the sheath protects the delicate stem from drying and predators during dormancy (Zahlbruckner, 1990).

The genus has considerable economic importance. The genus is prized for its spectacular flowers (Chase *et al.,* 2009) and was also considered to have potential in the cut-flower trade (Horn, 1962). The plant is used for livestock feed and herbal remedies for bacterial and fungal infections (Bringmann *et al*., 2008; Richardson *et al*, 2017). *Bulbinella* species in South Africa are utilised as a skin toner to remove impurities, production of antibacterial liquid and creams because of its healing properties (Schultz, 2013). In New Zealand, *B. hookeri* (Colenso ex Hook.) Cheeseman, locally known as '*riki*' or '*waoriki*' by the Maori, has medicinal use in the treatment of stomach pains (Riley, 1994). *Bulbinella* leaves are also used to plait baskets and floor mats by the Maori people (Goudling, 1971). *Bulbinella* species are not only limited to human beings concerning their use. For example, in New Zealand, browsers such as goats and sheep feed on species such as *B. anguistifolia* (Cockayne & Laing) L.B. Moore and *B. hookeri* in Goudland Downs's area (Milicich, 1993).

1.3: Motivation of the Study.

Despite the fact that South Africa is presently experiencing a remarkable increase in novel descriptions of its endemic diversity, a preliminary investigation into the history and nomenclature of *Bulbinella* (Moore, 1964; Milicich, 1993; Perry 1999) revealed that systematic studies in the South African and New Zealand groups are incomplete. Since then, there has been no update on the systematics of the genus. Perry's (1999) descriptive studies of species were largely based on superficial and aggregate characteristics, which showed very little variation between the different species. Subsequently, there is still a lack of proper diagnostic keys for *Bulbinella* because of the lack of clear diagnostic characters separating the different species. Such unreliable and restricted identification of species based on morphological characteristics is also a problem experienced in other genera such as *Albuca* L. and *Gethyllis* L. (Russell *et al*., 1985; Matsuki *et al.,* 2002).

The erosion of genetic diversity in plant species in the world has been increasingly severe due to several anthropogenic activities such as deforestation, and abiotic and biotic stresses (Wang *et al.,* 2007; Keneni, 2012). Similarly, climate changes have a possibility of diminishing the population viability of several species or possibly change habitats (Millennium Ecosystem Assessment, 2005; McClean *et al.,* 2005). This is especially so when a narrow genetic diversity leads to the vulnerability that consequently can lead to the extinction of species (Wang *et al.,* 2007; Keneni, 2012). The impact of such threats on *Bulbinella* is unknown but a rapid and accurate identification system among *Bulbinella* species is vital to initiate such studies, which will aid to determine the levels of genetic variation for conservation management purposes and to inhibit inbreeding of these endangered species (Oyler-McCance and Leberg, 2005).

Conservation of *Bulbinella* species is already urgent since even though *Bulbinella* species have characteristics aiding their survival, several factors pose an extinction threat to some species. According to field observations (Perry, 1999), there is an indication that land use in South Africa has reduced some populations to low levels and has probably exterminated others. The same phenomenon has occurred in New Zealand where *B. talbotii* L.B. Moore from Goudland Downs has been classified as locally extinct (Given, 1981). It is, therefore, imperative to be able to conduct accurate biogeographic assessments to determine up to date distributions. Furthermore, with genetic assessment of *Bulbinella* species it will be possible to select genes adaptable to climate change. The various factors threatening *Bulbinella* species are similar to threats against other species in the International Union for Conservation of Nature (IUCN) Red Data List (Debela, 2007; MACE, 2008).

A study by Moore (1964) revealed that the status of some *Bulbinella* species in New Zealand is nearing extinction. Almost half of these *Bulbinella* species are now listed in the IUCN Red Data List as being endangered, vulnerable, near threatened, critically rare, rare or declining (South African National Biodiversity Institution (SANBI), 2014). More species may become vulnerable or even risk extinction if *ex situ* and *in situ* conservation aspects are not taken into consideration. Equally important, is a complimentary study of the genetic status of these *Bulbinella* species to create an inventory of their genetic resources. It becomes imperative that the genetic diversity of *Bulbinella* genus should be better understood. This is because understanding the genetic diversity of these species is vital towards creating conservation priorities, proper utilisation of plant genetic resource and identification of unique and superior genotypes permitting efficient parental selection and development of elite lines for horticulture.

Bulbinella species have showy inflorescences consisting of many flowers, making them attractive garden or pot plants (Perry, 1999). Yet their exploitation and cultivation has been hampered by the lack of a strong foundational taxonomic and descriptive characteristic, and the complete lack of genetic (DNA) data. There also appears to be no studies of these species that focus on how to maximise their productivity. The aforementioned benefits that the species offer may encourage farmers to introduce the species in new areas. Knowledge on genetic diversity can allow specific plant varieties to be developed in order to satisfy the demand of the floriculture market (Maleka *et al.,* 2013). Hybrid species need to be recognised and the correct phylogeny of the species in *Bulbinella* is needed as a basis for selecting parents in crosses to breed exportable *Bulbinella* cultivars. The adoption and use of *Bulbinella* in floriculture market systems of South Africa may have considerable potential for income generation. Unfortunately, lack of adequate knowledge about germplasm conservation and genetic characterization of *Bulbinella* limits the prospects of utilising this valuable geophyte.

1.4: The advantages of complementing morphological studies with DNA sequence studies

It is evident that the species relationships and complexes in *Bulbinella* are poorly understood due to morphological homogeneity. Morphological characters may be influenced by environmental factors and the developmental stage of plant and may not distinctly distinguish closely related species (Tatineni *et al.,* 1996; Klich, 2002). Therefore, classifications relying solely on morphological characterisation can be erroneous resulting in many synonyms, species complexes and possible misidentifications of species (Avise, 1989). For this reason, it is highly beneficial to supplement taxonomic revision with extensive molecular data to aid in species identification and description (Hinrikson *et al.,* 2005; Steele *et al.,* 2010). DNA sequencing experiments are the most used to facilitate a better understanding of within- and between-species relationships (DeSalle and Amato, 2004; Rubinoff *et al.,* 2006; Pires and Marinoni, 2010).

Using molecular data has the following additional advantages. Molecular data provides additional characters for identification of plant species (Brown, 2002). Since many organisms have the presence of multiple characters during different life stages, identification of these organisms can be difficult and requires taxonomic expertise (Steele *et al.,* 2010). Identification should in some cases be made based on seeds or plant fragments, such as in samples under investigation (Steele and Pires, 2011).

Therefore, using genetic data in combination with morphological characteristics can resolve inconsistencies and provide refined taxonomic definitions (Oyler-McCance and Leberg, 2005).

Molecular data are essential for biodiversity and conservation assessments (DeSalle and Amato, 2004) since molecular data provide additional characters to identify the organism. Biodiversity is lost at an alarming rate and it is a formidable task for taxonomists to stay on the forefront of discovering and analysing new taxa. The taxonomic progress is currently very slow, and Smith *et al*. (2005) and von Staden *et al*. (2013a) suggested that the taxonomic process needs to be accelerated. Molecular techniques have been proven in previous studies to be a useful acceleration tool to the slow taxonomic process to assist in the biodiversity and conservation assessments (DeSalle and Amato, 2004; Smith *et al.,* 2005; Hajibabaei *et al.,* 2012).

A comprehensive knowledge of the relationship among species is essentially valuable in complementing conventional and molecular germplasm development programs aimed at increasing genetic diversity and genetic exchange (Burner, 1997). It is imperative to understand that different markers have different properties and will reflect different aspects of genetic diversity (Nesbitt *et al.,* 1995; Karp and Edwards, 1995). For a better understanding of the phylogenetic relationships, it is thus known that in many plant species the use of a single gene sequence in phylogenetic studies does not necessarily provides a better resolution (Liu *et al.,* 2015). It is, therefore, imperative to use more than one gene sequence to obtain a better inference from different genomes. In this regard, the use of genome sequence analysis and DNA sequencing of chloroplast and nuclear gene regions (*ITS*, *rbcL*, *matK* and *psbA-trnH*) on *Bulbinella* species will overcome potential problems arising from using single gene sequence data.

DNA barcoding is a downstream approach where once phylogenetic relationships have been established, samples can be identified by sequencing the differentiating genes defined as DNA barcode genes (Chase *et al*., 2007; Hajibabaei *et al*., 2012). Additional genes may be needed for proper phylogenetic resolution should the barcode genes prove inadequate (Uribe-Convers *et al*., 2016). It has the additional benefit that submitted DNA sequences needed for comparisons with new samples, are supplemented with photographic images, links to voucher specimens and ecological data (Ratnasingham & Hebert, (2007) and http://www.boldsystems.org/). Currently the recognized core barcode genes for land plants are *matK* and *rbcL,* the complementary *psbA-trnH* spacer and the *ITS* regions to the barcodes (Kress *et al*., 2009).

A phylogenomic approach enables the generation of a larger number of genes in one process that can then be applied in a phylogenetic study (Daubin *et al*., 2002; Foster *et al*, 2009; Uribe-Convers *et al*., 2016) or where the complete genomes of taxa are used for comparisons for example *Aloe maculata* All. and *A. vera* (L.) Burm. f. in Asphodelaceae family (GeneBankhttps://www.ncbi.nlm.nih.gov). This is particularly useful when fine scale resolution for below species questions is sought since a large number of genes can be generated in the analysis for example phlylogenomic studies of *Cardiocrinum cathayanum* (E.H. Wilson) Stearn and *Machilus yunnanensis* Lecomte by Yu *et al*. (2015). It is also useful for higher order questions, such as broad phylogenomic sampling and the sister lineage of land plants (Timme *et* *al.*, 2012). Phylogenomics also has the benefit that it reveals information on functionality when the roles or presence and absences of functional genes can be compared for example without functional genes such as *rpoA*, *rpoB*, *rpoC1* and *rpoC2*, a plant will be photosynthetically defective (Serino & Maliga, 1998). These approaches have been made possible with the advent of next generation sequencing techniques, where high throughput of samples or DNA fragments, and parallel sequencing of numerous samples or fragments, make timely production of such high numbers of sequences possible (Givnish *et al.,* 2010; Steele *et al.,* 2012; Xi *et al.,* 2012).

1.5: Aims and Objectives of the study

The revision by Perry (1999) provided the taxonomic framework and baseline for this study. The present study was aimed at constructing and elucidating the diversity and phylogenetic relationships of *Bulbinella* species from South Africa and New Zealand. We generated DNA sequence data from four gene regions (*ITS*, *matK,* *rbcL,* and *psbA-trnH*) for all of the species in *Bulbinella*. These include South African and New Zealand species. Due to the fact that South African species relationships needed more resolution, a subsequent phylogenomic analysis based on 34 protein-coding genes from the 16 South Africa species was done that were generated using a genome sequencing approach.

1.6: Statement of Research Questions

Based on this literature review the following research questions were addressed in this thesis:

1. Are the *Bulbinella* species from South Africa and New Zealand monophyletic or do they belong to different genera? The hypothesis is that the two taxa from the two countries could belong to two separate genera. The rationale for this theory is that South Africa and New Zealand is separated by an average of 11 575 km (www.distancefromto.net), intercepted by Australia. However, there are no *Bulbinella* species in Australia. Furthermore, there is a morphological difference between these groups in that, the leaves do not decay into prominent fibres at the base of the stem in New Zealand species, while this has been observed in South African species. Multigene DNA sequence comparisons will be used to test the hypothesis.

2. What are the phylogenetic relationships between the different representatives of the *Bulbinella* species from South Africa? Hereby current species morphological distinctions can be confirmed or taxonomic issues will be identified for future study. A phylogenomic approach will be used for this.

3. Due to the need to identify species for downstream applications in biodiversity, conservation and horticulture, can the generated sequences be developed into a tool to aid identification? A DNA barcode approach will be followed using the recognized barcode genes for plants that can then be used by others as a benchmark for species identification using DNA sequences.

1.7: Objectives

1. To generate a molecular phylogeny for *Bulbinella* from both South Africa and New Zealand, using DNA sequences from the plastid regions *rbcL, matK*, the *psbA-trnH* spacer and internal transcribed spacers (*ITS*) of nuclear ribosomal DNA.

2. To generate draft genomes from South African *Bulbinella* species to obtain a high number of genes for phylogenetic comparisons.

3. Genomic areas identified from the draft genomes will be used to compare species in phylogenetic analyses for finer resolution of the phylogenetic relationships between the South African species *(atpA, atpF, atpI, ndhI, psbI, ndhH, ndhF, rps*16, *rbcL, rpl*2, *rpl*23, *rpoC*1, *rpoC*2, *rps*7, *rps1.5, rps*19, *rps*2, *rps*7, *matK, ndhE, ndhB, ndhA, ccsA, atpH, orf*42, *orf*56, *psaC, rps*12, *ycf*15, *ycf*68, *psbA, rpoB* and *ndhD)*.

4. To generate tools based on the generated data to identify, conserve, and cultivate the diversity of *Bulbinella* species, and DNA sequences will be deposited as barcodes following international guidelines.

CHAPTER 2: GENERAL INTRODUCTION AND LITERATURE REVIEW[1]

2.1: Family Asphodelaceae

The family Asphodelaceae contains lily-related monocotyledons and has its main centre of diversity in southern Africa usually in arid habitats (Van Wyk *et al*., 1993; Smith and Van Wyk, 1998; Treutlein *et al*., 2003a, Bringmann, 2008; Klopper *et al*., 2010). Asphodelaceae is a petaloid, monophyletic family in the order Asparagales and consist of approximately 13 genera and more or less 800 species (Klopper *et al*., 2010). The family is amongst the most important families that have more than a hundred species (Procheş *et al*., 2006).

The presences of a trimerous flower with a superior ovary and the presence of arillate seeds have been used as evidence to support the monophyly of the family Asphodelaceae (Dahlgren *et al*., 1985, Smith and Van Wyk, 1998, Steyn and Smith, 2001, Treutlein *et al*., 2003a). Based on its vegetative and reproductive characters, the family Asphodelaceae is divided into two subfamilies, namely the Alooideae and the Asphodeloideae (Brummit, 1992; Treutlein *et al*., 2003a; Klopper *et al*., 2010). The recent most recognised morphological treatment is the framework of Dahlgren *et al.,* (1985).

Of interest to this review is the Asphodeloideae, which is a small homogeneous group comprising of nine genera with approximately 261 species (Bringmann, 2008; Klopper *et al*., 2010). Of these, the genus *Bulbinella* has disjunct outlier representatives in New Zealand (Chase *et al*., 2000; Bringmann, 2008; Klopper *et al*., 2010). The

[1] This chapter review has been published under the title, 'A review of *Bulbinella* (Asphodelaceae): distribution, conservation status and economic importance' in Botanical Sciences 95(2):155-168, 2017. DOI:10.17129/botsci.696

Asphodeloideae subfamily is quite diverse in form ranging from succulent through mesomorphic to xeromorphic, and it has varying extents of small to large chromosomes with a basic set of six chromosomes (2n=12) (Daru *et al*., 2013).

2.2: Derivation of the name *Bulbinella* and historical aspects

The genus *Bulbinella* dates from 1843 when Kunth erected the genus (Kunth, 1843). *Bulbinella* was named for its close resemblance to *Bulbine*, with the major difference mainly in the glabrous filaments which are always hairy in *Bulbine* (Boatwright and Manning, 2012). Before the study of Kunth, the species formed part of the then polymorphic genus *Anthericum L.* but the genus was discarded and the taxa divided among the known three genera *Phalangium* Mill., *Trachyandra* Kunth and *Bulbinella* Kunth (Perry, 1999; Boatwright and Manning, 2012). According to Gibb Russell *et al*. (1985), only four species of *Bulbinella* were documented in South Africa prior to 1987. However, South African *Bulbinella* species extracted from volumes of Index Kewensis totalled 21 (Perry, 1999).

According to Perry (1999), of these 21 South African *Bulbinella* species, two have since been placed in *Ornithogalum* L., four in *Trachyandra* Kunth and one has been identified as *Caesia contorta* (L.f.) T. Durand & Schinz. The various placings were given to the 14 remnant names by authors such as Kunth (1843), Baker (1872, 1876, and 1896) and Durand and Schinz (1894). Following the above studies, additional species have been described, resulting in the current recognition of 18 *Bulbinella* species and six sub-species in South Africa (Perry, 1999; Bringmann, 2008; Klopper *et al*., 2010). The 18 species are *Bulbinella nutans* (Thunb.) T. Durand &Schinz, *Bulbinella latifolia* Kunth & P.L. Perry, *Bulbinella punctulata* Zahlbr., *Bulbinella potbergensis* P.L. Perry, *Bulbinella* *eburniflora* P.L. Perry, *Bulbinella caudafelis* (L.f.) T. Durand & Schinz, *Bulbinella graminifolia* P.L. Perry, *Bulbinella barkerae* P.L. Perry, *Bulbinella elegans* P.L. Perry, *Bulbinella trinervis* P.L. Perry, *Bulbinella gracillis* Kunth, *Bulbinella divaginata* P.L. Perry, *Bulbinella nana* P.L. Perry, *Bulbinella chartacea* P.L. Perry, *Bulbinella ciliolata* Kunth, *Bulbinella elata* P.L. Perry, *Bulbinella calcicola* J.C. Manning & Goldblatt and *Bulbinella triquetra* (L.f.) Kunth. The subspecies are *Bulbinella nutans* subsp. *nutans*, *Bulbinella nutans* subsp. *turfosicola, Bulbinella latifolia* subsp. *doleritica*, *Bulbinella latifolia* subsp. *latifolia, Bulbinella latifolia* subsp. *denticulata* and *Bulbinella latifolia* subsp. *toximonata* (Perry, 1999).

Bulbine Wolf, *Kniphofia* Moench and *Bulbinella* Kunth are taxonomically related and form a monophyletic unit within the subfamily since they all produce knipholone-type compounds (Bringmann *et al*., 2008). The notion that *Kniphofia* is not related to the Alooideae is supported by the knipholone-type compounds which seem to be characteristic constituents for the three genera *Bulbine*, *Bulbinella* and *Kniphofia* (Van Wyk et al., 1995; Klopper et al., 2010). However, supplementary studies are essential to confirm the absence of this type of compounds in other genera of the Asphodeloideae (Van Wyk *et al*., 1995; Bringmann *et al*., 2008; Klopper *et al*., 2010).

2.3: Generic relationships of *Bulbinella*

A number of genera related to *Bulbinella* exist and these are *Asphodeline, Asphodelus, Eremurus, Jodrellia, Bulbine, Trachyandra* and *Kniphofia*. The ranges of species in *Asphodeline* genus (± 12 species) and *Asphodelus* genus (± 14 species) extend from the Mediterranean to western Asia in the northern hemisphere. *Eremurus* (± 40 species) is confined to the steppes of the high plateaus in central Asia. *Jodrellia* is a recently described genus from central Africa that is closely related to *Bulbine*. *Bulbine, Trachyandra* and *Kniphofia*, which comprise of about 70 species, occur in Africa (Chase *et al*., 2000; SANBI, 2009).

Bulbine Wolf (± 73 species) are shrubs, weedy perennials, dwarf geophytes, and soft annuals occurring in Africa and Australia, with 46 of the total species chiefly found in southern Africa (Chase *et al*., 2000; SANBI, 2009). It is a genus of succulent plants caulescent, largely branched, rhizomatous, and caespitose or solitary geophytes (Barnes *et al*., 1994). Some *Bulbine* species are ornamental plants and are sold in nurseries and garden shops, frequently as plant hybrids. With few exceptions, all *Bulbine* species have yellow flowers and the filaments are bearded with yellow pointed or clavate hairs (Hall *et al*., 1984).

According to Chase *et al*. (2000) and Treutlin *et al*. (2003a), *Kniphofia* Moench is best placed in Asphodeloideae and is sister to *Bulbinella* (Ramdhani *et al*., 2006). The species of *Kniphofia* are chiefly distributed in southern and eastern Africa (Ramdhani *et al*., 2006). Of these, 47 species are found in southern Africa. Two other species, *Kniphofia pallidiflora* and *Kniphofia ankaratrensis*, are indigenous to Madagascar and *Kniphofia sumarae* to Yemen (Ramdhani *et al*., 2006; Alasbahi *et al*., 2007). Most *Kniphofia* species in cultivation today are of hybrid origin whereas those naturally occurring are found growing near rivers or in damp or marshy areas and mountainous grasslands (Reid and Glen, 1993).

Kniphofia Moench has an enormous horticultural demand since some of its members have conspicuous inflorescences (Ramdhani *et al*., 2006). Generally, species of

Kniphofia are evergreen summer growing species while a few are deciduous that bear dense, erect spikes above the level of the leaves in either winter or summer depending on the species (Codd, 1968; Ramdhani *et al*., 2006). The leaves are non-succulent and usually borne in a rosette. *Kniphofia* flowers are small and tubular and fashioned in shades of various colours which are frequently visited by honey sucking sunbirds (Codd, 1968; Ramdhani *et al*., 2006).

Bulbinella Kunth (± 23 species) has been recorded in New Zealand (6 species) with the greatest diversity found in South Africa (17 species) (Ramdhani *et al*., 2006; Klopper *et al*., 2010). The genus is endemic and confined to the winter rainfall area with some in New Zealand in the central Otago region which enjoys a similar climate to the Cape Region of South Africa (Perry, 1999). In phylogenetic analyses, *Bulbinella* is monophyletic with *Eremurus*, *Kniphofia* and *Trachyandra*. This clade is sister to a clade made up by *Aloe, Bulbine*, *Haworthia*, and *Jodrellia* (Devey *et al*., 2006; Naderi Safar *et al*., 2014).

2.4: *Bulbinella* Morphology

The entire *Bulbinella* genus includes species that are deciduous geophytes ranging in height above the ground from about 0.2-1.2m (Perry, 1999). As hybridisation between species is not yet known to occur, *Bulbinella* plants come true from seed (Perry, 1999). The leaves which are produced annually die down at the end of each growing season to form sheaths which act as food reserves to enable the plant survive unfavourable conditions. This thicket formation (sheaths) is evidenced by three species which include *Bulbinella nutans*, *Bulbinella cauda-felis* and *Bulbinella triquetra* (Perry, 1999).

Bulbinella gracilis, Bulbinella nutans and *Bulbinella latifolia* have some degree of succulence and most leaves are glabrous with very few being sparsely and irregularly covered with fine longish hairs (Perry, 1999). The inflorescence is simple, the compact raceme of numerous star-shaped flowers usually in shades of yellow and less commonly white or orange and these variations are significant in the identification of *Bulbinella* species (Perry, 1999). There is similarity of floral structure in all *Bulbinella* species, yet with subtle differences in properties such as proportions colour, slight range in size and scents that are not easily definable (Perry, 1999). Expression of two or more different colour types occurs only in species such as *Bulbinella elegans* and *Bulbinella nutans* while the rest have flowers of one colour only (Perry, 1999).

The trilocular ovary is a very notable characteristic of the genus, with the stigma being apical, minutely papillate without copious fluid secretions (Dahlgren and Clifford, 1982). During dormancy, the sheath protects the delicate stem from drying and also predators (Zahlbruckner, 1990). The rootstock is rhizomatous with tuberous roots to perform the function of food storage and assist in perennation for the plant (Perry, 1999). The texture and colour of the outer walls of *Bulbinella* fruit may be of taxonomic significance with the seeds being three-angled of matt black or greyish black colour and the shape is very analogous in the diverse species (Perry, 1999).

2.5: Pollination Biology

The exact details of pollination in *Bulbinella* have not been sufficiently studied in their natural environment, so it is speculated that it has a cross-pollination system ensuring gene flow between plants (Perry, 1999). Since many organisms are able to perceive ultraviolet reflectance (Kevan and Phillips, 2001), a variety of crawling insects including honey bees which visit the inflorescences could be responsible for pollination. This has been observed chiefly in the orange flowered *Bulbinella latifolia* sub-species *doleritica* and *B. eburniflora* (Perry, 1999). According to Moar *et al*, (2011), sulcate pollen occurs with trichotomosulcate grains in species of *Bulbinella*.

Correspondingly, Faegri and Van der Pijl (1979) describe beetle-pollinated flowers as having few visual attractions, as exhibited by many species of *Bulbinella*, especially *Bulbinella eburniflora* with ivory coloured flowers and *Bulbinella barkerae* with off-white flowers (Perry, 1999). Scent may be connected with pollination and produce a somewhat musty odour as evidenced in *Bulbinella eburniflora* and *Bulbinella barkerae* species, whereas in other species the scent appears ephemeral (Perry, 1999).

2.6: Species recommended for cultivation

The adoption and use of *Bulbinella* in floriculture market systems of South Africa may have considerable potential for income generation. The advantages that the species offer may encourage farmers to introduce the species in new areas hence maximising its productivity. *Bulbinella* is fundamentally a genus of cold or cool, wet habitats and is confined to the winter-rainfall area of the Cape. However, most of the species cannot tolerate frost prone areas outdoors but are easily cultivated in cool greenhouses (Perry, 1999). Three species have been cultivated in the past, namely *Bulbinella nutans* var. *nutans*, *Bulbinella latifolia* var. *doleritica*, and *Bulbinella cauda-felis*. *Bulbinella latifolia* subspecies *doleritica* has since proved popular in cultivation in Israel because of the Mediterranean type of climate of the country (Perry, 1999).

Bulbinella latifolia subsp. *latifolia*, *Bulbinella elata* and the yellow flowered form of *Bulbinella nutans* subsp. *nutans* are most suitable for garden cultivation and are also the most valuable species for cut flowers (Perry, 1999). The smallest *Bulbinella* species, the spring-flowering *Bulbinella triquetra* with yellow flowers and autumn-flowering *Bulbinella divaginata*, could be grown in a rock garden, but are also the most suitable for container culture (Perry, 1999). Both the lemon-yellow and the cream coloured forms of *Bulbinella elegans* are well worth growing and they make neat plants and the venation on the leaf sheath adds to the significance of their identity (Perry, 1999). *Bulbinella gracilis*, as the name implies, is a graceful plant and probable would make a charming pot plant (Perry, 1999). *Bulbinella hookeri* and *Bulbinella rossii* are the most frequently cultivated species of the genus and have enjoyed most of the horticultural attention (Bryan and Griffiths, 1995).

2.7: Morphological Classification of *Bulbinella* Species

2.7.1: Summary of *Bulbinella* Species

Species are distinguishable groups of genotypes that remain distinctive in the face of probable or actual hybridisation and gene flow (Coyne *et al*., 2004; Mallet 2006; 2008). They are fundamental elements from which the larger groups are constructed (Russel, *et al*., 1985). Most of the species can be identified with certainty if enough morphological traits are available when identifying these species (Spies, 2004). A total of 23 species of *Bulbinella* is known, of which 17 are found in southern Africa, and 6 species in New Zealand (Perry, 1999). Unfortunately, the distribution areas overlap for some species in some parts of the distribution range, which implies that hybrids can easily be produced between different species (Spies, 2014).

Speciation and hybridization are two events that are currently still impeding the identification and classification of many plant species (Spies, 2014). However, in South Africa *Bulbinella* is clearly separated from related genera such as *Bulbine*, *Trachyandra* and *Kniphofia* by its simple compact raceme of stellate flowers, smooth filaments and ovarian shape (Perry, 1987). Since the genus has subtle morphological differences in an area, it has been classified into numerous species as shown in **Figure 1**. Below follows more detailed treatments of each species in *Bulbinella*.

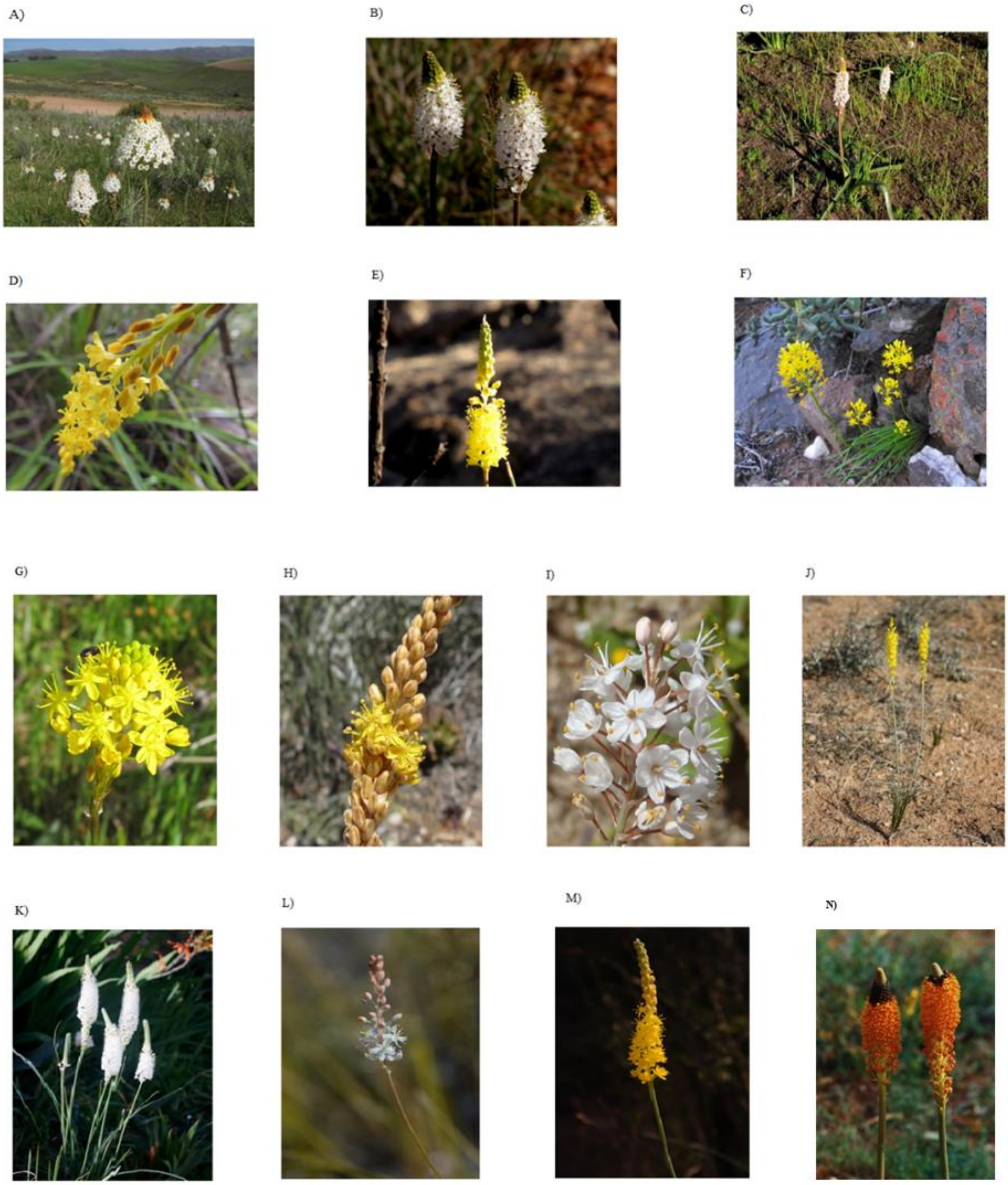

Figure 1: ***Bulbinella*** **Species of South Africa (A)** ***Bulbinella barkerae*****. (B)** ***Bulbinella cauda-felis*****. (C)** ***Bulbinella eburniflora*****. (D)** ***Bulbinella chartacea*** **(E)** ***Bulbinella elegans*****. (F)** ***Bulbinella gracillis*****. (G)** ***Bulbinella triquetra*****. (H)** ***Bulbinella calcicola*****.** ***(I)Bulbinella nutans (J) Bulbinella divaginata. (K) Bulbinella graminifolia (I) Bulbinella trinervis. (M) Bulbinella punctulata*** **[(Source: www.ispotnature.org)] (N)** ***Bulbinella latifolia*** **[(Source: www.dip.sun.ac.za)]**

2.7.2: Morphological Characteristics of *Bulbinella* in South Africa

These tufted, deciduous perennial, solitary plant species varies from 0.25m to 1m in height and their tubers are less uniform in appearance than those of the New Zealand species with swellings found adjacent to the root base (Milicich, 1993; Perry, 1999). The roots are somewhat fleshy to an elongated sausage shape over its entire length as an alternative to tubers (Milicich, 1993). In all South African species, the leaves are erect, but vary greatly from thick and fleshy to thin and deeply channel and often forms persistent fibrous leaf bases at the root stock (Milicich, 1993; Perry, 1999; Boatwright and Manning, 2012).

Pollination is made possible by insects, notably honeybees (Boatwright and Manning, 2012), with the flowering times varying for each species from 1-5months duration, coinciding with their respective wet seasons (Perry, 1999). The colour of the perianth segments varies both among and within some species in South Africa from white, some with a pink central stripe, through ivory, cream and yellow to bright orange (Perry, 1999; Boatwright and Manning, 2012). Most species do prefer moist, cool habitats and a peaty, acid, sandy soil (Boatwright and Manning, 2012).

2.7.2.1: Bulbinella nutans (Thunb.) **T. Durand & Schinz**

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa

Bulbinella nutans **(Fig 1I)** and *Bulbinella latifolia* **(Fig 1N)** are closely similar to each other, but *B. nutans* can be distinguished by its slightly smaller stature, narrower, erect leaves and shorter inflorescences (Perry, 1999; Boatwright and Manning, 2012). These species are mostly found on clayey soils that are seasonally wet (Perry, 1999; Boatwright and Manning, 2012).

Figure 2: Distribution map for *Bulbinella nutans* (Thunb.) T. Durand and Schinz. (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Depending on the diverse habitat preference and also the size of leaves, the species is divided into two subspecies, namely subsp. *nutans* and subsp. *turfosicola* (Perry, 1999). The subsp. *nutans* has the widest leaves and broadly conical inflorescence (Perry, 1999) and is found from the Cape Peninsula northwards as far as Loeriesfontein and eastwards to Swellendam (Boatwright and Manning, 2012). The subsp. *turfosicola* has a late spring to summer-flowering time and is found on dark peaty soils of seepage areas in mountains of the Table Mountain Group **(Fig 2)** (Perry, 1999).

2.7.2.2: *Bulbinella latifolia* Kunth & P.L. Perry

Conservation status and criteria: Least Concern, Vulnerable D1+2 [Raimondo *et al.* (2009)]

Provincial distribution: Northern Cape, Western Cape, South Africa

Young cultivated plants of *Bulbinella nutans* **(Fig 1I)** and *Bulbinella latifolia* **(Fig 1N)** show marked differences in their habit even when grown side by side with consistent differences in length and width of their roots (Perry, 1999).

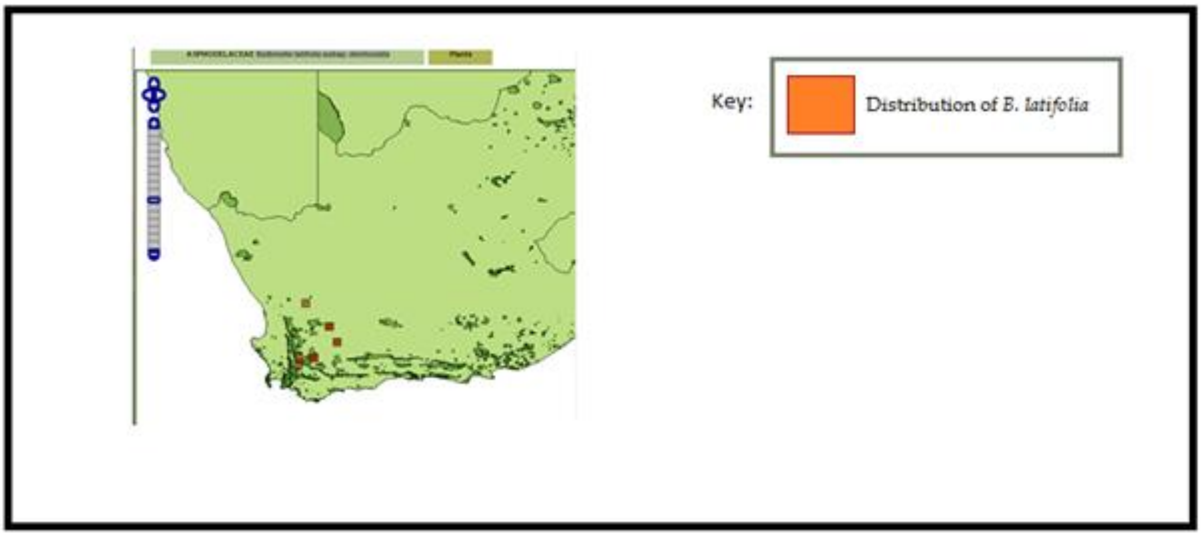

Figure 3: Distribution map for *Bulbinella latifolia* Kunth P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Bulbinella latifolia **(Fig 3)** occupy a diversity of habitats often along streams or dams on granite, peat and clay where it forms large seasonal stands in seasonally wet areas (Perry, 1999; Boatwright and Manning, 2012).

2.7.2.3: *Bulbinella punctulata* Zahlbr.

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Western Cape, South Africa.

Bulbinella punctulata **(Fig 1M)** area unique species due to the small number of their leaves which are comparatively long and narrow, and they may also be documented by their long narrow inflorescence of yellow flowers (Perry, 1999).

Figure 4: Distribution map for *Bulbinella punctualata* Zahlbr (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

A further characteristic which evidently separates *Bulbinella punctulata* from other species is the loose net-like part of the sheath with the inner cataphyll extending for some distance up the leaves (Perry, 1999). The species are confined to the Cederberg range **(Fig 4)**, where they grow on sandy soils or in damp flats of Restioveld (Perry, 1999).

2.7.2.4: *Bulbinella potbergensis* P.L. Perry

Conservation status and criteria: Critically Endangered B1ab (iii) +2ab (iii) [Raimondo *et al*. (2009)]

Provincial Distribution: Western Cape, northern side of the Potberg range, South Africa.

Bulbinella potbergensis is a very rare species so far found only on the low Koppies near the foot of Potberg range (Perry, 1999) **(Fig 5)**.

Figure 5: Distribution map for *Bulbinella potbergensis* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Bulbinella potbergensis grows well on clayey silcrete with stones at an altitude of about 150m among clumps of the Cape reed **(Fig 5).** The single long leaf and neatly reticulate sheath make it unique but it is closely related to *Bulbinella punctulata* (Perry, 1999).

2.7.2.5: *Bulbinella eburniflora* P.L. Perry

Conservation status and criteria: Vulnerable B1ab (iii, v) +2ab (iii, v) [Raimondo *et al.* (2009)]

Provincial Distribution: Northern Cape, Bokkeveld Escarpment, South Africa.

The hispido-ciliate, canaliculated leaves which vary in size are distinct features which separate *Bulbinella eburniflora* **(Fig 1C)** from closely resembling species (Perry, 1999). Another characteristic that makes *Bulbinella eburniflora* distinct is the ivory-white flowers which habitually have a strong musty odour (Perry, 1999).

Figure 6: Distribution map for *Bulbinella eburniflora* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

The fibrous sheath in *Bulbinella eburniflora* is fine, soft and somewhat reticulate, whereas in *Bulbinella ciliolata* it is straight and loose and in *Bulbinella elegans* intricately reticulate (Perry, 1999). The species has been found on flats of soft fine silty loam and sandier soils mainly in Renosterveld (Perry, 1999) **(Fig 6).**

2.7.2.6: *Bulbinella caudafelis (L.f.)* T. Durand & Schinz

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa.

Bulbinella cauda-felis **(Fig 1B)** is a widespread species found frequently on clayey and sandy soils among Renosterveld or Karoo-type vegetation (Perry, 1999). They penetrate into the drier habitats on the northern and eastern margins of Cape **(Fig 7)** (Perry, 1999). *Bulbinella cauda-felis* is a very variable species complex in which it is not easy to find clear-cut distinguishing features (Perry, 1999).

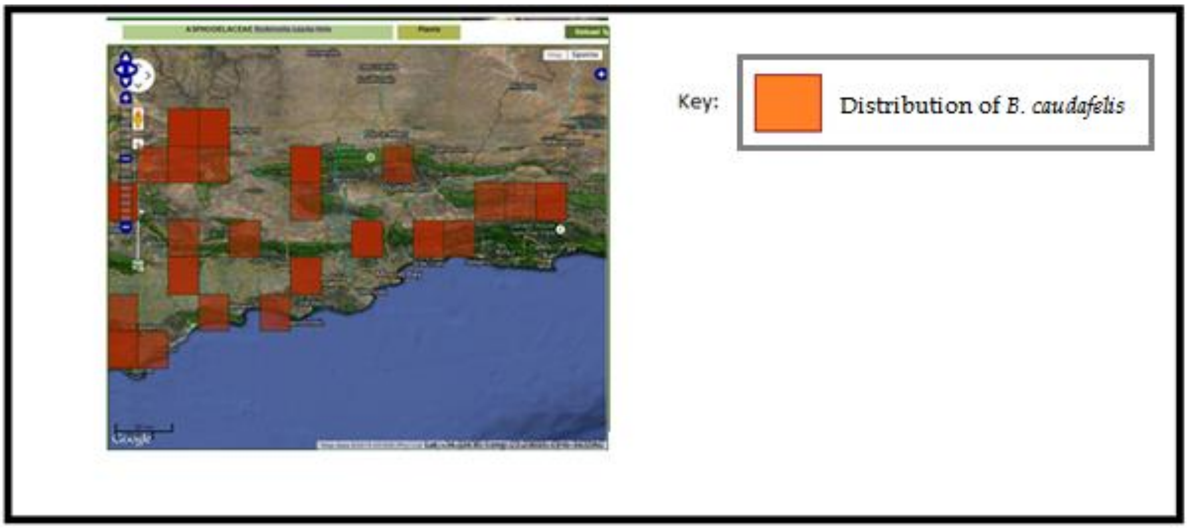

Figure 7: Distribution map for *Bulbinella caudafelis* (L.f.) T. Durand and Schinz. (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

According to Perry, (1999), the species has narrow racemes of pinkish-white flowers, large dull black seeds and thin walled, pale fawn capsule which are considered as significant diagnostic characters. The species could be confused with *Bulbinella triquetra* because of the narrow leaves but most commonly the leaves always have a dilated sheath and somewhat glaucous appearance. The diverse populations of these species flower in November and December (Perry, 1999).

2.7.2.7: *Bulbinella graminifolia* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa.

Bulbinella graminifolia **(Fig 1K)** is closely related to *Bulbinella cauda-felis* **(Fig 1B)** but is distinguished by its considerably finer, reticulate fibrous sheath (Perry, 1999).

Figure 8: Distribution map for *Bulbinella graminifolia* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Further to that, the fruit and the seeds of *Bulbinella graminifolia* **(Fig 1K)** are just about half the size of those of *Bulbine cauda-felis* and the inflorescence of *Bulbinella graminifolia* is smaller, more narrowly cylindrical with flowers purer white (Perry, 1999). The species occur on stony, clayey or loamy, damp, south facing hillsides and is confined largely to the Clanwilliam area **(Fig 8)**, where it occurs in Renosterveld or among Karroid bushes (Perry, 1999).

2.7.2.8: *Bulbinella barkerae* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Western Cape, South Africa

The species was named in honour of Miss W. F Barker (Perry, 1999). *Bulbinella barkerae* **(Fig 1A)** is straightforwardly separated from the other species with ciliate margins, on locality and also on the broader and few leaves (Perry, 1999).

Figure 9: Distribution map for *Bulbinella barkerae* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

The spreading of leaves with regularly ciliate margins, the smaller greyish green fruits, the seeds with a broadish wing extension and the strong-smelling flowers are characteristics of *Bulbinella barkerae* which separates it from *Bulbinella cauda-felis* (Perry, 1999). *Bulbinella barkerea* is confined to the Caledon, Bredasdorp and Riversdale districts **(Fig 9)** and found growing on shale flats or slight slopes mainly on stony, sandy ground at the foot of the Riviersonderend Mountains (Perry, 1999).

2.7.2.9: *Bulbinella elegans* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa

Bulbinella elegans **(Fig 1 E)** has a broader leaf which developed a more intricate system of conducting tissues resulting in a basal sheath with more prominent reticulate veins (Perry, 1999).

Figure 10: Distribution map for *Bulbinella elegans* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

It appears to be most closely related to *Bulbinella triquetra* though it is a larger type (Perry, 1999). *Bulbinella elegans* possess the dense reticulate fibrous sheath which separates it from *Bulbinella ciliolata* which has a loose straight fibrous sheath. Furthermore, *Bulbinella elegans* shave dead fibres which are solidly compact and intertwined, different from the shorter, straighter and looser fibres of *Bulbinella triquetra* (Perry, 1999).

The species thrive in drier areas and flower colour is dependent on distribution, with the white type occurring on sandy soils of mountain Renosterveld in the Sutherland and Laingsburg Districts (Perry, 1999) **(Fig 10)**. On the other hand, a lemon-yellow form appears to be confined to western mountain Karoo vegetation of the doleritic and dwyka clays in the Nieuwoudtville area (Perry, 1999) **(Fig 10)**.

2.7.2.10: *Bulbinella trinervis* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Eastern Cape, Western Cape, South Africa

Owing to the similar narrow leaves, *Bulbinella trinervis* **(Fig 1L)** may be confused with *Bulbinella triquetra* **(Fig 1G)** particularly those populations flowering afterwards in the season in November and December (Perry, 1999).

Figure 11: Distribution map for *Bulbinella trinervis* (Baker) P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

According to Perry (1999), the features that clearly separate *Bulbinella trinervis* from *Bulbinella triquetra* is the non-sheathing leaf bases, small bracts and also the smaller seeds. Furthermore, the bracts are broad and truncate without the more typical attenuate apex making *Bulbinella trinervis* very distinctive in *Bulbinella* (Perry, 1999). Another distinguishing character is the white flowers of *Bulbinella trinervis* that are produced in autumn whereas *Bulbinella triquetra* have yellow flowers produced in spring (Perry, 1999). These species have established on clay, on rocky lower mountain slopes, or sandy soils among fynbos vegetation in the western part of southern Cape excluding the Peninsula (Perry, 1999) **(Fig 11)**.

2.7.2.11: *Bulbinella gracillis* Kunth

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa.

The patent pedicels in the fruiting stage are unique to *Bulbinella gracilis* **(Fig 1F)** and *Bulbinella nana* and the absence of dead leaf remains forming a fibrous sheath around the stem and leaf bases. This is not seen in any other *Bulbinella species* in South Africa except in *Bulbinella gracilis* (Perry, 1999).

Figure 12: Distribution map for *Bulbinella gracillis* Kunth (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Regardless of low and erratic rainfall (150mm/yr) *B. gracillis* may establish in dampish areas either among the rocks of dried river beds and flood plain ravines. The species are found in the Northern Cape **(Fig 12)** from the Richtersveld as far south as Nuwerus (Perry, 1999).

2.7.2.12: *Bulbinella divaginata* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa

Bulbinella divaginata **(Fig 1J)** is a conspicuously autumn-flowering species.

Figure 13: Distribution map for *Bulbinella divaginata* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

The distal swollen regions are not found in *Bulbinella divaginata* but in *Bulbinella triquetra* roots (Perry, 1999). The membranous white cataphylls surrounding the base of the leaves is a crucial diagnostic characteristic (Perry, 1999). The species is found in a variety of soil types from fine clay to sandy, and predominantly in the hillier or mountainous areas of Northern and Western Cape in Namaqualand (Perry, 1999), **(Fig 13)**.

2.7.2.13: *Bulbinella nana* P.L. Perry

Conservation status and criteria: Vulnerable D2 [Raimondo *et al*. (2009)]; Rare [Hilton-Taylor (1996)].

Provincial Distribution: Northern Cape, Namaqualand, Stein Kopf and Springbok, South Africa.

It is the smallest of all the *Bulbinella* species forming dainty, delicate plants and is known from two collections from the Richtersveld area **(Fig 14)** of the Northern Cape (Perry, 1999). The species has a close resemblance with *Bulbinella gracilis* but are separated by the more numerous and very fine filiform leaves compared with the more succulent ones of *Bulbinella gracilis* (Perry, 1999).

Figure 14: Distribution map for *Bulbinella nana* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Bulbinella nana also has few seeds formed in cultivation which was markedly similar to the distinctive seeds of *Bulbinella gracillis.* Lastly, it has more prominent basal sheath fibres and distinct veining in the cataphylls which is not so obvious in *Bulbinella gracilis* (Perry, 1999).

2.7.2.14: *Bulbinella chartacea* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Western Cape, South Africa

The basal sheathing fibres clearly distinguishes *Bulbinella chartacea* **(Fig 1D)** from all other species, being very loose, straight and papery (Perry, 1999). Both *Bulbinella chartacea* and *Bulbinella trinervis* flowers at the same time of year often in similar areas, but *Bulbinella trinervis* has white flowers and is found on lower slopes while *Bulbinella chartacea* has yellow flowers (Perry, 1999).

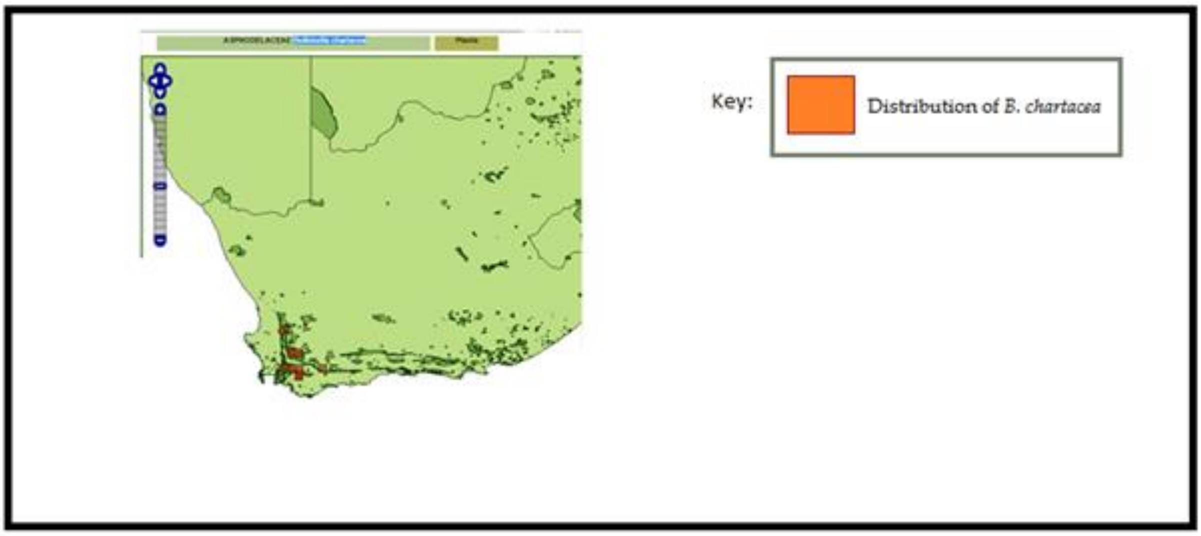

Figure 15: Distribution map for *Bulbinella chartacea* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

It occurs in rocky areas and has a comparatively limited distribution mainly in the Riviersonderend Mountains and ranges to the north of Worcester (Perry, 1999) **(Fig 15)**.

2.7.2.15: *Bulbinella ciliolata* Kunth

Conservation status and criteria: Least Concern [Raimondo *et al.* (2009)]

Provincial Distribution: Northern Cape, South Africa.

Bulbinella ciliolata is easily distinguished from *Bulbinella elegans* species by the fibrous sheath which is loose and straight whereas in *Bulbinella elegans* it is compactly reticulate. Its leaves and inflorescence are similar to those of *Bulbinella elegans* but tend to be narrower and more numerous (Perry, 1999).

Figure 16: Distribution map for *Bulbinella ciliolata* Kunth (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

The species are restricted to northern Namaqualand **(Fig 16)** on sandy loams of the granite hills, especially in damper depressions or by streamlets in the vicinity of Springbok and Kamieskroon brokenveld (Perry, 1999).

2.7.2.16: *Bulbinella elata* P.L. Perry

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa

Bulbinella elata has two colour variations. The cream-flowered form is restricted from the West Coast north through Clanwilliam to Calvinia. The yellow-flowered form is known from two populations on the escarpment below the Roggeveld: one on

Bloukrans Pass and the other in a shaded kloof near to the north of Ouberg Pass in the Sutherland District (Perry, 1999) **(Fig 17)**.

Figure 17: Distribution map for *Bulbinella elata* P.L. Perry (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Although this taxon is closely related to *Bulbinella latifolia* and *Bulbinella nutans,* it has flat, spreading, coriaceous, noncanaliculate leaf blades, which are thinner and more delicate when pressed than those of *Bulbinella latifolia*. In nature, *Bulbinella elata* normally flowers earlier in the season than the forms of *Bulbinella latifolia* and *Bulbinella nutans. Bulbinella elata* species prefers clayey or granitic soils (Boatwright and Manning, 2012).

2.7.2.17*: Bulbinella calcicola* J.C. Manning & Goldblatt

Conservation status and criteria: Critically Endangered A3c [Raimondo *et al*. (2009)]

Provincial Distribution: Western Cape, South Africa

Bulbinella calcicola **(Fig 1H)** is a recently described species (Manning and Goldblatt, 2010) which is most similar to *Bulbinella triquetra* but differs in its broader, channelled leaves with narrowly cylindrical racemes and flowers that are orange-tipped (Manning and Goldblatt, 2010).

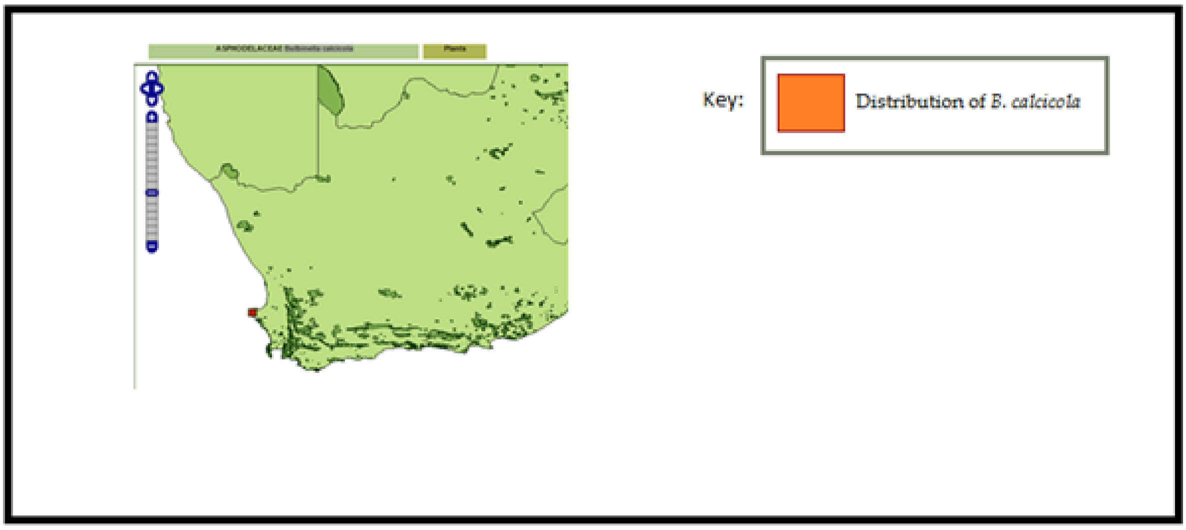

Figure 18: Distribution map for *Bulbinella calcicola* J.C. Manning and Goldblatt (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Bulbinella calcicola is restricted to the limestone outcrops around Jacobsbaai close to Saldanha (Manning and Goldblatt, 2010) **(Fig 18)**.

2.7.2.18: *Bulbinella triquetra* (L.f.) Kunth

Conservation status and criteria: Least Concern [Raimondo *et al*. (2009)]

Provincial Distribution: Northern Cape, Western Cape, South Africa

Bulbinella triquetra **(Fig 1G)** *is* a widespread species which extends its habitats to damper shaded slopes on clayey soils in Karroid vegetation from the Cederberg to the Cape Town area and east to the Caledon area (Perry, 1999), **(Fig 19)**. *Bulbinella triquetra* are spring-to-early summer-flowering with the leaves having completed development at flowering.

Figure 19: Distribution map for *Bulbinella triquetra* (L.f.) Kunth (Source: https://www.pacificbulbsociety.org/pbswiki/index.php)

Bulbinella triquetra have narrow leaves with denticulations, trigonous and finely denticulate margins. Both *Bulbinella divaginata* and *Bulbinella trinervis* have similar sized and narrow leaves except that *Bulbinella divaginata* leaves are almost terete (Perry, 1999). *Bulbinella triquetra* have yellow flowers similar to *Bulbinella divaginata* but they are evidently separated by the sheathing leaf bases in *Bulbinella triquetra*, whereas in *Bulbinella divaginata* the fibrous sheath is formed from separate cataphylls (Perry, 1999).

2.8: Morphological Characteristics of *Bulbinella* in New Zealand

2.8.1: Distribution and Habitat

Bulbinella has an interesting and unusual, highly disjunct distribution of an average of 11 575km between South Africa and New Zealand (Boatwright and Manning, 2012). New Zealand has created a great deal of diversity in vegetation types as a result of its climate and geology (Hamish and Hutching, 2007). All New Zealand *Bulbinella* species (*Bulbinella angustifolia* (Ckn. & Laing) L.B. Moore, *Bulbinella gibbii* Cockayne, *Bulbinella hookeri* (Hook.) Cheeseman, *Bulbinella rossii* (Hook.f.) Cheeseman, *Bulbinella talbotii* L.B. Moore and *Bulbinella modesta* L.B. Moore) occur in separate non-overlapping geographical areas

The species thrives on permanent swamps, the river banks and seepage sites in wet grassland (Milicich, 1993). *Bulbinella hookeri* and *Bulbinella rossii* are the most frequently cultivated species of the genus and have enjoyed most of the horticultural attention (Bryan and Griffiths, 1995) (**Fig 20**)

2.8.2: Morphology

All the six species have a crown with a rosette of up to 12 strap-shaped leaves (Moore, 1964). Their erect stems have leaf insertions crowded over a short length and varying in height (Moore, 1964). All the flowers are borne on flexible pedicles, subtended by small, leaf-like bracts and have a star-like appearance with two whorls each of three perianth segments (tepals) and two whorls each of three anthers (Moore, 1964).

Their ovaries are green in flowers and their capsules change to brown when drying prior to dehiscence. The capsules are triangular in cross section and each may enclose up to six seeds (Milicich, 1993). The roots or tubers are tough; function as storage organs and are resistant to rotting or fungal attack (Milicich, 1993).

2.8.3: Pollination Biology

All New Zealand *Bulbinella* species have yellow flowers which produce a faint scent and none of them has the feathery anthers which is a characteristic of wind-pollinated species (Moore, 1964; Milicich, 1993). The insects observed on flowers of *Bulbinella hookeri, Bulbinella gibbisi, Bulbinella angustifolia* and *Bulbinella modesta* include honey bees, flies and bugs, signifying that insects are likely to be involved in *Bulbinella* pollen transport (Milicich, 1993).

2.8.4: Features of the Individual Species.

2.8.4.1: *Bulbinella angustifolia* (Ckn. & Laing) L.B. Moore

Conservation status and criteria: Least Concern/ Not Threatened (Milicich, 1993).

Distribution: Endemic. Common south of Waiau (South Island) in the eastern hills of Canterbury, Otago and Southland, **Fig 21**.

Overall the size of the *Bulbinella angustifolia* **(Fig 20A)** plants is smaller than that of *Bulbinella hookeri* **(Fig 20C)**. The species is hermaphroditic and its flowering occurs during November and December (Moore and Edgar, 1970). Most plants produce racemes having 50 flowers or less but ones with more flowers do occur (Moore, 1964; Milicich, 1993).

2.7.4.2: *Bulbinella gibbii* Cockayne

Status and Criteria: *Bulbinella gibbsii* var. *gibbssii* (At Risk - naturally uncommon) and *Bulbinella gibbsii* var. *balanifera* (Not Threatened) (Milicich, 1993).

Distribution: Endemic and restricted to Stewart Island.

The species are closer to *Bulbinella rossii* than to *Bulbinella hookeri* but altogether a smaller plant with much slenderer shape and very much shorter and more open raceme (Moore, 1964). *Bulbinella gibbsii* var. *gibbssi* plants are smaller than those on the mainland and produced 40 or fewer flowers per raceme. Nonetheless, both varieties of *Bulbinella gibbsii* are gynodioecious and *Bulbinella gibbsii* var. *balanifera* shows a widely disjunct distribution pattern (Moore *et al*., 1970). Their flowering times begin in December and the inflorescences are prominently cone-shaped when the lower most flowers were just open (Moore, 1964; Milicich, 1993). *Bulbinella gibbsii* var. balanifera has wide yellow flower clusters.

2.8.4.3: *Bulbinella hookeri* (Hook.) Cheeseman

Conservation status and criteria: Least Concern /Not Threatened (Milicich, 1993). **Distribution**: Endemic. North Island: (Urewera Country, Mount Egmont, parts of the Volcanic Plateau) and the Ruahine Range; South Island: north of Waiau, North Canterbury, Marlborough and Nelson, **Fig 21**.

Bulbinella hookeri **(Fig 20C)** is hermaphroditic, with a columnar habit and its flowering occurs between November and January (Moore and Edgar, 1970). The plant is deciduous during winter months and racemes of the flowers are usually easily visible above the erect leaves, contain more than 50 flowers. The plant requires a range of 2-5years to reach its full growth with a height of 0.4m (Moore, 1964; Milicich, 1993).

2.8.4.4: *Bulbinella rossii* (Hook.f.) Cheeseman

Conservation status and criteria: At Risk (Vulnerable) (Milicich, 1993). **Distribution**: Endemic to Auckland and Campbell Island, **Fig 21**.

The species is dioecious and it is a most magnificent plant reaching a height of more than 1m (Moore, 1964). It is only *Bulbinella rossii* **(Fig 20D)** that possesses fibrous leaf bases and it is therefore considered to bear the closest physical resemblance to plants of the South African genus (Perry, 1987). Flowering is common during December (Moore and Edgar, 1970). *Bulbinella rossii* inflorescence is cylindrical in shape and contains more than 50 flowers with short pedicels (Moore, 1964; Milicich, 1993).

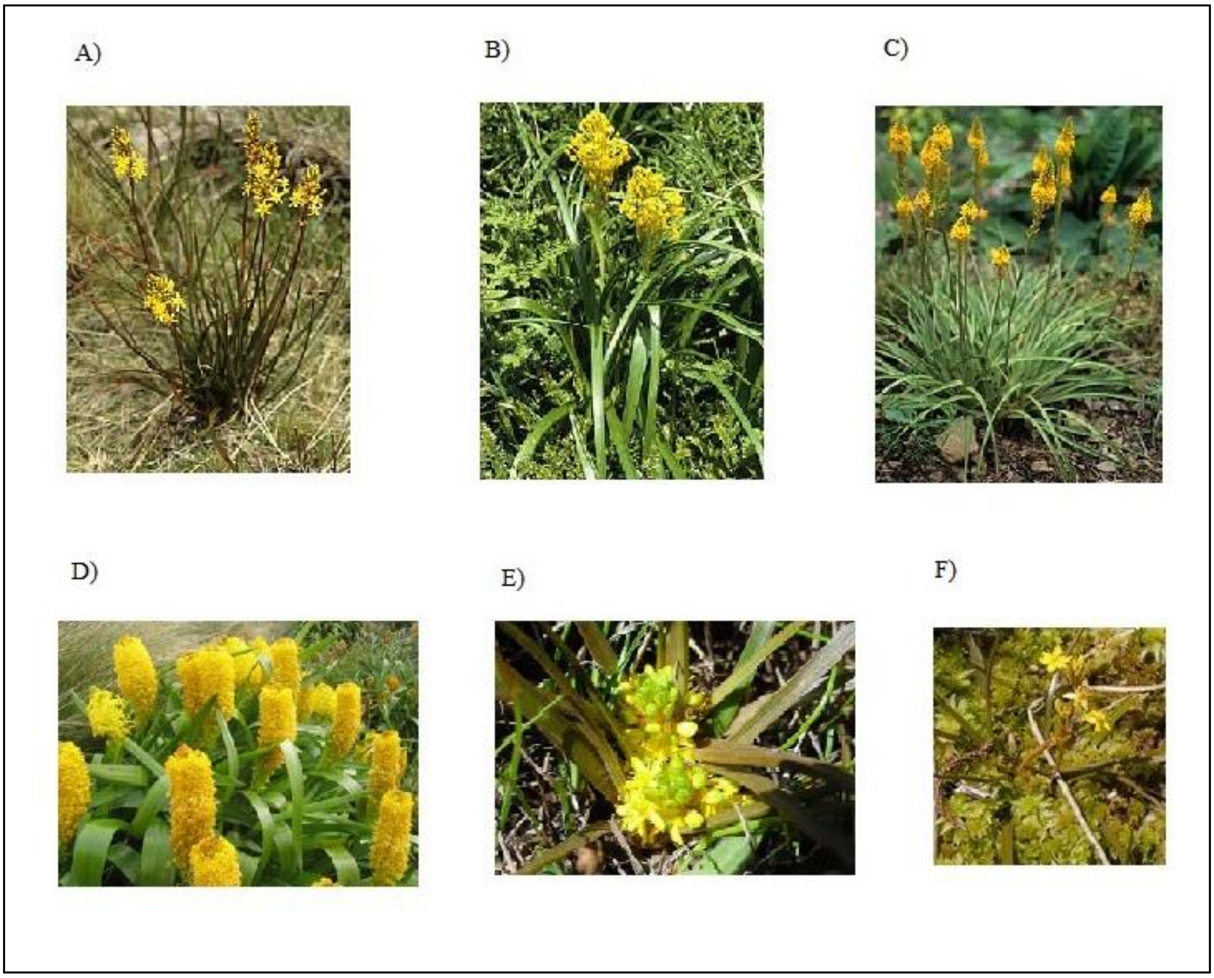

Figure 20*:* ***Bulbinella*** **species of New Zealand. [(A)** ***Bulbinella angustifolia,*** **(B)** ***Bulbinella gibbsii*** **var.** ***balanifera,*** **(C)** ***Bulbinella hookeri,*** **(Source: www.hebesoc.org). (D)*****Bulbinella rossii,*** **(E)** ***Bulbinella talbotii,*** **(F)** ***Bulbinella modesta,*** **(Source: www.nzpcn.org.nz)].**

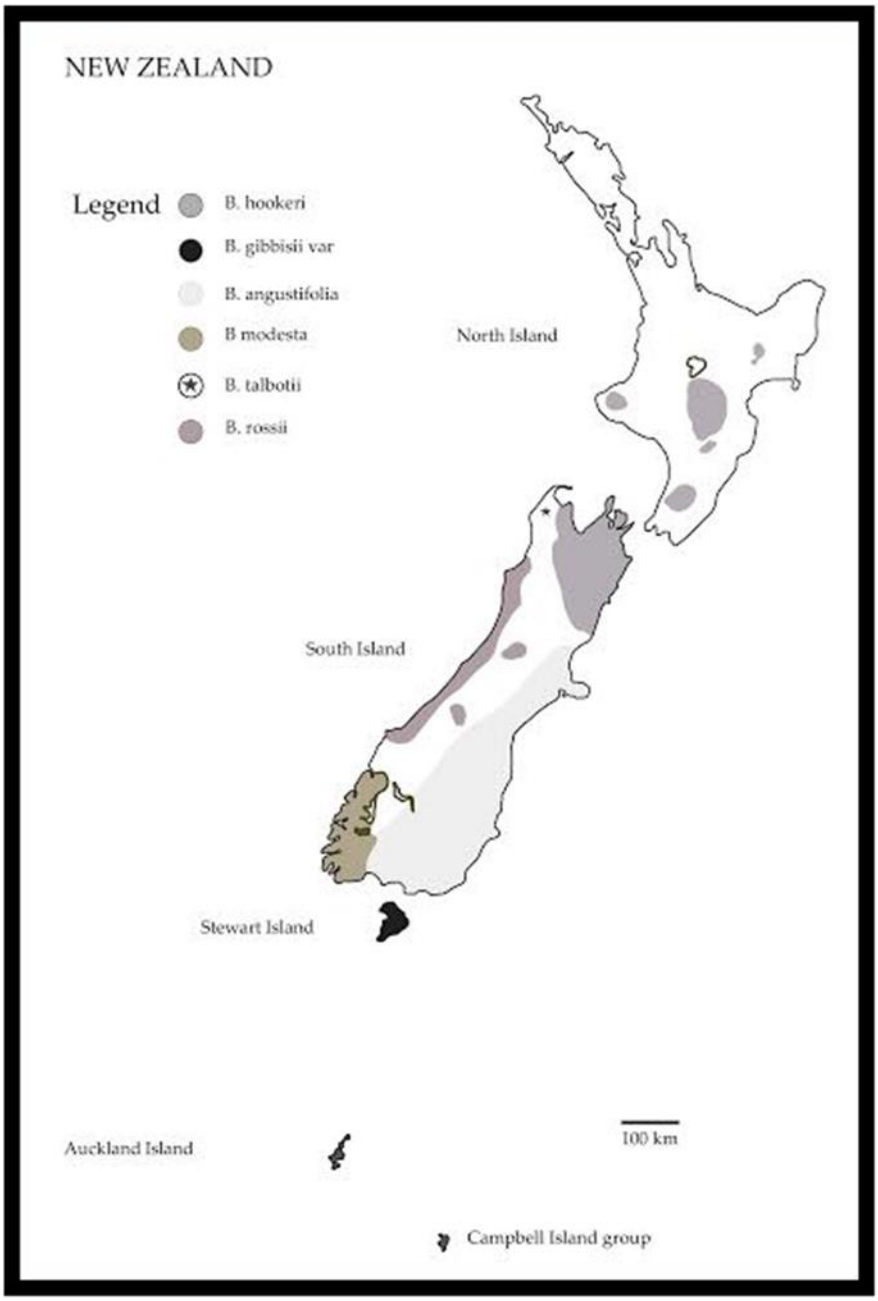

Figure 21: Distribution Map of *Bulbinella* Species in New Zealand. [Source: Milicich, 1993]

2.8.4.5: *Bulbinella talbotii* L.B. Moore

Conservation status and criteria: At Risk (Naturally uncommon) (Milicich, 1993). **Distribution:** Endemic. South Island, north-west Nelson, Gouland Downs, **Fig 21**.

This species differs from all described species by their low habit with leaves spreading horizontally from the crown (Moore, 1964). *Bulbinella talbotii* **(Fig 20E)** is much smaller even than *Bulbinella modesta* but both are hermaphroditic (Milicich, 1993). The root is swollen proximally in fusiform shape and its flowering occurs during December and January (Moore and Edgar, 1970). The peduncles are so short that they make inflorescences barely visible between the leaf bases, even at fruiting (Milicich, 1993). Most racemes have only about 10 flowers. The species are locally abundant in open, boggy areas (Milicich, 1993). The chromosome number is 2n = 14 (Moore and Edgar, 1970).

2.8.4.6: *Bulbinella modesta* L.B. Moore

Conservation status and criteria: At Risk (Vulnerable) (Milicich, 1993). **Distribution:** Endemic. West Coast of the Island from Buller District as far south as Jackson Bay, **Fig 21**.

Bulbinella modesta (**Fig 20F)** differs from all described New Zealand species by its short lax raceme (Moore, 1964). *Bulbinella modesta* is hermaphroditic and its flowering occurs during December or January (Moore and Edgar, 1970). Peduncles are spindly and delicate and the racemes of most populations have 10-20 flowers. The leaves are similar in length to those of *Bulbinella hookeri*, but considerably thinner and have a prostate growth habit (Moore, 1964; Milicich, 1993).

2.9: ECONOMIC IMPORTANCE OF *BULBINELLA (KNIPHOFIA* AND *BULBINE)*

2.9.1: Background of Geophytes

Ancient man discovered and identified the value of certain wild plants and intensely altered them into valuable cultivated plants (Alam *et al*., 2013). These plants have various uses such as decoration of the surrounding as ornaments, for food and trade, for religious ceremonies and medicinal roles (De Hertogh and Lenard, 1993; Alam *et al*., 2013). Flowering geophytes plants were important to mankind throughout the centuries because they form part of civilisation and culture (Rossi, 1990; Hessayon, 1999, Alam *et al*., 2013). Despite the small percentages of geophytes, they form an integral part of the world floriculture industry (De Hertogh and Lenard, 1993a; Van Wyk *et al*., 1997; Louw, 2002; Van Uffelen and De Groot, 2005). Even though there are countless ornamental plants known today, these ornamental geophytes have their unique recognition due to their colourful, showy flowers (Bodley *et al*., 1989; Perry, 1999).

Bulbine, *Bulbinella* and *Kniphofia* are essential geophytes; however, little information is available of the importance of these plants on the markets in southern Africa (Bodley, 1989; Kleynhans and Spies, 2011). There has also been a major decrease in taxonomic revisions of these genera and not much attention has been given to these indigenous geophytes of South Africa to date, particularly of the species of *Bulbinella*. These geophytes are predominantly noteworthy for the reason that they produce a range of biochemical compounds such as anthraquinones, knipholone and isoknipholone (Fennell and Van Staden, 2001).

2.9.2: Economic Importance

In South Africa, herbal medicine has obtained popularity (Obici *et al*., 2008) because the rich heritage of floral biodiversity is found in this country (Louw, 2002). Geophytes have proven to contain a range of unique biologically active compounds (Louw, 2002). Traditional treatments involve mainly the use of these plant extracts (Akerele, 1993; Saggu, 2007). *Bulbinella* is one of the indigenous geophytes plants of importance to South African traditional healers. However, there is still a lack of scientific research regarding some of its genetic profile and unique pharmacological compounds.

Bulbinella is taxonomically related to *Bulbine* and *Kniphofia* (Tsukamoto *et al*., 1989; Kuroda, 2003). In addition to them being drought-resistant, *Bulbinella* species in South Africa are also valuable plants indeed due to their various medicinal properties. A literature survey regarding the secondary metabolites of *Bulbinella* species showed that they produce numerous anthraquinone derivatives, including phenyl anthraquinones, by conventional TLC and HPLC analysis (Van Wyk *et al*., 1995; Kuroda, 2003; Bringmann *et al*., 2008).

These phenyl anthraquinones produced from plant species of the Asphodelaceae are extensively useful as herbal remedies for innumerable ailments which arise from bacterial and fungal infections (Bringmann *et al*., 2008). The extracts from these geophytes plants exhibit higher levels of fungal inhibitions than other herbs such as ginger and hot peppers (Louw, 2002). For example, *Bulbine* species are generally used in the treatment of ringworms, wounds, rashes, and sores (*Be. frutescens*, *Be. asphodeloides*, *Be. tortifolia*). Leaf, root, or tuber decoctions are used for the treatment of diarrhoea and dysentery (*Bulbine asphodeloides*), eczema (*Bulbine latifolia*, *Bulbine* *natalensis*), venereal diseases (*Bulbine alooides*, *Bulbine asphodeloides*, *Bulbine latifolia*), and rheumatism (*Bulbine alooides*, *Bulbine narcissifolia*) (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996).

The phenyl anthraquinones are a new class of antiplasmodial substances (Abegaz *et al*., 2002) that are found in several *Bulbinella* species such as *B. nutans* roots; *B. divaginata*, *B. elata*, *B. nutans* var. *nutans*, *B. nutans* var. *turfosicola*, *B. punctulata*, *B. latifolia*, *B. trinervis*, and *B. triquetra* roots (Dagne and Yenesew, 1994, Bringmann *et al*., 2008). Researchers have reported the co-occurrence of isofuranonaphthoquinones from the roots of *Be. capitata* to have antioxidant and also mild antiplasmodial properties (Bezabih *et al*., 1997; Majinda *et al*., 2001; Ntie-kang, 2014).

The phenyl anthraquinones and isofuranonaphthoquinones which are extracted from the same *Bulbinella* and *Bulbine* species have antiparasitic and antioxidant activity (Abegaz *et al*., 2002; Habtemariam, 2007). In addition, 10, 7′-bi-chrysophanol is present in *Bulbine*, *Bulbinella* and *Kniphofia* and is used by the Basotho, Griqua, and white people of southern Africa for wound healing and as a mild purgative (Smith and Van Wyk, 1998; Qhotsokoane and Karuso, 2001). The *Bulbinella* leaves are long, fairly thick, and contain a natural healing sap. This sap contains glycoproteins which have soothing and protective qualities hence helps to treat bites from mosquitoes, bees or wasps (Afolayan and Yakubu, 2009).

The *Bulbinella* herb is exceptional for slowing down bleeding; drying up acne, soothing cold sores, chapped lips and cracked heels, sunburn and it gives relief from eczema symptoms (Schultz, 2013). *Bulbinella* derivates are of paramount importance, for example, Bulbineloneside A, 4'-O-demethylknipholone-6'-O-ß-D-xylopyranoside

(*Bulbineloneside* B), knipholone, and isoknipholone have lately been stated to show good antitumoral activities against HSC-2 cells (Dahlgren *et al*., 1985; Chase *et al*., 2000; Kuroda *et al*., 2003; Bringmann *et al*., 2008).

The roots of *Kniphofia foliosa* are orally administered for the healing of abdominal cramps in countries such as Ethiopia (Dagne and Steglich, 1984; Berhanu *et al*., 1986). Plant infusions of *Kniphofia buchananii*, *Kniphofia parviflora*, *Kniphofia laxiflora*, and *Kniphofia rooperi* are used in South Africa as snake deterrents and for chest ailments (Hutchings, 1996). According to Habtemariam (2007), antioxidant properties may accelerate wound healing, hence the reported activities gives evidence on the use of *Kniphofia foliosa* in folk medicine for the cure of lesions (Habtemariam, 2007).

Bulbinella nutans is a plant native to the western area of Cape Province in South Africa, but commercially sold at markets in Japan (Kuroda, 2003; Bringmann *et al*., 2008). It has recently been investigated although no ethnomedical uses have been reported (Kuroda, 2003). The broad role of these plants in folk medicine suggests their worthwhile pharmacological potential and justifies further investigation (Bringmann *et al*., 2008).

The Asphodelaceae have proved to be outstanding especially for their traditional antimicrobial uses in South Africa (Hutchings *et al*., 1996; Kornienko *et al*., 2008). This is demonstrated by *Bulbine frutescens*, an ornamental herb that grows widely in Botswana which has been used medicinally to enhance the healing of wounds (Watt and Breyer-Brandwijk, 1962; Abegaz, 2002). According to Dyson, (1998), *Bulbine frutescens* leaf gel cures insect bites, wounds, rashes, acne, blisters, burns, ulcers, cracked lips, cold sores, acne and ringworm.

According to Van Staden and Drewes (1994), the anthraquinones, knipholone and isoknipholone isolated from roots, are some of the chemical constituents of *Bulbine frutescens*. The roots strengthen the general immune system of the body and also help in the healing of diarrhoea, gall bladder colic, urinary disorders and venereal disease in humans (Van Wyk *et al*., 1995). Chrysophanol is found in most genera of the Asphodelaceae and can, therefore, probably be used as a chemical marker (Klopper *et al*., 2010).

Goudling (1971), presented evidence that *Bulbinella* leaves were made into plaited baskets and floor mats by the Maori people in New Zealand. Although *Bulbinella* tissues are reported to be distasteful to livestock (Moore and Irwin, 1978, Salmon 1985; Webb *et al*., 1990), some species such as *Bulbinella hookeri* and *Bulbinella angustifolia* are fed on by browsers in Goudland Downs's area (Milicich, 1993). Recently, *Bulbinella* has been utilised as a skin toner because it removes impurities and has been used in the production of antibacterial liquid and creams because of its healing properties (Schultz, 2013).

Bulbine natalensis is widely distributed in the eastern and northern parts of South Africa where it is traditionally used as the testosterone booster and is consumed as a mixture of stem powder and milk for the management of male sexual dysfunction (Van Wyk, 1997; Afolayan and Yakubu, 2009). Correspondingly, its leaf sap is extensively used in the treatment of wounds, burns, rashes, itches, ringworms, and cracked lips (Afolayan, 2009). To suppress vomiting, diarrhoea, convulsion, venereal diseases, diabetes, and rheumatism, the infusion of the *Bulbine natalensis* roots is taken orally (Pujol, 1990; Afolayan, 2009).

Bulbine abyssinia is a succulent member of the genus that occurs from the Eastern Cape and is useful because of its ethno-medicinal value as it is often used in traditional medicine to treat rheumatism, dysentery, bilharzia and diabetes (Cromwell and Anthony, 2015).

Despite the above-mentioned medicinal properties, *Bulbinella talbotii* miniature species from Goudland Downs has been classified as going locally extinct in New Zealand (Given, 1981). Even though *Bulbinella* species are widely distributed in South Africa, a significant number of species are considered as vulnerable or under least concern (von Staden *et al*., 2011). This may probably lead to extinction if conservation aspects such as ex-situ and in-situ are not taken into consideration. There is, therefore a call for more research on the genetic profiling of *Bulbinella* species to have a rapid inventory of its genetic resources and set appropriate conservation measures.

2.10: Conservation of Biodiversity

Data on the conservation status show that all *Bulbinella* species are vulnerable. *Bulbinella calcicola* J.C. Manning & Goldblatt is critically endangered (Raimondo *et al.*, 2009), and all the other species should rather be regarded as endangered (Raimondo *et al.*, 2009). Despite their vulnerability, *Bulbinella* species in South Africa are still harvested for their various medicinal properties (Van Wyk *et al.*, 1995; Kuroda, 2003; Bringmann *et al.*, 2008).

Table 2.1: Red list Assessments of the South African and New Zealand species (Milicich. 1993; Raimondo *et al.*, 2009)

STATUS	SPECIES
Least Concern	*B. barkerae, B. cauda-felis, B. chartacea, B. ciliolata, B. divaginata, B. elata, B. elegans, B. gracilis, B. graminifolia, B. latifolia subsp. denticulata, B. latifolia subsp. latifolia, B. nutans subsp. nutans, B. nutans subsp. turfosicola, B. punctulata, B. trinervis, B. triquetra, B. anguistifolia* and *B. hookeri.*
Vulnerable	*B. eburniflora* VU D2, *B. latifolia* subsp. *doleritica* VU B1ab (v) +2ab (v) *B. latifolia* subsp. *toximontana* VU D1+2 *B. nana* VU D2, *B. rossi* and *B. modesta.*
Critically Endangered	*B. calcicola* CR A3c *B. potbergensis* CR B1ab(iii)+2ab(iii) and *B. talbotii*

Habitat destruction due to property developments and plant removal by traditional healers for use as *muthi* (medicine), as well as the removal of beautiful or rare plants for horticultural purposes, poses a threat to the survival of *Bulbinella* in nature. Thus, it is the horticultural potential and medicinal properties of *Bulbinella* that have contributed to its threatened status. A great need exists to have a more thorough understanding of the species status and a means to more rapidly identify them. This will form an integral starting point for the selection of species worth of protecting from medicinal markets.

2.10.1: Biological Diversity

Biodiversity refers to the richness and variety of life forms on earth, the ecological roles they perform and the genetic diversity they contain, and it comprises of three levels which are genes, species and ecosystems (Hawksworth, 1995; Fulekar, 2010; Rao and Hodgkin, 2002; Antofie, 2011; Kasso and Mundanthra, 2013). Biological diversity is a universally known value in natural resource management and is indeed more of a continuum predominantly with plant species that tend to hybridise more freely than do animals (Young and dePamphilis, 2000).

A species is a unit that is universal to both the biological diversity and taxonomic classification systems and there is diversity because of either genetic variations or environmental influences or a combination of both within species (Young *et al*., 2000). For example, a species may possibly comprise of two or more subspecies that naturally have some genetic dissimilarities from one another (Young *et al*., 2000). This could be the case for *Bulbinella gibbii* as *Bulbinella gibbsii* var. *gibbssi* and *Bulbinella gibbsii* var. *balanifera*, or *Bulbinella nutans* which has subspecies subsp. *nutans* and subsp. *turfosicola* (Perry, 1999). The genetic differences within morphologically similar species or subspecies are recognised through assessment using either allozymes or molecular markers (Mondini, 2009; Yadav and Srivastava, 2014). Therefore, species diversity becomes central in the evaluation of diversity and used as a point of reference in biodiversity conservation (Kasso *et al*., 2013).

Lately, the use of molecular techniques in studying genetic diversity has contributed to better understanding of the extent and distribution of genetic diversity in a number of essential plant species (Hodgkin *et al*., 2001; Rao and Hodgkin, 2002; Batugal *et al*., 2005). These methods, coupled with Eco geographic surveys present information on species distribution as well as intraspecific diversity (Rao and Hodgkin, 2002). The advent of molecular techniques such as Next Generation Sequencing (NGS) can assist in improved species identification and biodiversity assessment of *Bulbinella* in South Africa and New Zealand (Lahaye *et al*., 2008; Maria *et al*., 2011). These molecular methods of analysing diversity are correspondingly imperative because they can refine old-fashioned descriptive taxonomy with the newest technology. In this case, molecular methods purpose to resolve some of the major remaining questions in the phylogeny of *Bulbinella* in South Africa and New Zealand.

2.10.2: Genetic Variation

Genetic diversity is defined as the amount of genetic differences among individuals of a variety, or population of a species (Rao, 2005; Bindroo and Moorthy, 2014). It is also defined as the raw material upon which natural selection acts to bring about adaptive evolutionary change (Hammer and Teklu, 2008). According to Hedrick *et al*., 2010), genetic variation is the genetic diversity found within species and is ubiquitous throughout nature.

Genetic diversity results from the many genetic differences between individuals and may manifest in differences in DNA sequence, in biochemical characteristics, in physiological properties or in morphological characters such as flower colour or plant form (Rao, 2002; Batugal, 2005). Genetic variation will be lost over a period of time in isolated populations and this loss will occur more rapidly in small populations than in large ones (Furlan *et al*., 2012).

The variation that underpins genetic diversity arises from mutations and gene flow. Mutations are changes found in the DNA sequence of an organism (Fairbanks and Andersen, 1999; Solomon, 2002) while gene flow is the transfer of alleles or genes from one population to another or an indication of any movement between populations which result in genetic exchange (Hedrick, 2000). Consequently, allele movement will be observed between local populations and it has been noted that mutations and gene flow can have a significant influence on the evolutionary development of a specific species (Solomon, 2002).

Genetic drift results in the random loss of genetic variants and migration introduce new variants of existing ones (Kohn, 2006). The genetic variants that alter the protein sequence of genes can adversely affect fitness (Sunyaev, *et al*. 2001; Reumers, *et al*. 2005). The knowledge of the amount of genetic diversity and the spatial distribution of the diversity is critical for a correct diagnosis of the status, threats and viability of populations (Dunham *et al*., 1999; Escudero *et al*., 2003; Torres *et al*., 2003; Eliades, 2008). Data on the extent, structure and distribution of genetic diversity is necessary for conservation and use of genetic diversity (Rao and Hodgkin, 2002; Mondini *et al*., 2009).

Biodiversity is lost at an alarming rate and it is a formidable task for taxonomists to stay on the forefront of discovering and analysing biodiversity. Some species are threatened or endangered at the International level and are listed on the Red List of IUCN, for example, *Bulbinella potbergensis* and *Bulbinella calcicola* (Raimondo *et al*., 2009). According to the IUCN, threatened species are defined as species with a high risk of extinction within a short time frame (Mace *et al*., 2008). The systematic relationships among species and subspecies groups in the *Bulbinella* genus are not entirely understood and the rate of extinction might largely increase with time (Primack, 2006; Raimondo *et al*., 2009). Many species are currently threatened despite our limited and incomplete knowledge about them (Debela, 2007). Species are being lost at a rate that far exceeds the origin of new species (IUCN, 2007).

South Africa is described as being mega-diverse because of the level of endemism of the vegetation (DEAT, 2005; Berjak *et al*., 2011). Nevertheless, the terrestrial ecosystems of South Africa are fragile (Barnard and Newby, 1999) and its biodiversity is rapidly diminishing due to continuing escalation of the human population and land conversions for settlement, agriculture and industries (Barnard and Newby, 1999; Millennium Ecosystem Assessment, 2005a).

The accelerating and potentially catastrophic loss of biodiversity is irreversible and the extinction rates are destined to accelerate markedly (Millennium Ecosystem Assessment, 2005a; Naughton-Treves *et al*., 2007; Frankham, 2010; Berjak *et al*., 2011). Invasion by alien plant species contributes to extinction of species (DEAT, 2005) and climate change is equally predicted to be the major driver of extinction in the future due to lags in the ability of species to adjust their physiology and life histories to match new climate regimes (Thomas *et al*., 2004; Bellard *et al*., 2012).

Among these predicted extinct species are indispensable geophyte plants. However, some of these geophytes are harvested without permits and the enforcement of the existing legislation is ineffective in hampering the local and international trade of the bulbs (McCartan and Van Staden, 1999; Spies, 2004). The bulbs of these species are sold at an inclining price but there is a decline in their availability and size (Cunningham, 1988; Spies, 2004). These actions are reducing the density, distribution and genetic diversity of wild populations (McCartan and Van Staden, 1999).

Bulbinella species occupy peripheral areas of Cape regions in small populations and there is an increased chance of becoming extinct in future (Grassi *et al*., 2004). It is clear that a healthy level of genetic variation is essential for species survival (Woodruff, 2001). With the use of molecular techniques in genetic studies of endangered species, conservation genetics has developed into a distinct discipline. Therefore, an estimation of the genetic variation of these *Bulbinella* species under discussion would be instrumental in the conservation of these species.

Due to these high rates of biodiversity losses, conservation of plants becomes a high priority, nonetheless only when a genus is properly revised can it be effectively conserved (Frankham, 2010). During the Rio Earth Summit of 1992, there has been a renewed recognition of the importance of descriptive (alpha) taxonomy as the basis for effective conservation of biodiversity (Brooks and Kennedy, 2004). The IUCN Red List seeks to challenge the extinction crisis, providing indispensable facts on the state of, and trends in, wild species. Hence it is used as an evaluating tool by conservationists to assess which species necessitate focused conservation attention (Vié *et al*., 2008).

The population trends of *Bulbinella* species are decreasing or are unstable due to habitat loss caused by development and mining (von Staden *et al*., 2011). Currently, the Threatened Species Programme is systematically completing full assessments for all taxa with an automated status (Foden and Potter, 2005; Goldblatt and Manning, 2000). Subsequently listing of these species or sub-species as endangered provides a scientific formulation for national and international legal protection and may lead to remedial actions for recovery (Frankham, 2010). Threatened species are also protected from trade by 172 countries that have signed the convention of international trade in endangered species of wild fauna and flora (CITES, 2007).

2.10.3: Significance of Genetic Diversity

Genetic diversity ensures the species' ability to adapt to changing environmental conditions over time (Stock, 2008). It is not the entire species that adapts in concert but particular populations over time (Young, 2001). Hence focusing, recognising and managing the diversity levels within species are an essential consideration in conserving biological diversity (Moritz, 2002).

Genetic variation provides the raw material for adaptation and is, therefore, critical to continued evolutionary change (Templeton, 2001; Hammer, 2008). It also allows the species to exist in substantially differing environments through the species' ability to colonise new areas and occupy new ecological niches (Young, 2001; Febbraro *et al*., 2013). Furthermore, there is considerable evidence that levels of genetic diversity are positively related to a species' ability to produce substantial and robust progeny and persist in the long term, though the cause-effect connections are not all understood at present (Young, 2000).

Studies of genetic diversity using molecular techniques may reveal important relationships based on sequence similarity and differences and thus shows a much more detailed analysis of taxonomic relationships (Koonin, 2003; Noor *et al*., 2007). According to Grassi *et al*., (2004), gene exchange amongst different populations can be beneficial because it will lead to an improved allele pool which will increase the effective population size. Thus, the genetic diversity of a small population can be improved by the addition of new individuals of the same species (Van der Westhuizen *et al*., 2010).

Genetic diversity is the basis for survival and adaptation and allows continuation and advancement of the adaptive processes possible and ultimately evolutionary success (Rao, 2002; Stock, 2008; Ulukan, 2011; Vigueira *et al*., 2013). The changes in the environment force organisms to adapt in order to survive. A population with a high level of genetic variation has more alleles to "choose" from and therefore has a better chance to survive in an event of environmental pressure. A small population size can be an indication of a low level of genetic diversity found in this particular population (Grassi *et al*., 2004).

The genetic variation in plant populations is structured in space and time (Rao, 2005) and the description of the extent and distribution of the different aspects of genetic diversity in species, is an essential prerequisite to determining what to conserve, and where and how to conserve it (Rao, 2002; 2005; Batugal, 2005). The genetic richness decreases when alleles become lost from the gene pool in a specific population and when diversity is very low, all the individuals that are nearly identical and are at risk (Greenbaum and Portillo, 2014). On the other hand, in a population with high genetic diversity, probabilities are higher that some individual species will have a genetic makeup that permits them to survive (Batugal, 2005).

The breeding system of the species is vital in determining the differences between populations from different geographic locations (Utelli, 1999; Rao and Hodgkin, 2002; Ness *et al*., 2010). For instance, self-pollinated species show much better dissimilarities between populations often with quite diverse alleles in diverse populations (Tachida and Yoshimaru, 1996; Rao and Hodgkin, 2002). Outcrossing helps plant populations maintain high levels of genetic diversity (Rao and Hodgkin, 2002). Genetic variation declines in proportion to the severity of the bottleneck when an outbreeding population passes through a bottleneck (Amos and Balmford, 2001; Briskie and Mackintosh, 2004).

The knowledge of spatial genetic structures provides a valuable tool for inferring these causal factors and also the underlying genetic processes such as differential selective pressures, gene flow and drift (Escudero, 2003). Hybridization between widespread and rare taxa may contribute to the extinction of endangered species (Francisco-Ortega *et al*., 2000). Habitat fragmentation diminishes the size and upsurges the spatial isolation of plant populations and is the significant threat to the maintenance of biodiversity in many terrestrial ecosystems (Kasso and Mundanthra, 2013). Small populations are likely to become extinct because they are prone to genetic drift and inbreeding depression (Frankham, 2010; Francisco-Ortega *et al*., 2000).

2.10.4: Conservation of Biodiversity

The plight of individual species often continues to be overlooked. However, major advances have been made in conserving them since plant genetic resources are among the most essential of the world's natural resources (Rao and Hodgkin, 2002; Tisdell, 2011). Conserving biodiversity has economic, social and cultural value and it is integral to the biological and cultural inheritance of many nations (Kasso and Mundanthra, 2013). Biodiversity conservation involves the management of human use of biodiversity in order to obtain the ultimate sustainable benefit to present and future generations (Borokini *et al*., 2010; Kasso and Mundanthra, 2013).

One of the fundamental issues in systematic conservation planning is to define how much needs to be protected (Eeley *et al*., 2001; Sanderson *et al*., 2002). Conservation of biodiversity in nature comes to be critical during the last years, trying to alleviate the pending extinction of the biosphere by humans (Nevo, 1998). There have been increasing efforts to develop improved *in-situ* and *ex-situ* conservation methods which would permit dynamic conservation of plant populations (Jarvis, 1999; Rao and Hodgkin, 2002).

The current application of new molecular techniques has made the analysis of genetics in endangered species feasible and genetic analysis has become widely used in conservation research (Hedrick, 2001; Oliveira *et al*., 2006). The primary international conservation body, IUCN, recognises the need to conserve the biological diversity at all three levels which are genetic diversity, species diversity and ecosystem diversity (Mcneely *et al*. 1990; de Klemm and Shine, 1993). Genetics is the central consideration at all levels, being the sole issue in the first, having an important role in species viability, and a role in ecosystem viability (Bangert *et al*. 2005; Lankau and Strauss, 2007).

Genetically sound conservation efforts necessitate the understanding of the processes by which species show genetic variation in local populations (Kreivi *et al*., 2005; Gaafar *et al*., 2014). In order to realise the full value of *Bulbinella* species in South Africa and New Zealand, studies on the extent and distribution of genetic diversity need to be integrated with information on habitat, the degree of threat and physical and human geography (Rao, 2002).

The maintenance of biodiversity is justified for four reasons; the economic value of bio resources, ecosystem services, aesthetics and rights of living organisms to exist (Scherr and McNeely, 2008; Naeem *et al*., 2014). The goal of plant genetic resource conservation is to preserve as broad a sample of the existing genetic diversity of the targeted species plus currently recognised genes, traits and genotypes (Veteläinen *et al*., 2009). Red Lists at the global or sub-global level (IUCN, 2001; 2003) comprise data not only on threats to species but also on species extent and occurrence, and habitats at different temporal and spatial scales (IUCN, 2003). Henceforth they are probably the main source of information for conservation planners (Lamoreux *et al*., 2003).

2.10.5: Conservation Techniques for Genetic Resources

Effective conservation of biodiversity is mainly based on accurate species delimitation (Coetzer *et al.*, 2015). From the data on conservation status (IUCN), it is evident most *Bulbinella* species are regarded as endangered (Raimondo *et al.*, 2009), except for *Bulbinella hookeri* and *Bulbinella angustifolia* which are vulnerable while *Bulbinella calcicola* are critically endangered (Raimondo *et al*. (2009) **(Table 2.1).** In an attempt to control or eliminate the erosion of *Bulbinella* genetic diversity; there are two major alternative conservation techniques that should be taken into consideration, which is *in situ* and *ex-situ* conservation (Kasso *et al*., 2013).

As accentuated by Prance (1997) it is better to save both species and ecosystems integrating *in-situ* and *ex-situ* conservation. The prime aim of conservation biologists is to know the risk of extinction for given species and to find out where resources for protected species and ecosystems can best be allocated (Plassmann, 2004; Robbirt *et al.* 2006; Gentili *et al*., 2011). The considerations of plant genetic conservation comprise the estimation of genetic diversity by means of molecular markers which provide genetic information of direct value in key areas of conservation both *ex-situ* and *in-situ* (Rao, 2001; 2002).

In-situ conservation refers to the conservation of ecosystems, natural habitats and important genetic resources in wild populations (Kasso *et al*., 2013). It is a dynamic system often associated with traditional subsistence agriculture, which permits the biological resources to evolve and change over time through natural or human-driven selection processes (Holsinger and Anon, 2005; Dulloo *et al*., 2010; Kasso *et al*., 2013). *In situ* consists of the legal protection of the area and habitat in which the species grows ((Jarvis, 1999; Hayward, 2012). The advantage is that the evolutionary dynamics of the species are maintained while its drawback is the cost and the social and political difficulties which occasionally arise (Hammer and Teklu, 2008).

The *in-situ* technique allows evolution to continue and increases the amount of diversity that can be conserved (Rao and Hodgkin, 2002; Hammer and Teklu, 2008). The optimum reserve size of the *in-situ* preservation approach is dependent on the effective population size and unique population genetic structure of each species (Lee *et al*., 2002, Greene *et al*., 2014). It is imperative to ensure that appropriate populations are identified and managed in such a way that populations survive and continue to evolve. The populations preserved *in-situ* constitutes part of ecosystems and both intra- and interspecific diversity must be conserved over time at suitable levels (Rao, 2001).

On the other hand, *ex-situ* conservation is a technique to conserve biological diversity, its natural habitats, and tracing all levels of biodiversity for instance genetic, species, and ecosystems (Kjaer *et al*., 2001; Borokini *et al*., 2010; Antofie, 2011; Kasso *et al*., 2013). It also refers to the conservation of genetic resources off-site in gene banks, often in long-term storage as seed, shoots, *in vitro* culture, plants and aims at maintaining the genetic integrity under human supervision (Holsinger and Anon, 2005; Niino, 2006).

The objective of *ex-situ* conservation is to maintain the accessions without a change in their genetic constitution and these sites (Botanic Gardens) become educational centres to the public for biodiversity conservation in the world (Kasso *et al*., 2010). Molecular markers may, therefore, be used and molecular data on diversity may lead to the identification of useful genes contained in collections while providing essential information to develop core collections (Rao and Hodgkin, 2002) that accurately represent the entire collection.

2.10.6: Conclusion

The genus *Bulbinella* lacks a proper taxonomic key, its revisions are out of date and its biodiversity and evolutionary histories need to be assessed for conservation purposes. Without this knowledge, even the simple task of deciding what groups or types should be conserved becomes more or less impossible. Systematic studies of *Bulbinella* plants in South Africa and New Zealand were incomplete since the descriptions of species were largely based on superficial and aggregate characteristics, which show very little variation between the different species. However, molecular systematics of nuclear or chloroplast gene regions possibly provide a better understanding of the phylogenetic relationships of the species than that of morphological approaches (Liang and Hilu, 1997; Small *et al*., 2004). The combinations of explicit methods for phylogenetic analysis of *Bulbinella* species would reveal genetic variation between and within these species.

The use of molecular techniques to compliment morphological and taxonomic studies will be of great benefit to study the systematics of the genus *Bulbinella*. This is especially so because of the similarities and overlap in morphology for some of the species, the different forms of the species, and the fact that often incomplete plants lacking diagnostic features are found.

The most widely used technique to aid morphology is DNA sequence comparisons. A number of genes have been used to delimit species relationship for plants, namely the *matK, rbcl, psbA-trnH* and *ITS* (Chapter 4: "4.2.1"). New approaches using high throughput sequencing techniques with Next Generation Sequencers also makes it possible to compare entire genomes, and for this especially chloroplast genomes have proven useful (Chapter 5: Appendix "II").

No phylogenetic studies have been conducted on species of *Bulbinella* before. The purpose of this research thesis is to establish multigene phylogenies for the genus for the first time. Moreover, a multigene and phylogenomic approach will prove invaluable not only to strengthen the taxonomy of the genus, but also to aid identifications for users of the plants in, for instance, medical applications or the ornamental industry, and to aid biodiversity and conservation efforts to protect the diversity and germpool of this beautiful genus.

CHAPTER 3: MATERIALS AND METHODS

3.1: Sample Collection

Leaf samples from twenty-six morphologically and geographically distinct *Bulbinella* specimens from different provinces of South Africa (17 specimens) and New Zealand (9 specimens) were collected (**Table 3.1**) by various collectors for molecular studies. Where possible, more than one sample per species was collected from different geographical areas. During sample collection, plants were photographed for identification purposes. Special care was taken to include all the relevant information with each collection tied with a unique collection number including the collection site and the province from which it was collected (**Table 3.1**).

Leaf samples were preserved in 1.5 ml tubes with silica gel (Chase & Hills, 1991) and stored at room temperature. For some species only, seeds could be supplied by collectors or suppliers (Silverhill Seeds, Seeds for Africa, Summerfields). *Bulbine* and *Kniphofia* specimens were also collected (**Table 3.1**) as outgroups due to their close relatedness to *Bulbinella* within the Asphodelaceae (Chase *et al.*, 2000; Devey *et al.*, 2006).

Table 3.1: Samples used during this study, including sequences from GenBank.

Species	Collection Number	Locality/Source	Genbank
Bulbine latifolia [a]	Ramdhani 61 UDW	Durban, South Africa	EU707290
Be. latifolia [b, c]	Spies B002	Western province, South Africa	
Be. semibarbata [a,b]	Chase 8019	Australia	JQ039294
Be. semibarbata [a,b]	K Dixon s.n. (KPBG)	Australia	HM640528,
Be. semibarbata [a,b]	K Dixon s.n. (KPBG)	Australia	HM640646
Be. wiesei [a,b]	1995-3501	South Africa	AF234350
Bulbinella anguistifolia	OTA 038740	Cultivated ex. Flagstaff, Otago, New Zealand	
B. cauda-felis **(seeds)** [c]	Silverhill 9183	Nieuwoudtville, Northern Cape, South Africa	
B. cauda-felis [c]	Spies 9295	Nieuwoudtville, Northern Cape, South Africa	
B. cauda-felis [c]	Spies 9192	Capeseeds, South Africa	
B. cauda-felis [a]	UCI Arb. 359	Grahamstown, South Africa	JX903194
B. chartacea [c]	Stedge & Musara 863	Cederberg Nature Reserves, Western Province, South Africa	
B. ciliolata [c]	Stedge & Musara 872	Nieuwoudtville Flower Reserve. Northern Cape, South Africa	
B. divaginata [c]	Stedge & Musara 877	c. 22 km NW of Sutherland, Northern Cape, South Africa	

B. elata **(seeds)** [c]	Silverhill 9298	Cederberg, Western Province, South Africa	
B. elegans **(seeds)** [c]	Silverhill 9299	Middelpos area, Northern Cape, South Africa,	
B. erbuniflora **(seeds)** [c]	Silverhill 9297	Nieuwoudtville, Northern Cape, South Africa	
B. erbuniflora [c]	9184	Nieuwoudtville, Northern Cape, South Africa	
B. gibbii **var. *balanifera***	OTA 066755	Sutton Salt Lake, Otago, New Zealand	
B. gibbii **(narrow leaves)**	OTA 032761	West Cape, Fiordland, New Zealand	
B. gibbii **var. *gibbii***	OTA 33054	Mt. Anglem, Stewart Islands, New Zealand	
B. gracillis	Stedge & Musara 873	58 km W of Calvinia, Northern Cape, Namakwa, South Africa	
B. graminifolia **(seeds)**	Silverhill 9185	Cederberg, Western Province, South Africa	
B. hookeri	OTA 018327	Mt Arthur, Nelson, New Zealand	
B. latifolia [c]	Stedge & Musara 860	NW of Darling along R315, Western Province, South Africa	
B. latifolia **var. *granitus*** [c]	Spies 9191	Capeseeds, Northern Cape, Western Cape South Africa,	

B. modesta	OTA 062695	Hapulea Estuary, Westland, New Zealand	
B. nana	Stedge & Musara 879	Bains Kloof Pass, Western Cape, South Africa,	
B. nana [a]	BGW, 303/92, Van Wyk- JRAU	South Africa	AJ511419
B. punctualata	Silverhill 9146	Cederberg range, Western Cape South Africa	
B. rossi	OTA 031504	Campel Islands, New Zealand	
B. rossi	OTA 065322	Enderby Islands, Auckland Islands, New Zealand	
***B. rossi* (flowers)**	Not accessioned	Enderby Islands, Auckland Islands, New Zealand.	
B. trinervis [c]	Stedge & Musara 875	c. 17 km of Calvinia, Northern Cape, South Africa	
B. triquetra	Spies 9309	Summerfields, Northern Cape, South Africa	
Kniphofia praecox [a,b]	JRAU van Wyk 4119	Grahamstown, South Africa	AJ512276
K. praecox [a,b]	Pearse, W.D. 210980	Grahamstown, South Africa	KM360836
K. praecox [a,b]	JRAU Van Wyk	Grahamstown, South Africa	AJ511424
K. praecox [a,b]	Ramdhani 529 GRA	Grahamstown, South Africa	EU707255
K. praecox [b]	Spies 078	Western Province, South Africa	

K. stricta [a,b]	SR279	Grahamstown, South Africa	HQ646907

[a]- Sequences obtained from the Genbank.

[b]- Outgroups.

[c]- Specimen used for genome sequencing

OTA- Voucher numbers refer to specimens in the University of Otago (New Zealand).

s.n. = unnumbered collections with no herbarium voucher

SR- Voucher numbers refer to specimens collected by S. Ramdhani

GRA- Voucher numbers refer to specimens from Grahamstown, Rhodes (South Africa)

UDW- Voucher numbers refer to specimens from University of Durban Westville (South Africa)

JRAU- where specimens are held at Rhodes, South Africa.

BGW- refer to burrow-dwelling ground wanderer (plants)

KPBG- Voucher numbers refer to specimens from Kings Park and Botanical Garden in Perth (Australia)

3.2: Molecular Techniques

3.2.1: DNA Extraction.

Total genomic DNA was extracted from silica dried *Kniphofia, Bulbine* and *Bulbinella* seeds or leaves (**Table 3.1**), using the Qiagen Plant DNA Extraction Kit protocol (Qiagen, Maryland, USA). However, a modified protocol was introduced that yielded more consistently amplifiable DNA from the species. The modified protocol was as follows. The samples (10 g of dried tissue) were pulverised using the TissueRuptor-handheld rotor-stator homogeniser at 120V, 60Hz (Qiagen) and the fine powder was transferred into 2 ml Eppendorf micro tubes. A total volume of 400 µl API Buffer and 4 µl of 100 mg/ml RNase A was added to the tube. The contents were vigorously vortexed and incubated at 65°C for 60 min, while mixed three times by overturning during incubation. A total volume of 130 µl of P3 Buffer was added to the tube and the contents were mixed by hand shaking, where after it was incubated on ice for 60 min. It was then centrifuged (14 500 rpm, 10 min) at room temperature. The QIAshredder Mini spin column was put in a new 2 ml microtube, the supernatant was added and it was centrifuged at 14 500 rpm for 5 min at room temperature. The filtrate was transferred to a new 2 ml microtube. The volume of the filtrate was determined and 1.5x this volume of AWI Buffer was added to the filtrate and mixed with a pipette. A total volume of 650 µl of the mixture was transferred into a DNeasy Mini spin column (placed within a 2 ml collection tube) and was centrifuged for 5 min at 8 000 rpm. The flow-through was discarded and the step was repeated with the remaining sample. The spin column was then placed into a new 2 ml collection tube and 500 µl of AW2 Buffer was added and centrifuged for 5 min at 8 000 rpm. The flow through was discarded, 500 µl of AW2 Buffer was added to the DNeasy Mini spin column and centrifuged at 14 500 rpm for 2 min at room temperature. The DNeasy Mini spin column was transferred to a new 1.5 ml microtube and 100 µl of AE Buffer was added in the column. The contents were incubated for 5 min at room temperature and then centrifuged at 8 000 rpm for 3 min at room temperature. The DNeasy Mini spin column was removed from the tube and the eluted genomic DNA solution was preserved in the tube at -20°C.

3.2.2: DNA Precipitation

The extracted DNA were purified using a glycogen and ammonium acetate protocol as follows. A tenth volume of 3 M sodium acetate (pH 5.2) was added to the eluted genomic DNA solution and mixed by vortexing briefly. Up to 1 µl of glycogen and also 2 to 2.5 volumes (calculated after salt addition) of ice-cold 100% ethanol was added to the solution, mixed by vortexing, and the mixture was incubated overnight. The mixture was centrifuged for 15 min at 10 000 rpm and the supernatant was removed. The pellet was rinsed twice with 60 µl of cold 70% (v/v) ethanol, centrifuged at 10 000 rpm for 10 min and the supernatant was removed. The DNA concentration was determined by an UV spectrophotometer (Thermo Fisher Scientific, Wisconsin, USA) at an absorbance of A260/280. The purity and integrity of the extracted gDNA were confirmed using 1% agarose gel electrophoresis against known concentrations of unrestricted lambda DNA (Thermo Fisher Scientific, Bio-Rad, USA). High quality DNA concentration (at least 20 ng/µl; A260/230>1.7; A260/280= 1.8~2.0) was used for Sanger sequencing.

3.2.3: Polymerase Chain Reaction (PCR)

Standard PCR reactions were set up for four DNA-barcoding regions of plants (*matK, rbcL, psbA-trnH* and *ITS*). The primers used for amplifying the nuclear *ITS*, and *rbcL, matK*, and chloroplast *psbA-trnH* DNA regions, are shown in **Table 3.2**. A total of 104 reactions were prepared for the sampled species, and the DNA was amplified in a Thermal Cycler 2720 (Applied Biosystems, California USA) using cycling conditions described below. The PCR products were sequenced in both directions using the same set of primers for the respective PCR reactions. The HiFi Hot Start ReadyMix DNA Polymerase (pre-mixed enzyme and buffer) (KAPA Biosystems, Massachusetts, USA) were used for PCR reactions according to the manufacturer's protocol given below.

The PCR mixture contained forward and reverse primer in a volume of 20 µl reaction mixtures consisting of 10 µl HiFi ReadyMix (Hot Start Ready mix), 1.0 µl (0.3 µM) forward primer, 1.0 µl (0.3 µM) reverse primer; 1.0 µl template DNA (20.0 ng/µl) and 7 µl nuclease-free water. The PCR amplifications were performed using a G-storm 9700 PCR (Somerton Biotechnology Centre, Somerset, United Kingdom) with the following thermal cycle conditions. For the three chloroplast regions, DNA was initially denatured at 95°C for 3 min, followed by 34 cycles of denaturing at 95°C for 20 s, primer annealing at various temperatures for each gene (*matK* 52°C; *rbcL* 55°C; *psbA-trnH* 57°C) for 15 s, and elongation at 72°C for 30 s, with a final 1 min elongation step at 72°C. Reaction conditions for the *ITS4* and *ITS 5a* were as follows: one cycle at 98°C for 5 min; 35 cycles consisting of 98°C for 10 s, primer annealing at 50°C for 30 s, and 72°C for 2 min; and one cycle at 72°C for 1 min. The PCR products were purified with a PureLink® PCR Micro Kit (ThermoScientific, Canada) according to the manufacturers' protocol and quantified with a spectrophotometer (Nano Drop ND-1000, Thermo Fisher Scientific, Wisconsin, USA).

Table 3.2: Universal primers used for the amplification of the *ITS4, matK, rbcL* and *psbA-trnH* gene regions.

DNA region	Primer sequence 5′-3′	References
Internal Transcribed Spacers *(ITS)*		
ITS5a	CCTTATCATTTAGAGGAAGGAG	Chen *et al.*, 2010
ITS4	TCCTCCGCTTATTGATATGC	White *et al.*, 1990
Ribulose-bisphosphate carboxylase* gene *(rbcL)		
rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	CBOL Plant Working Group, 2009
rbcLa-R	GTAAAATCAAGTCCACCRCG	
Maturase Kinase (matK)		
matK-1RKIM-f	ACCCAGTCCATCTGGAAATCTTGGTTC	CBOL Plant Working Group (2009)
matK-3FKIM-r	CGTACAGTACTTTTGTGTTTACGAG	
psbA-trnH* intergenic region *(psbA-trnH)		
*PsbA*3_Fwd	GTTATGCATGAACGTAATGCTC	Sang *et al.*, 1997
*TrnHf*_05 Rev	GCGCATGGTGGATTCACAATCC	Tate & Simpson, 2003

3.2.4: DNA Sanger Sequencing

Sequencing was done at the Genetic Analysis Services, Otago, New Zealand. The purified PCR products were sequenced after 1:5 dilutions with sterile water. Amplified regions were sequenced in both directions using the ABI Prism Big Dye Terminator *v* 3.1 Cycle Sequencing Kit, according to the protocol provided with few modifications. The component and volumes for the sequencing PCR reactions were 1 µl of 5x sequencing buffer, 0.5 µl premix (Applied Bio Systems, Life Technologies), 3 µl of 10 µM primer, 3 µl dH_2O, 5% dimethyl sulfoxide (DMSO) and 2 µl purified PCR product. For the *ITS* the premix was adjusted to 1 µl due to high GC content. The final PCR reaction was set up to 10 µl. The cycle sequencing steps were as follows: Initial denaturation at 96°C for 1 minute, followed by 25x cycles of 96°C for 10 seconds (with a ramp seed of $3°Cs^{-1}$), 48°C for 15 seconds; and 60°C for 4 minutes; and a last cycle of 72°C for 1 minute. Cycle sequencing products were purified using the Ethylene Diaminetetra Acetic Acid (EDTA)/Ethanol precipitation method (Sambrook *et al.*, 2001). The purified sequencing products were analysed using Sanger Sequencing on an ABI 3500xl Genetic Analyser (Applied Biosystems, California, USA).

3.2.5: Sequence Alignment and Data Analysis

The forward and reverse sequences were sampled, assembled into contigs and edited using Sequencher 4.8 (Gene Codes, Michigan, USA) followed by manual adjustment and trimming of the ambiguous ends with Geneious (Biomatters Ltd., Auckland, NZ) using the default alignment parameters. Data sets for each gene region were compiled with the new sequences and supplemented with sequences from GenBank (http://www.ncbi.nlm.nih.gov). The final dataset comprised of 94 taxa, of which 86 were in-group and 8 outgroup taxa (**Table 3.1**). Outgroup taxa were selected from the genera *Bulbine* and *Kniphofia* that were previously shown to be closely related to *Bulbinella* in the Asphodelaceae (Chase *et al*., 2000, Treutlin *et al*., 2003, Ramdhani *et al*., 2006). The datasets for each of the different genes contained corresponding sequences of each gene for the same specimen, and species, where possible.

The sequence datasets of *matK, rbcL, psbA-trnH* and the *ITS* region, respectively, were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE vs. 3.8.31; Edgar, 2004) as implemented in Molecular Evolutionary Genetics Analysis (MEGA) 6.0.1 (Tamura *et al*., 2013), and then checked manually to ensure homology. Discrepancies in sequence alignments and base pair differences between sequences were manually checked against the original electropherograms. The post-trimmed sequence lengths were at least 80% of the original read length and a sequence which covered more than 70% overlap in general between the forward and reverse sequences was considered for the various sequences.

3.2.6: Phylogenetic Analysis

The sequencing data of the four gene regions (*ITS, rbcL, matK* and *psbA-trnH*) were initially analysed separately. Because the results for the individual gene regions were shown to be in general agreement about relationships, they were combined into a single data matrix. In the individual and combined gene analyses, data were partitioned by the gene with model parameters unlinked across partitions. Phylogenies were constructed using Maximum likelihood (ML) analyses conducted in Garli v2 (Zwickl, 2006), and Bayesian Inference (BI) using Mr Bayes v3.2 (Ronquist *et al*., 2012). For these analyses the optimal model of nucleotide substitution for each gene region was selected based on the Akaike information criterion (AIC) (Akaike, 1974) implemented in jModelTest v.2.1 (Darriba *et al*., 2012). The branch support was assessed using 1000 bootstraps replicates (BS) with consensus topologies generated using PHYLIP v. 3.695 (Felsenstein, 1989; 2009).

For the Bayesian Inference (BI), analyses were run two times independently for 10,000,000 generations, sampling trees every 1000 generations. Each Bayesian run consisted of three heated chains at default temperature of 0.200 and one cold chain were used. The first 25% of samples (25,000 trees) were discarded from the cold chain as burn-in. To ensure that Markov Chain Monte Carlo algorithm (MCMC) chains had reached convergence, Tracer v1.5 (Rambaut and Drummond, 2007) was used to verify that the appropriate estimated sample sizes (ESS) for all parameters were above 200 (Drummond, 2006). The posterior probability (PP) values for the nodes were calculated in Mr Bayes. A 50% majority rule consensus tree was constructed in PHYLIP after burn-in was removed. Tree visualization was carried out using FigTree v1.4.0 (Rambaut, 2012). Clades with a bootstrap value higher than 50% and the Bayesian posterior probability of 0.5 were considered as a proper cut off value for a monophyletic grouping (Fazekas *et al*., 2008).

3.3: Partial Chloroplast Phylogenomic Analysis of South African Species

3.3.1 DNA Extraction and Precipitation

The focus was on the South African population for genome analyses and the number of taxa sequenced was 14 South African *Bulbinella* species and two outgroups (*Bulbine* & *Kniphofia*) (**Table 3.1**). Total genomic DNA was extracted from 100 mg leaf tissue from silica dried samples of *Bulbinella*, using the Qiagen DNeasy Minikit (Qiagen, Germantown, Maryland, USA). The DNA extraction protocol is the same as the one described in **Section 3.2.1** except that two extractions per sample were performed. DNA was eluted with 25 µl elution buffer for each extraction, which was then combined for a total of 50 µl per sample. The quality and concentration of pooled DNA samples were quantified with a Nano drop (ThermoScientific, Delaware, USA) and gel electrophoresis, since 20 µl of DNA at a concentration of 50 ng/µl is recommend for the Illumina sequencing (White Scientific, USA). The samples below this concentration threshold were concentrated using glycogen/ethanol precipitation, while those over the threshold were diluted with elution buffer from the Qiagen DNeasy Minikit. The extracted DNA was purified using glycogen and ammonium acetate protocol as described in **Section 3.2.2.**

3.3.2: Illumina Sequencing

High-quality DNA (concentration >50 ng/µl; A260/230>1.7; A260/280 = 1.8~2.0) was sequenced using the Illumina HiSeq 2000 (GA II) platform at the Agricultural Research Council, Pretoria, South Africa. The current Nextera protocol calls for pure DNA template, an accurate assessment of input concentration and a column clean-up

(Lamble *et al.*, 2013). The Nextera sequencing follows a common library-preparation procedure. Pre-library normalisation of gDNA was performed using the AxyPrep Mag PCR Normalizer Kit (Axygen Biosciences) and the concentration of the normalized samples was determined by Qubit (Invitrogen) following the manufacturer's specifications. The Illumina method included DNA fragmentation (sonication to shearing), followed by DNA end-polishing or A-tailing, and finally platform-specific adaptor ligation (Caruccio, 2011). The library preparation followed the TruSeq DNA Sample Preparation Guide protocol (Illumina, Inc., 2010), except where noted. The total gDNA was prepared with the TruSeq DNA PCR-Free HT Sample Preparation Kit (21 Samples), where each sample was digested with an enzyme and adapters were ligated to the ends using a PCR-free method. Each sample was prepared with unique adapters making multiplexing of the samples possible. The adapter ends were automatically removed by the Illumina HiSeq 2000 (GA II) which also construes reads based on adapter ends into separate files. Sequencing yielding paired-end (2x125bp) reads was performed following the Illumina Nextera 2012 protocol (Illumina, Inc., San Diego, California).

3.3.3. Bioinformatics Analyses of Genome Data

3.3.3.1. Data Quality-trimming and Filtering

The quality of the sequencing read were assessed using FastQC (http://www. bioinformatics.bbsrc.ac.uk/projects/fastqc/) and quality filtering performed using PrinSeq-lite v0.20.4 (Schmieder and Edwards, 2011). All data sets were pre-processed to remove any sequences with a mean quality score below 20. Remaining sequences were trimmed to obtain an average quality score of ≥25 using a 7 nt window with a 4 nt step. Any sequences containing N's were removed.

3.3.3.2. Filtering chloroplast reads from genome data

Reads representing chloroplast genome sequences were filtered from whole genome sequence data in the dataset using the filter_by_blast.py command in the seq_crumbs (https://bioinf.comav.upv.es/seq_crumbs/) package. Filtering was performed using the Refseq plastid database (https://www.ncbi.nlm.nih.gov/genome/organelle/) with similarity and e-value cut-offs of 90% and 0.1, respectively. Paired-end sequence integrity was kept using the paired-end option.

3.3.3.3. Chloroplast draft genome assembly and annotation

Filter reads were assembled with SPAdes v3.8.0 (Bankevich, 2012) using default settings and the paired-end flag (--12). The quality of the assemblies was assessed using QUAST v3.2 (Gurevich *et al.*, 2013) to obtain number and length of contigs as well as N50 and N70 values. Contigs were further assembled into scaffolds using LINKS v. 1.3 (Warren *et al.*, 2015). Individual scaffolds were uploaded to Dual Organellar GenoMe Annotator (DOGMA) for annotation (Wyman *et al.*, 2004).

3.3.4: Phylogenetic Analysis

Out of the total partial genome data obtained, 34 gene regions that were completely sequenced were selected (**Table 4.1**) and analysed separately. The DNA sequence data for the 34 genes were then combined into a single data matrix after individual phylogenetic tree showed satisfactory levels of congruence. Clades with a bootstrap value higher than 50% and the Bayesian posterior probability of 0.5 were considered as a proper cut off value for a monophyletic grouping (Fazekas *et al*., 2008). The phylogenetic analyses were done as in **Section 3.2.6**.

3.3.5: Preparation for Barcode submissions

Sequence data of the 4 barcoding gene regions were prepared for submission to the BOLD (Barcode for Life Data Systems) database (Hajibabaei *et al*., 2005; Ratnasingham & Hebert, (2007); http://www.boldsystems.org/). This was done for the *Bulbinella* species listed in **Table 3.1** in order to prepare an identification tool for other users working with *Bulbinella* species. According to the instructions of BOLD, datasets including image and specimen data, the tracefiles and sequences were prepared and will be uploaded at the completion of examination of the thesis.

CHAPTER 4: RESULTS AND DISCUSSION OF VARIOUS DNA REGIONS

4.1 Phylogenetic Analyses of nuclear and chloroplast genes

A molecular phylogeny for *Bulbinella* was generated with Maximum Likelihood and Bayesian Inference analysis using DNA sequences from the plastid regions *rbcL*, *matK* with one spacer, and *psbA-trnH*, and the Internal Transcribed Spacers of the nuclear ribosomal DNA. Results of the ML and BI were superimposed to one tree unless the trees differed significantly in topology. Four separate sets of analyses were carried out for the four gene regions (*ITS, rbcL, matK* and *psbA-trnH*) and then combined into a single data matrix. For these analyses, the optimal model of nucleotide substitution for each gene region was used (**Table 4.1**). The Bootstrap support is shown as a percentage of trees found that contain that group of the taxa. We use the following explanations for categories of bootstrap support: weak, 50±74%; moderate, 75±84%; strong, 85±100 %. All percentages of less than 50% were not reported for the reason that there was no significance (no internal support) in a group being found in less than 50% of the replicates. For Bayesian Inference Posterior Probabilities (PP), the following scale was used to evaluate; >0.85 is strongly supported; 0.75-0.84 moderately supported and <0.74 is weakly supported. Bootstrap percentages and Posterior Probabilities are indicated above the branches, but groups with bootstrap percentages less than 50% or below 0.5 probability were specified by (*).

Table 4.1: Gene Regions: The Akaike Information Criterion (AIC) Values obtained with JMODELTEST.

GENE	MODEL	Sub models finals
matK	GTR+G	6-gamma
rbcL	HKY+G	2-gamma
psbA-trnH	HKY+G	2-gamma
ITS	GTR+G	6- gamma
matK, rbcL, psbA-trnH & ITS	GTR+G	6-gamma

DNA sequences were obtained for the following DNA regions, *matk, rbcL, psbA-trnH* and *ITS* **(Table 4.2**). The results **(Table 4.2)** were used to determine phylogenetic relationships in *Bulbinella*. In order to discuss the results certain terms and the philosophy behind these terms have to be clarified.

Table 4.2: DNA regions sequenced and used during this study

Species	Collection Number	Genbank	*Matk*[d]	*rbcl*[d]	*psbA-trnH*[d]	*ITS*[d]
Bulbine latifolia [a]	Ramdhani 61 UDW	EU707290	x	x	X	x
B. semibarbata [a,b]	Chase 8019	JQ039294	x	x	x	x
B. semibarbata [a,b]	K Dixon s.n. (KPBG)	HM640528	x	x	x	x
B. semibarbata [a,b]	K Dixon s.n. (KPBG)	HM640646	x	x	x	x
B. wiesei [a,b]	1995-3501	AF234350	x	x	x	x
Bulbinella anguistifolia	OTA 038740		x	x	x	x
***B. cauda-felis* (seeds)** [c]	Silverhill 9183		x	x	x	x
B. cauda-felis [c]	Spies 9295		x	x	x	x
B. cauda-felis [c]	Spies 9192		x	x	x	x

B. cauda-felis **[a]**	UCI Arb. 359	JX903194	x	x	x	x
B. chartacea **[c]**	Stedge & Musara 863		x	x	x	x
B. ciliolata **[c]**	Stedge & Musara 872		x	x	x	x
B. divaginata **[c]**	Stedge & Musara 877		x	x	x	x
B. elata **(seeds) [c]**	Silverhill 9298		x	x	x	x
B. elegans **(seeds) [c]**	Silverhill 9299		x	x	x	x
B. erbuniflora **(seeds) [c]**	Silverhill 9297		x	x	x	x
B. erbuniflora **[c]**	9184		x	x	x	x
B. gibbii **var.** ***balanifera***	OTA 066755		x	x	x	x
B. gibbii **(narrow leaves)**	OTA 032761		x	x	x	x
B. gibbii **var.** ***gibbii***	OTA 33054		x	x	x	x
B. gracillis	Stedge & Musara 873		x	x	x	x
B. graminifolia **(seeds)**	Silverhill 9185		x	x	x	x

B. hookeri	OTA 018327		x	x	x	x
B. latifolia [c]	Stedge & Musara 860		x	x	x	x
B. latifolia* var. *granitus [c]	Spies 9191		x	x	x	x
B. modesta	OTA 062695		x	x	x	x
B. nana	Stedge & Musara 879		x	x	x	x
B. nana [a]	BGW, 303/92, Van Wyk- JRAU	AJ511419	x	x	x	x
B. rossi	OTA 031504		x	x	x	x
B. rossi	OTA 065322		x	x	x	x
***B. rossi* (flowers)**	Not accessioned		x	x	x	x
B. trinervis [c]	Stedge & Musara 875		x	x	x	x
B. triquetra	Spies 9309		x	x	x	x
Kniphofia praecox [a,b]	JRAU van Wyk 4119	AJ512276	x	x	x	x
K. praecox [a,b]	Pearse, W.D. 210980	KM360836	x	x	x	x

K. praecox [a,b]	JRAU Van Wyk	AJ511424	x	x	x	x
K. praecox [a,b]	Ramdhani 529 GRA	EU707255	x	x	x	x
K. stricta [a,b]	SR279	HQ646907	x	x	x	x

[a]- Sequences obtained from Genbank.

[b]- Outgroups.

[c]- Specimen used for sequencing

OTA- Voucher numbers refer to specimens in the University of Otago (New Zealand).

s.n. = unnumbered collections with no herbarium voucher

SR- Voucher numbers refer to specimens collected by S. Ramdhani

GRA- Voucher numbers refer to specimens from Grahamstown, Rhodes (South Africa)

UDW- Voucher numbers refer to specimens from University of Durban Westville (South Africa)

JRAU- where specimens are held at Rhodes, South Africa.

BGW- refer to burrow-dwelling ground wanderer (plants)

KPBG- Voucher numbers refer to specimens from Kings Park and Botanical Garden in Perth (Australia)

[d] Genbank numbers will be added once sequences are submitted to Genbank after examination. Numbers under the column "Genbank" reflect already published sequences.

4.1.1: Systematics

Systematics is defined as the scientific study of the diversity and history of life and has deduced relationships among plant groups based upon a wide variety of biological characters (May 1990; Hidayat and Pancoro, 2006). Systematics can improve biodiversity science, conservation and policy in four ways: by solidifying species concepts; identifying lineages worth of conservation; setting conservation priorities and evaluating the effects of hybridization on the biology and conservation, especially those of rare species (Soltis and Gitzendanner, 1999; Gravendeel, 2000; Hendry *et al.*, 2010). Therefore, systematics plays an important role in conservation and planning (Steele and Pires, 2011).

Systematics can be used to direct the exploration for plants with potential commercial importance, for example, the discovery of a new or exotic species or drug plants (Judd *et al.*, 1999; Daly et al., 2001; Spies, 2004). The basic activities of systematics are to make sense of classifications in light of evolution and to delve into the dynamic aspects of nature. Systematic also attempt to assist in the understanding of and communication about the natural world, hence classification and naming have been implemented since ancient times to deal with information about the natural world (Judd *et al.*, 1999; Spies, 2004).

Systematics is dedicated to discovering, organising, and interpreting biological diversity (Spies, 2004). Therefore, the systematics determines a previously unknown species and provides the world with a diagnostic description of the newly known plant or animal (Anonymous, 2010). At the root of all these tasks, the primary result of systematics is the satisfaction of the inherent human drive to arrange and to classify things and it incorporates the following tasks, taxonomy, classification and phylogenetic analysis (Anonymous, 1994; Spies, 2004).

4.1.2: Taxonomy

Taxonomy is the science of circumscribing, discovering, naming, describing, and grouping individuals into species, arranging these species into larger groups and giving these groups names, thus producing a classification (Seberg *et al*. 2003; Wheeler, 2005; Crisci, 2006). It is classifying taxa among which the species is the fundamental unit, (Seberg *et al*. 2003; Dayrat, 2005; Wheeler, 2005; Crisci, 2006). Therefore, taxonomy provides a framework for the meaningful expression and synthesis of biological information (Spies, 2004).

Taxonomy provides the necessary underpinning for many aspects of management of genetic resources as it permits clear and unequivocal communication between conservationists allowing them to exchange material and to describe its properties on the basis of a shared understanding of identity (Rao, 2002). The taxonomy includes two main tasks and the first primary task of taxonomists, commonly known as alpha-taxonomy (Mayo *et al*., 2008), is to circumscribe, describe and name species.

The circumscription of a species encompasses testing hypotheses based on available data at a given time, comprising traditionally morphological, anatomical and ethological characters, and develops predominantly as science progresses (de Meeûs *et al*., 2003; Seberg *et al*., 2003; Will and Rubinoff, 2004; Esselstyn, 2007).

The naming and the description of species are conventions that follow the rules of the International Codes of Nomenclature such as the application of the so-called binominal description of an organism by its genus and species (Winston, 1999; Seberg *et al*., 2003). There is, therefore, a need to establish and maintain effective mechanisms for the stable naming of biological taxa. To ensure that a species can have a name that is unambiguous and globally understood, and is legitimately attached to a type specimen, regardless of its scientific status, the rules should be developed based on the work of Linnaeus (1753) (Mallet and Willmott 2003; Seberg *et al*., 2003; Bowman, 2005; Krishnankutty and Chandrasekaran, 2007; Glover *et al*., 2009; Rainbow, 2009).

Since there was no common methodology for classifying taxa, this obviously led to different classifications for the same group of organisms based on the characters studied or according to the relative importance given to them by taxonomists (Tassy, 1986; Wiley *et al*., 1991). This brought the second principal task of taxonomists to classify organisms into diverse taxa arranged in a hierarchical structure such as species, genus, family, order, class, phylum and kingdom (Tassy, 1991; Lewin 1999; Crisci, 2006).

The goal of the biological classification is to reflect phylogenetic relationship and this has triggered the researcher to update the phylogenetic relationship of *Bulbinella* species in South Africa and New Zealand. It is also imperative to update the taxonomic revisions of South African genera in order to achieve Target 1 of the Global Strategy for Plant Conservation and in that way South Africa will fulfil its commitments to the Convention on Biodiversity (von Staden *et al*., 2013). These updates are accompanied by molecular data (DNA-barcodes and chloroplast genomes) to assist in biodiversity assessments. *Bulbinella* is one of the South African genera that was revised to generate molecular phylogeny using Illumina sequencing based on 34 chloroplast protein-coding genes (genome sequence analysis) and DNA sequencing of four gene regions (*ITS, rbcL, matK* and *psbA-trnH*).

4.1.3: Classification

Classification is the grouping of species, ultimately on the basis of evolutionary relationships and is used to organise information about plants (Judd *et al*., 1999). Current day classification is based on the so-called binomial system introduced by Linnaeus in his Species Plantarum (1753) (Erkens, 2007). To classify and group things appears to be a fundamental human instinct (Sivarajan, 1991).

In order to understand plant diversity, one must have a good quality and reliable system of classification that can be used as a reference system of information (Erkens, 2007). Current classifications usually do not represent phylogenies, but rather the product of a long human history, which makes systematics a history-bound discipline (Judd *et al*., 1999). However, one of the reasons why it is necessary to classify is it has predictive value.

4.1.4: Phylogenetics

Phylogenetics is the discovery of evolutionary relationships (hence its history of descent from their common ancestors including the order of branching and sometimes of divergence) among and within a group of species (Unda, 2006; Patwardhan *et al*., 2014). The use of DNA-sequence data is now the routine to solve phylogenetic problems and it’s an attempt to reconstruct the evolutionary history of those sequences (Patwardhan *et al*., 2014). The crucial goal is to use sequence data from several gene regions to provide information about the phylogenetic history of organisms (Brown, 2002; Small *et al*., 2004; Delsuc *et al*, 2005; 2007; Patwardhan *et al*., 2014).

Phylogeny aims to reflect the evolutionary history and relationships of a particular taxon (Klopper *et al*., 2010) whereas evolution duly considers the phylogeny of the taxa as well as the evolutionary processes and ecological adaptiveness of evolutionary divergence (Mayr and Bock 2002; Klopper *et al*., 2010). The ideal would be to take account of a classification system that precisely reflects both the phylogenetic relationships and the sum of character state evolution among all plants (Klopper *et al*., 2010).

In the Asphodelaceae, the phylogenetic relationships amongst and within genera in the family, are still unresolved (Treutlin, 2003; Daru *et al*., 2013). There is a lot to be done, to fully document character state diversity, evolution and adaptive radiation in the family (Klopper *et al*., 2010). Hence there is still considerable uncertainty regarding the current infrageneric phylogenetic affinities and relationships amongst the *Bulbinella* genus in South Africa and New Zealand.

4.1.5: Molecular Systematics

Systematic studies give insight into the history of groups of organisms and the evolutionary processes that produce diversity among species (Weaver, 2002). Molecular systematics is the use of any molecular data (DNA and RNA) to infer relationships among individuals and species and or determine the evolutionary history of a taxon (Judd *et al.*, 1999). Numerous molecular techniques have been functional in the studies of phylogeny species evolution and have been useful to enhance the understanding of the distribution and extent of genetic variation within and between species (Mondini *et al.*, 2009).

Molecular data is more reliable in determining phylogenetic relationships than morphological data primarily because they revealed gene-level changes, which were thought to be less subject to convergence and parallelism than morphological traits (Johnson and Hall, 2005; Patwardhan, 2014). Molecular systematic is an immensely useful tool to help resolve relationships among and within taxa on various levels and evolutionary relationships of organisms (Liang, 1997; Dowell, 2008).

Molecular analyses have not yet produced *Bulbinella* multigene phylogenies. In this regard, many of the phylogenetic and taxonomic problems associated with Asphodelaceae are due to the fact that the family is characterised by a combination of characters, most of which also occur in other Asparagoid families (Chase *et al.*, 2000; Klopper *et al.*, 2010). Therefore, none of them in isolation or possibly not even in combination are sufficient to distinguish Asphodelaceae from other Asparagales families (Chase *et al.*, 2000). As a rule, molecular data ought not to be used in isolation, but always combined with existing knowledge on the morphology of the group in question (Klopper, *et al.*, 2010).

Molecular data have indicated that a re-evaluation of the long-established taxonomic concepts is needed (Chase *et al.*, 2000, Treutlein *et al.*, 2003a). Nevertheless, more taxa and more evidence need to be included in phylogenetic analyses and comparative studies of character evolution. Only a combination of data from micro- and macro morphology will provide a clear picture of the true phylogeny and evolution of the group and none of these characters should be used in isolation (Smith and Steyn, 2004).

Morphological similarities were traditionally used to try and deduce relationships among plant groups (Spies, 2004) and additional criteria were similarities with respect to plant secondary metabolites, isozymes, and other protein systems (Spies, 2004). Molecular data are subject to the same problems as morphological data but has more molecular characters available. This promotes the interpretation of the data and molecular data are, therefore, widely used for generating phylogenetic hypotheses (Judd *et al*., 1999; Spies, 2004).

The entire methods that permit a direct assay of mutational differences at the level of DNA have great promise for systematic biology (Clegg and Durbin, 1990; Spies, 2004). Molecular genetics and biochemistry are becoming more and more essential as tools for understanding evolution, consequently resulting in a rapid incline in applying macromolecular techniques and data for plant systematic studies (Judd *et al*., 1999; Crawford, 2000; Spies, 2004). Molecular data have, in many cases, supported the monophyly of groups that were recognised based on morphology (Judd *et al*., 1999; Mayr, 2003; Wahlberg *et al*., 2005).

In addition, DNA-based biodiversity identification tools such as DNA-barcoding and systematics have been proven to be a useful acceleration tool to the slow taxonomic process to assist in the biodiversity conservation process (DeSalle and Amato, 2004; Smith *et al*., 2005; Hajibabaei *et al*., 2012). The sequencing information should also reveal genetic variation between species and allow for the reconstruction of the phylogenetic relationship within the genus *Bulbinella*. The objectives were to put across the systematic relationships among species in the *Bulbinella* genus. Therefore, chloroplast protein-coding genes (genome sequence analysis) and DNA sequencing of both nucleus and chloroplast gene regions (*ITS*, *rbcL*, *matK* and *psbA-trnH*) were used for genetic analyses during this study.

4.2: MOLECULAR ANALYSIS USED DURING THIS STUDY

Molecular methods have had a profound impact and have become crucial in most studies on genetic diversity and other key features affecting genetic diversity patterns. Equally, it is imperative to understand that different markers have different properties and will reflect different aspects of genetic diversity (Nesbitt *et al*., 1995; Karp and Edwards, 1995). The discrepancy between the marker analyses may be interrelated to the quantity of genome coverage characteristic of a particular marker system in species and its efficiency in sampling variation in a population (Staub *et al*., 1997; Hodgkin *et al*., 2001).

Through their progression, PCR, DNA sequencing and Data analysis have developed into most indispensable techniques which can be used for the characterisation and assessment of germplasm and genetic diversity (Lin *et al*., 1996; Jones *et al*., 1997). Recently, a series of techniques and genetic markers have been introduced that determines genetic variation within and between species. Nevertheless, no single technique is universally the ultimate (Mueller and Wolfenbarger, 1999; Renau-Morata *et al*., 2005).

The information generated using various markers can provide with important information on detection of redundancy in germplasm collections (Rao and Hodgkin, 2002). Currently, taxonomy is in crisis in Southern Africa since there has been an absolute decline in the number of taxonomists in recent years and the discipline is significantly under-supported (Parnell, 1993; Guerra-Garcia *et al*., 2008). This has caused a major decrease in taxonomic revisions of plants as evidenced in South Africa where 273 priority genera have been identified (Von Staden *et al*., 2013). Among the 273 genera where the taxonomy is poorly defined, *Bulbinella* with 23 species, has been selected for this study (Von Staden *et al*., 2013). This genus lacks a proper taxonomic key, its revisions are out of date and its biodiversity and evolutionary history needs to be assessed for conservation purposes. Without this knowledge, even the simple task of deciding what groups or types should be conserved becomes more or less impossible.

4.2.1: Choice of Gene Regions

Systematic studies of *Bulbinella* plants in South Africa and New Zealand were incomplete since the descriptions of species were largely based on superficial and aggregate characteristics, which show very little variation between the different species. However, molecular systematic of nuclear or chloroplast gene regions possibly provide a better understanding of the phylogenetic relationships of the species than that of morphological approaches (Liang, 1997; Small *et al*., 2004). The combinations of explicit methods for phylogenetic analysis of *Bulbinella* species would reveal genetic variation between and within these species.

The high proportion of data used in plant molecular phylogenetic studies develops from chloroplast DNA and nuclear DNA (Small *et al*., 2004). Most plant cells comprise of three diverse types of genomes namely nuclear; plastid and mitochondrial, each of these is inherited in a different manner (Harding *et al*., 1991). It is, however, imperative to sequence and compares more than one gene from all three genomes to ensure a more reliable organismal phylogeny (Qui *et al*., 1999) hence a combination of plastid regions together represent a variable plant barcode (Chase *et al*., 2007). In this regard, the use of genome sequence analysis, and DNA sequencing of chloroplast and nuclear gene regions (ITS, *rbcL*, *matK* and *psbA-trnH*) of *Bulbinella* species will overcome potential problems arising from using single gene sequence data.

Bulbinella species are flowering plants which display implausible diversity in habit, morphology, anatomy, physiology, and reproductive biology (Perry, 1999) and this variation have to be resolved and strongly supported by a phylogenetic framework. Conrad *et al*. (2003) argued that, through analysing genes found in the chloroplasts region, it would be possible to predict phylogenetic relatedness between *Bulbinella* species in South Africa and New Zealand. Plastid genomes are somewhat conserved in structure and sequence such that comparisons across green plants are practicable and would also help to identify related organisms (Barker and Wolf, 2010).

Generally, the genes located in the chloroplast region of the majority of plants are maternally inherited (Judd *et al*., 1999; Spies, 2004). According to (Judd *et al*., 1999), the nucleus is inherited biparentally with its inheritance and control of expression being studied the most. It is the largest genome and contains the majority of horticultural important genes (Harding *et al.*, 1991). The nuclear genome is, however, less frequently used in systematic botany for the reason of its complexity and repetitive properties (Liang, 1997; Bora, 2010).

The different genes have specific advantages and disadvantages; hence the biosystematics is confronted with a wide range of choices. Furthermore, different genes develop at distinctly different rates and hence present varying degrees of genetic resolution amongst plant groups (Hidayat and Pancoro, 2006). Two most essential criteria should be applied: firstly, the suitable genome must be selected to best deal with the exact biosystematics question at hand and secondly, the suitable molecular method must be chosen (Spies, 2004; Hidayat and Pancoro, 2006).

When these criteria are applied, the chloroplasts genome tend to be the molecule of choice, principally if the goal is to look into relationships at or above the family level or species level (Clegg and Durbin, 1990). In contrast with the chloroplasts genome, the use of DNA mitochondria (mtDNA) for biosystematics studies in plant is very restricted due to the fact that it is large in size so that it is more difficult to isolate and purify (Hidayat and Pancoro, 2006). In addition, because it is circular and rearranges itself regularly in structure, size, configuration, and gene order; it, therefore, cannot be used to infer relationships between species (Douglas, 1998; Bora, 2010).

Therefore, the genes that are often used in sequencing studies include the chloroplast genes *rbcL*, *psbA-trnH*; *matK* and the nuclear the internal transcribed spacer region

(*ITS*) (Hoot *et al*., 1995, Judd *et al*., 1999). All these genes provide optimal phylogenetic results at different taxonomical levels (Bousquet *et al*., 1992) and above (Chase *et al*., 1993). The major gene regions used for barcoding are *matK* and *rbcL* and these have exhibited usefulness in resolving phylogenetic relationships at various levels in the same family of Asphodelaceae (Small *et al*., 2004; Daru, 2013).

4.2.1.1: Maturase Kinase (m*atK*)

This region has proven as yet one more gene with probable significance to plant molecular systematics and evolution (Selvaraj *et al*., 2008; Takundwa *et al*., 2012). The coding region of *matK* is normally located within an intron of the chloroplast *trnK* gene (Duffy *et al*., 2009; Zoschke, 2009; Hao *et al*., 2010). Being a coding region and its very high evolutionary rate (*matK*), has made it useful in phylogenetic reconstructions at high taxonomic levels and has also been used effectively in addressing systematic questions at low taxonomic levels, such as genus or species (Chase *et al*., 2007; Lahaye *et al*., 2008; Dong *et al*., 2012; Petitjean *et al*., 2014).

The *matK* codes for a maturase protein and is very useful in DNA barcoding for the identification of plant families (Jing Yu and Zhou, 2011, Gao *et al*., 2008; Selvaraj *et al*., 2008, 2013; Ali *et al*., 2014). The *matK* gene has higher variation than any other chloroplast genes, thus in accordance with the detailed analysis of the *matK* sequence data which is available in GenBank and also preliminary studies (Liang and Hilu, 1997).

The high proportion of the *matK* gene might endow with more phylogenetic information on *Bulbinella* species and this emphasises the efficacy of the *matK* gene in systematic studies. Henceforth imply that comparative sequencing of *matK* is possibly suitable for phylogenetic reconstruction at subfamily and family levels (Liang and Hilu, 1997; Patel *et al*., 2014). Recent studies have shown the usefulness of this gene for resolving intergeneric and interspecific relationships among family Asphodelaceae (Klopper *et al*., 2010; Daru *et al*., 2013).

Sequences of the *matK* region were obtained for 22 *Bulbinella* specimens (11 South African species and 5 New Zealand *Bulbinella* species). The complete alignment included 900 nucleotide positions. The resultant phylogenetic tree (**Figure 22**) shows that New Zealand and South African species had four groupings designated as clades 1, 2, 3 and 4. *Kniphofia praecox* (AJ511424) and *Bulbine semibarbata* (HM640646) were used as outgroups.

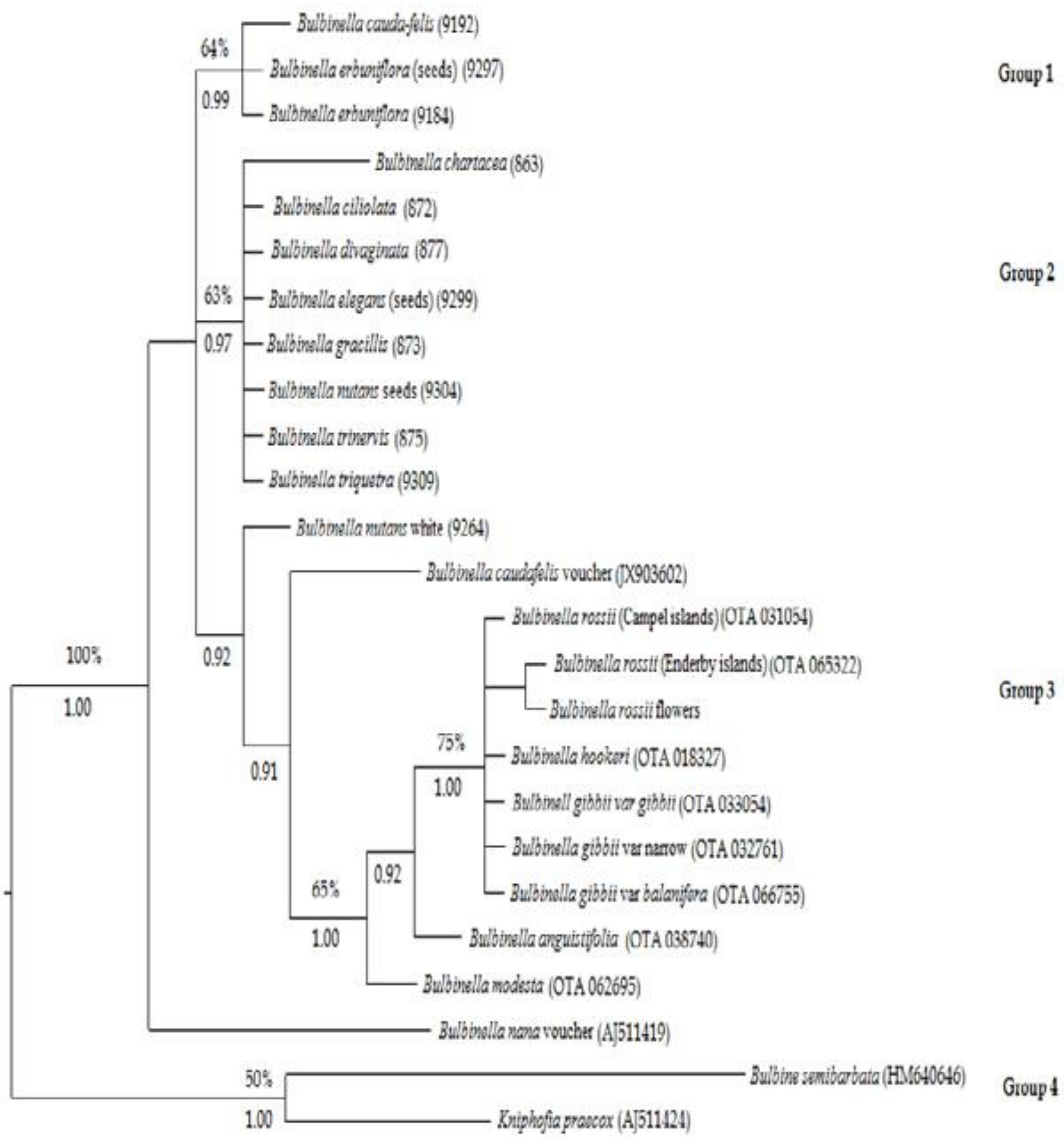

Figure 22: Reconstruction of a phylogenetic tree from *matK* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (*depicts MLB and PB values <50%). *Kniphofia praecox* and *Bulbine semibarbata* were presented as outgroups.

Species of the genus *Bulbinella* formed a monophyletic clade, including the South African and New Zealand species. Within this clade the New Zealand specimens form a paraphyletic clade with the rest of the genus. The species formed three clades. The first clade (clade 1) had three South African species, *B. caudafelis* (9192), *B. erbuniflora* seeds (9297) and *B. erbuniflora* (9184) with a strong Bayesian posterior probability (PP= 0.99) but weak support in ML (BS =64%). The second clade had a weak bootstrap support (BS = 63%) but strong Bayesian posterior probability (PP= 0.97) and was composed of eight South African species, namely *B. nutans* seeds (9304), *B. elegans* (9299), *B. divaginata* (877), *B. chartacea* (863), *B. trinervis* (875), *B. triquetra* (9309), *B. ciliolata* (872), and *B. gracillis* (873). Clade three contained all nine New Zealand specimens based on strong posterior probability support (PP =0.99) and moderate bootstrap support (BS=65%). These included *B. gibbii* var. *gibbii* (OTA 033054), *B. gibbii* var. *balanifera* (OTA 066755), *B. gibbii* var. narrow (OTA 032761), *B. hookeri* (OTA 018327), *B. rossii* flowers, *B. rossii* Enderby (OTA 065322), *B. rossii* Campbell Islands (OTA 031504), *B. anguistifolia* (OTA 038740) and *B. modesta* (OTA 062695). The members of NZ group appeared closely related but with little divergence (**Figure 22**). *B. caudafelis* (9295), *B. nana* voucher (AJ511419), *B. caudafelis* voucher (JX903602) and *B. nutans* white (9264) did not show any grouping. The fourth clade consist of *K. praecox* (AJ511424) and *Be. semibarbata* (HM640646) based on strong posterior probability support (PP =1.00) and very weak bootstrap support (BS=50%). The following South African species were excluded from analysis due to either alignment difficulties and or poor PCR amplifications: *B. elata* (9298), *B. punctualata* (9146), *B. latifolia* (860), *B.*

latifolia var. *granitus* (9191), *B. graminifolia* seeds (9185), *B. caudafelis* seeds (9183) and *B. nana* (879).

4.2.1.2: Ribulose-bisphosphate carboxylase gene (*rbcL*)

The *rbcL* gene has been extensively sequenced from several plant taxa and the consequential data base has significantly assisted studies of plant phylogeny (Chase *et al*. 1993; Gielly, 1994). It is the most common protein encoding plastid gene that has been used to provide sequence data for plant phylogenetic analyses (Chase *et al*., 1993). The gene has been proposed as a potential barcode despite the fact that it has been commonly used to resolve evolutionary relationships at the generic level and above (Kress *et al*., 2005; Chase *et al*., 2005; Newmaster *et al*., 2006; Chase, 2007; Arca *et al*., 2012). The single copy of the *rbcL* gene is free from length mutations except at the far 3' end and has a somewhat conservative rate of evolution (Liang, 1997; Bora, 2010; Patel *et al*., 2014).

The purpose of the *rbcL* gene is to code for the large subunit of ribulose 1, 5 bisphosphates carboxylase/oxygenase (Liang, 1997; Bora, 2010). The *rbcL* gene is periodically too conserved to explicate relationships between closely related genera (Gielly, 1994). Nevertheless, it is apparent that the ability of *rbcL* to resolve phylogenetic relationships below the family level is often poor because it evolves too slowly for species-level identifications (Ge *et al*., 2002; Shaw *et al*., 2005).

Sequences of the *rbcL* region were obtained for 25 *Bulbinella* specimens and the complete alignment included 550 nucleotide positions. *Kniphofia praecox* (KM360836) and *Bulbine semibarbata* (HM640528) were used as the outgroups. From both the ML and BI analysis (**Figure 23**) three very weakly dissolved clades were observed.

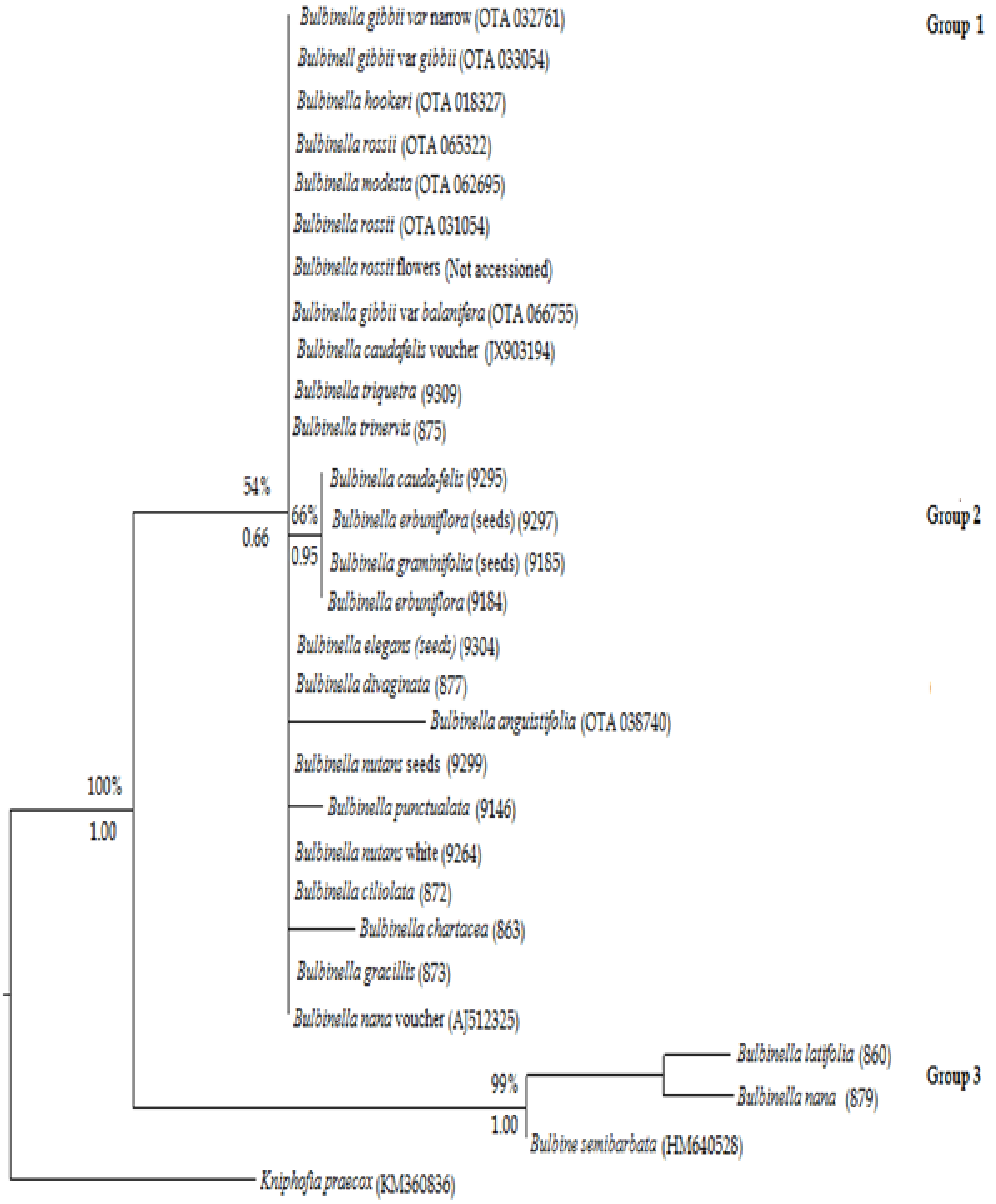

Figure 23: Reconstruction of a phylogenetic tree from *rbcL* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (*depicts MLB and PB values <50%). *Kniphofia praecox* and *Bulbine semibarbata* were presented as outgroups.

The 18 South African specimens including *B. nutans* (9304), *B. nutans* (9264), *B. divaginata* (877), *B. trinervis* (875), *B. nana* (AJ512325), *B. elegans* (9299), *B. triquetra* (9309), *B. caudafelis* (JX903194), *B. gracillis* (873), and *B. ciliolata* (872). The group also included 8 New Zealand specimens, namely *B. gibbii* var. *balanifera* (OTA 066755), *B. gibbii* var. narrow (OTA 032761), *B. hookeri* (OTA 018327), *B. modesta* (OTA062695), *B. rossii* Campbell Islands (OTA 031504), *B. rossii* flowers, *B. gibbii* var. *gibbii* (OTA 033054) and *B. rossii* Enderby (OTA 065322) with weak support (BS =54%; PP = 0.66) forms a polytomy. The second clade consisted of South African species including *B. erbuniflora* (9184), *B. graminifolia* (9185), *B. erbuniflora* (9297) and *B. caudafelis* (9295) (PP=0.95, BS=66%). Clade 3 included *B. nana* (879), *B. latifolia* (860) and the outgroup *Be. semibarbata* (HM640528) with strongest support of both ML & BI (PP=1.00, BS=99%). The following species was excluded from analysis due to either alignment difficulties and or poor PCR amplifications; namely *B. caudafelis* seeds (9183) and *B. elata* (9298) from South Africa. *Bulbinella chartacea* (863), *B. punctualata* (9146) and *B. anguistifolia* (OTA 038740) did not show any grouping and is also part of polytomy.

4.2.1.3: *psbA-trnH* spacer

Another plastid DNA region proposed for phylogenetic studies of *Bulbinella* is the non-coding intergenic *psbA-trnH* spacer, as a good barcode candidate for land plants (Kress *et al*., 2005; Shaw *et al*., 2007). It has the highest percentages of variable sites (Shaw *et al*., 2007). This variation means that this inter-genic spacer is a critical tool in plant molecular phylogenetic as it can offer high levels of species discrimination studies at the low taxonomic level and as suitable for DNA barcoding studies (Kress *et al*., 2005; Shaw *et al*., 2007; Degtjareva *et al*., 2012).

However, the consortium for the barcoding of life (CBOL) disregarded *psbA-trnH* because of its complex molecular evolution (CBOL, 2009) and as it does not consistently provide bidirectional unambiguous sequencing reads (CBOL, 2009). It was then proposed by Kress and Erickson (2007) to combine the original *psbA-trnH* barcode with *rbcL*, following analyses from Newmaster *et al*., (2006). Since the plastid genome is evolving so slowly in relation to other genomes, more than one barcode may be required to provide sufficient variation for this technique to work (Kress *et al*., 2005; Newmaster *et al*., 2006; Taberlet *et al*., 2007; Chase *et al*., 2007).

Sequences of the *psbA-trnH* region were obtained for 17 *Bulbinella* specimens (11 South African spp. and 4 New Zealand *Bulbinella* spp.). The complete alignment included 650 nucleotide positions. Sequences of *psbA-trnH* for *Kniphofia praecox* used in the other analyses were not available in GenBank and *Kniphofia stricta* (HQ646907) and *Bulbine semibarbata* (JQ039294) were thus used as outgroups. From the findings (**Figure**

24) it showed that both New Zealand species and South African species had three groupings designated as clade 1, 2 and 3.

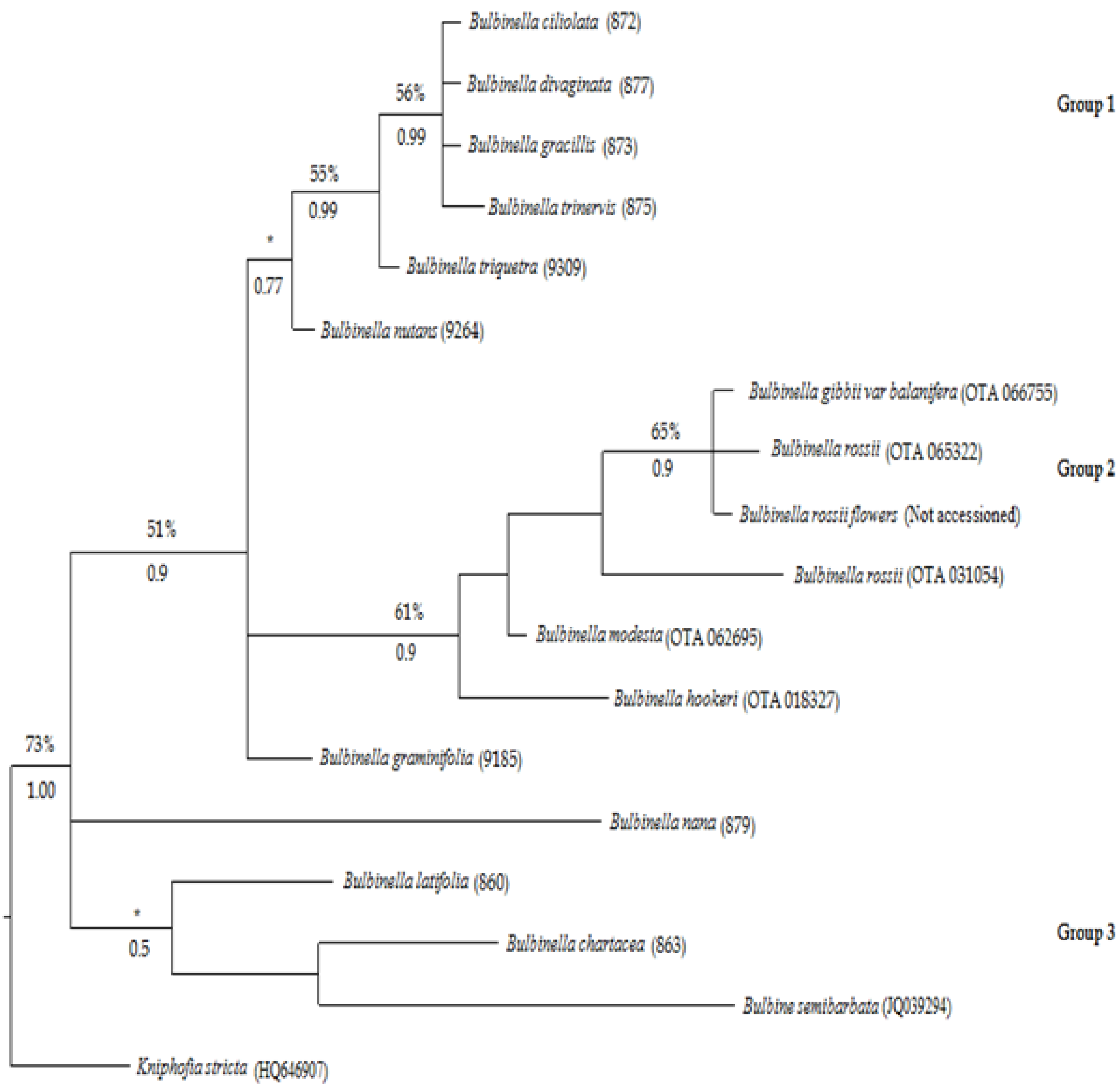

Figure 24: Reconstruction of a phylogenetic tree from *psbA-trnH* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (* depicts MLB and PB values <50%). *Kniphofia stricta* and *Bulbine semibarbata* were presented as outgroups.

Clade 1 included South African species, namely *B. triquetra* (9309), *B. divaginata* (877), *B. gracillis* (873), *B. ciliolata* (872) and *B. trinervis* (875) (BS=55%; PP=0.99). *B. nutans* white (9264) grouped basally to this group and could either be separate or form part of a greater group that includes the *Bulbinella* species. Clade 2 formed a clade with six New Zealand specimens which are *B. hookeri* (OTA 018327), *B. rossii* from separate locations (OTA 031504; OTA 065322), *B. modesta* (OTA 062695), *B. rossi* flowers (not accessioned), and *B. gibbii* var *balanifera* (OTA 066755) (PP=0.99; BS=61%). Clade 3 included *B. chartacea* (863), *B. latifolia* (860) and *Be. semibarbata* (JQ039294) with only posterior probability support (PP=0.5). *Bulbinella nana* (879) and *B. graminifolia* (9185) did not strictly group in the any of the clades. The following species were excluded from analysis due to either alignment difficulties or poor PCR amplifications and included *B. anguistifolia* (OTA 038740), *B. gibbi* var *gibbi* (OTA 033054) and *B. gibbii* narrow (OTA 032761) from New Zealand, and *Bulbinella elata* (9298), *B. elegans* (9299), *B. caudafelis* (9295), *B. caudafelis* (9192) *B. caudafelis* (9183), *B. nutans* (9304), *B. punctualata* (9146) and *B. erbuniflora* (9297) from South Africa.

4.2.1.4: Internal Transcribed Spacers (ITS)

The Internal transcribed spacer gene is a benefit to plant systematic (Linder, 2000), as it has shown broad utility across photosynthetic eukaryotes and fungi by improving the quality of plant phylogenetic reconstruction in species-level molecular systematic (Kress *et al*., 2005; Dong, 2012).

According to Kress *et al*., (2005), for closely related taxa, *ITS* was found to evolve more rapidly than many plastid regions and is also subject to concerted evolution (Feng *et* *al*., 2013; Dong, 2015). The use of ITS sequences is generally accepted for the molecular analysis of plants, but its primary purpose is to identify species rather than to discriminate varieties (Kyiashchenko and Berlin, 2011; Rajapakse *et al*., 2012). *ITS* 2 becomes a potentially useful as a standard DNA barcode to identify medicinal plants (Kress *et al*., 2005; Gao, 2010; Chen *et al*., 2010; Kyiashchenko and Berlin, 2011; Rajapakse *et al*., 2012) and as a barcode to identify animals (Prasad *et al*., 2009). The ITS region can be amplified in two smaller fragments (*ITS1* and *ITS2*) adjoining the 5.8S locus, which has proven largely significant for degraded samples (Kress *et al*., 2005; Bhattarai *et al*., 2010; Bruhn, 2011; Selvaraj *et al*., 2013).

Due to insufficient sequence variation and its small number of nucleotide sites, the *ITS* is not suitably phylogenetically informative for a few recently evolved angiosperm lineages (Linder, 2000; Christelova *et al*., 2011). Even with its known limitations, ITS is a prime candidate as an effective locus for DNA barcoding in plants (Kress *et al*., 2005). The nuclear genome may possibly offer for plant barcoding because the plastid genome has been more readily exploited (Kress *et al*., 2005; Gao *et al*., 2010; Vernooy *et al*., 2010). In the current research, the researcher analysed the sequences of both the chloroplast genomes (*matK*, *rbcL* & *psbA-trnH*) and the nuclear genome (*ITS*) mainly of rare or taxa that are presumed extinct especially *Bulbinella* species in South Africa and New Zealand.

Sequences of the *ITS* region were obtained for 20 *Bulbinella* species (10 South African spp. and 3 New Zealand *Bulbinella* spp.). The complete alignment included 750 nucleotide positions. Outgroups were *Kniphofia praecox* (EU707255) and *Bulbine wiesei*

(AF234350). New Zealand species and South African species formed four groupings (**Figure 25).**

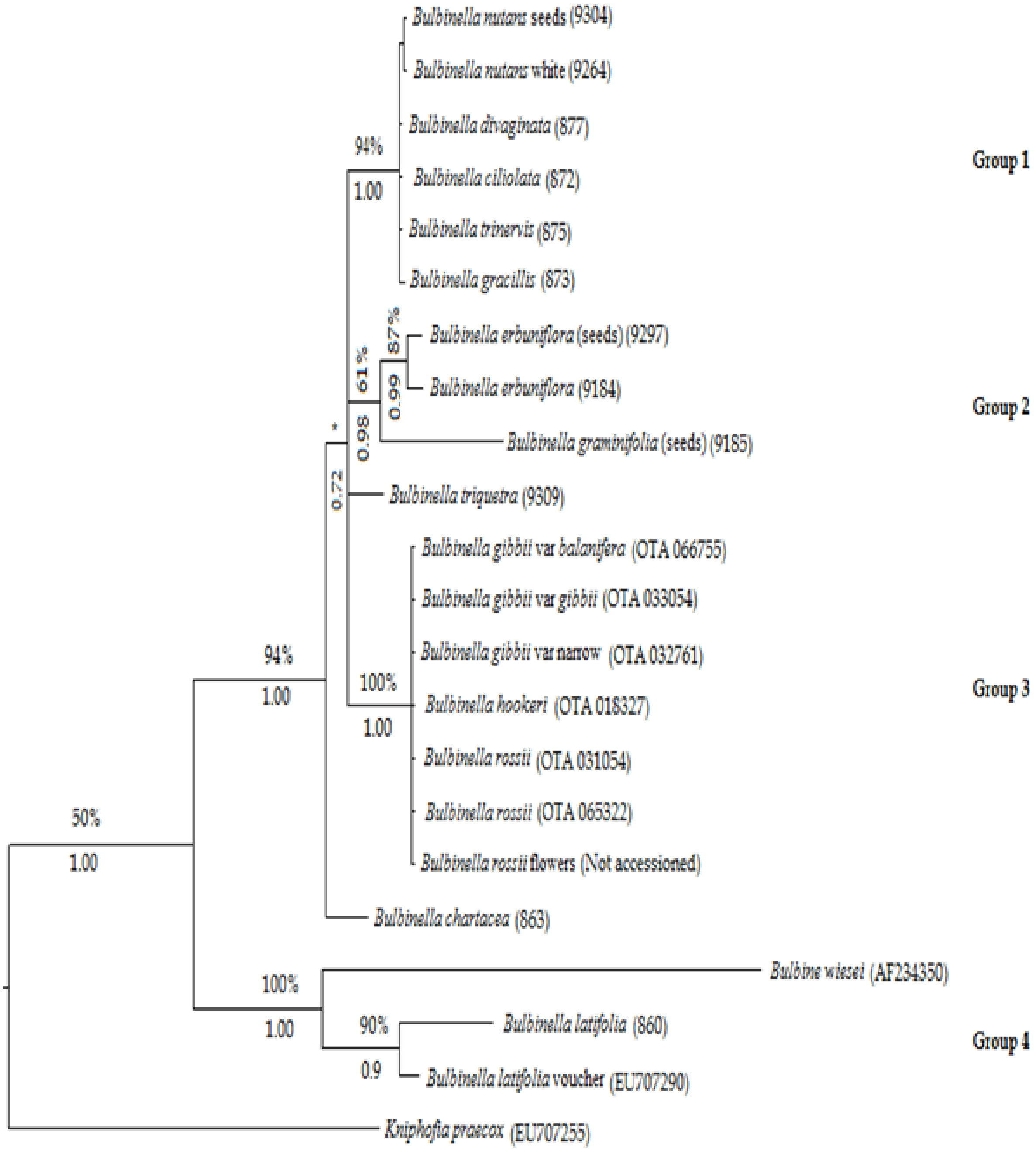

Figure 25: Reconstruction of a phylogenetic tree from *ITS* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (* depicts MLB and PB values <50%). *Kniphofia praecox* and *Bulbine wiesei* were presented as outgroups.

Four main clades were observed besides one of the outgroups (*Kniphofia praecox)*. Clade 1 consisted of some of the SA species, namely *Bulbinella nutans* white (9264), *B. nutans* seeds (9304), *B. gracillis (873)*, *B. trinervis* (875), *B. divaginata* (877), and *B. ciliolata* (872) (PP=1.00, BS=94%). Clade 2 consisted of South African species, namely *B. graminifolia* (9185), *B. erbuniflora* seeds (9297) and *B. erbuniflora* (9184) (PP= 0.98, BS=61%). Clade 3 included all seven NZ specimens forming a paraphyletic clade including *B. hookeri* (OTA 018327), *B. rossii* from Campbell Islands (OTA 031504), *B. rossi* from the Enderby Islands (OTA 065322), *B. rossi* flowers, *B. gibbii* var *balanifera* (OTA 066755) and *B. gibbii* var *gibbii* (OTA 033054) (PP=1.00, BS=100%). The fourth clade had the strongest statistical support (BS =100%; PP =1.00) and comprised of one *Bulbine* species, namely *Be. wiesei* (AF234350), and *B. latifolia* voucher (EU707290) and *B. latifolia* (860) from South Africa. *B. chartacea* (863) and *B. triquetra* (9309) did not form part of any groupings. The following species were excluded from analysis due to either alignment difficulties and or poor PCR amplifications. These were *B. anguistifolia* (OTA 038740) and *B. modesta* (OTA 062695) from New Zealand and *B. elata* (9298), *B. elegans* (9299), *B. caudafelis* (9183), *B. caudafelis* (9295), *B. caudafelis* (9192), *B. nana* (879) and *B. punctualata* (9146) from South Africa.

4.2.1.5: Combined analysis of *matK, rbcL, psbA-trnH* and *ITS.*

In the combined gene (*matK, rbcL, psbA-trnH* and *ITS)* analyses, groupings obtained (**Figure 26**) generally reflected what was observed in the individual trees. Sequences of the combined plastid (*matK, rbcL, psbA-trnH)* and nuclear genes (*ITS*) included 28 *Bulbinella* specimens from both South Africa and New Zealand. The complete alignment included 2811 nucleotide positions. In the combined analyses, data were partitioned by the gene with model parameters unlinked across partitions. The resultant phylogenetic tree (**Figure 26**) consisted of five groupings designated as Clade 1 up to 5. *Kniphofia praecox* (AJ511424) and *Bulbine wiesei* (HM640646) were used as outgroups. These five clades were formed with weak support of both BI and ML.

Clade 1 consisted of all nine New Zealand specimens, including *B. gibbii* var. *gibbii* (OTA 033054), *B. gibbii* var. *balanifera* (OTA 066755), *B. gibbii* var. *narrow* (OTA 032761), *B. hookeri* (OTA 018327), *B. rossii* flowers, *B. rossii* Enderby (065322), *B. anguistifolia* (OTA 038740), *B. modesta* (OTA 062695) and *B. rossii* Campbell Islands (OTA 031504) (PP=0.92; BS=69%). South African *Bulbinella* species belonging to the second clade were based on weak support (BS=61%, PP=0.63) and included *B. caudafelis* (9192), *B. erbuniflora* seeds *(9297), B. erbuniflora* (9184), and *B. graminifolia seeds* (9185). *B. caudafelis* voucher (JX903194), *B. nutans* white (9264) and *B. triquetra* (9309) formed the third clade (BS=61% only). The fourth clade had weak statistical support (BS=74%, PP=0.67 only) and included six South African specimens, namely *Bulbinella nutans* seeds (9304), *B. elegans* (9299), *B. divaginata* (877), *B. trinervis* (875), *Bulbinella ciliolata* (872), and *B. gracillis* (873). Clade 5 consisted of *B. nana* (879), *Be. latifolia* voucher (EU707290), *B. latifolia* (860) and *Be. wiesei* (BP=88%, PP=0.71). The following species, namely *B.*

chartacea (863), *B. punctualata* (9146), and *B. nana* voucher (AJ511419) from SA, did not group in any group.

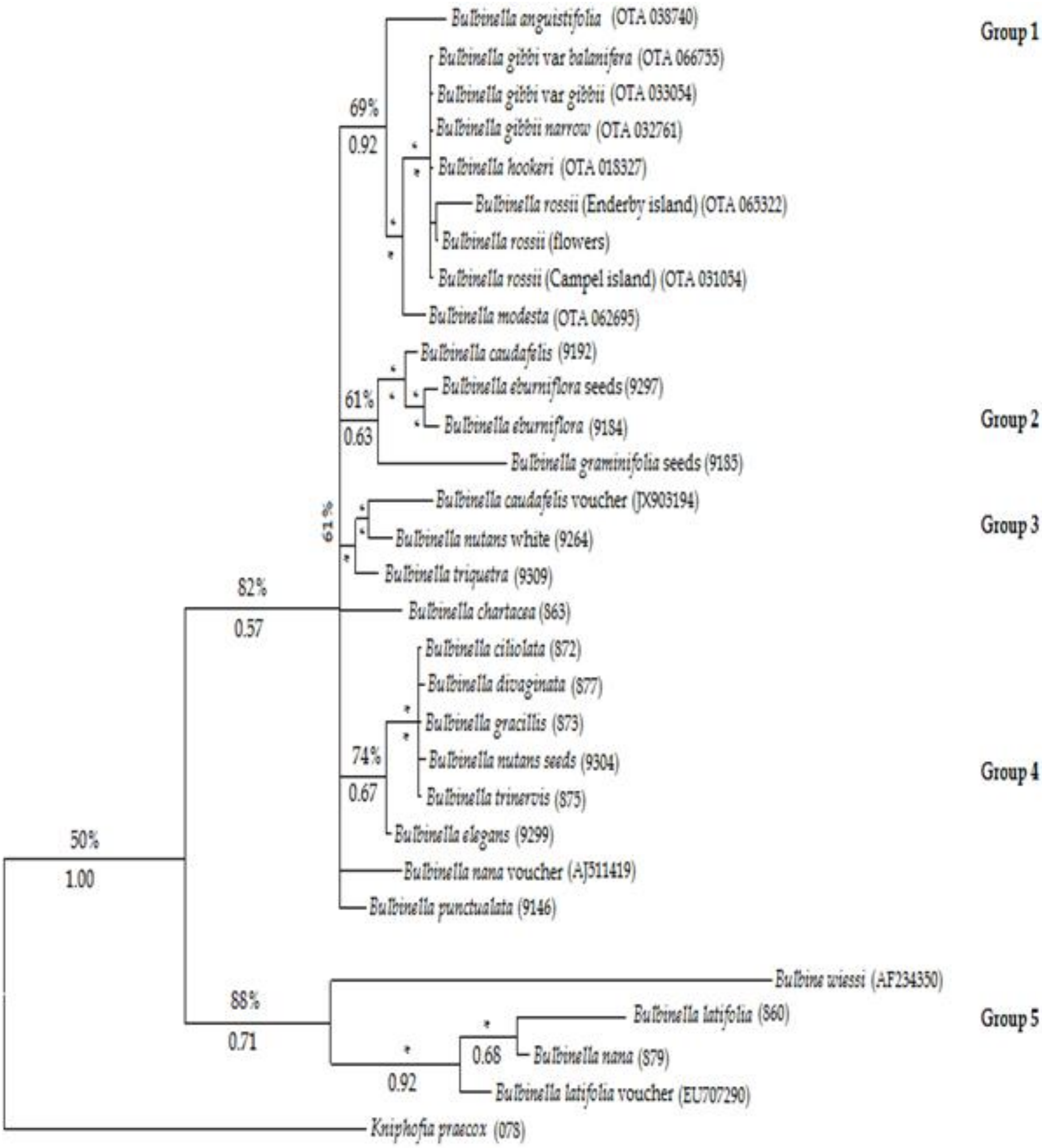

Figure 26: Reconstruction of a phylogenetic tree from combined (*matK, rbcL, psbA-trnH* & *ITS*) sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (*depicts MLB and PB values <50%). *Kniphofia praecox* and *Bulbine wiesei* are presented as outgroups.

4.2: General conclusions from the multigene analyses

The New Zealand specimens all consistently grouped in their own group as a paraphyletic group within the genus from the South African species in most of the gene analyses (separate and combined) **(Figure 22-26)**. Only the *rbcL* gene did not support the separate grouping, but this gene appears to be too conserved for species level differentiation for *Bulbinella* **(Figure 23)**. However, the New Zealand clade always grouped well within the general group represented by *Bulbinella* species in all of the separate and combined gene analyses, and never basal to South African *Bulbinella* species. Based on the analyses of the various genes, the New Zealand species appeared to be nested in *Bulbinella* and do not represent a separate genus.

Some New Zealand species are well supported based on gene sequences and represent distinct species. For instance, *B. modesta* and *B. anguistifolia* have separate groupings in most of the genes investigated. However, other species appear to be conspecific. In this regard, all the specimens and variations of *B. rossii (*Enderby Islands*), B. rossii* flowers*, B. rossii (*Campel Islands*)*, *B. gibbii* var. *gibbii*, *B. gibbii* var. *balanifera, B. gibbii* narrow and *B. hookeri* (except in the *psbA-trnH* tree), grouped together without any strong internal support separating the species in any of the genes sequenced.

Some South African species varied in the groupings of the collected specimens but a number of species consistently grouped together. Those grouping separately appear to represent robust species based on the collections used and available gene data. *Bulbinella elegans* (9299), *B. punctualata*, *B. chartacea*, *B. triquetra*, and *B. graminifolia* had separate groupings in the combined analyses, supported by the majority of the individual genes. However, other South African species appear to be synonymous to other species. These included *B. gracillis* (873), *B. trinervis* (875), *B. divaginata* (877), and *B. ciliolata* (872) had similar groupings than the New Zealand species *B. rossii*, *B. gibbii* and *B. hookeri*, where there were no bootstrap supports to distinguish these species, even in the combined analysis.

Specimens labeled as the same species did not always group together. These paraphyletic groupings included *B. caudafelis* (specimens 9192, 9295, and 9183), *B. nana* (AJ511419, 879), *B. latifolia* (860; EU707290; 9191), *B. erbuniflora* (9297, 9184), and *B. nutans* (9264; 9304). In these cases, it will be difficult to determine which grouping truly reflects the phylogenetic position of the species.

Based on the results of the different genes there were a level of constant grouping for certain South African species together, besides the constant grouping of the New Zealand species. The first group consisted of specimens of *B. caudafelis* (9192) and *B. eburniflora* (9297, 9184), and mostly *B. graminifolia* (9185) if present. Specimens of *B. gracillis* (873), *B. trinervis* (875), *B. divaginata* (877), *B. ciliolata* (872) and *B. nutans* (9304) also generally associated with each other. A third grouping were that of specimens *Bulbinella* (860) and sequence EU707290 of *B. latifolia* and specimen 879 of *B. nana*, which always grouped basally to the general *Bulbinella.* clade, and close to *Bulbine* species. Outside these groups, *B. triquetra* (9309), *B. chartacea* (863), *B. caudafelis* (9295), *B. caudafelis* voucher (JX903602) and *B. nutans* white (9264) grouped with different species or separately for each gene.

The results showed that the genes with the better resolution to distinguish *Bulbinella* species were *matK, ITS* and *psbA-trnH.* The *rbcL* gene region was too conserved to accurately distinguish between some species. Of the core DNA barcodes (*matK* + *rbcL*), only *matK* thus had sufficient resolution. However, both sequence sets were uploaded as barcodes into BOLD. Combinations of the four genes also did not significantly improve resolution since bootstrap and posterior probability support values remained low. However, trees obtained with *matK, ITS* and *psbA-trnH* generally showed the same trends as those observed in the combined analyses, and these genes on their own will give a fairly accurate reflection of phylogenetic relationships.

4.3: Relationships between *Bulbinella, Bulbine* and *Kniphofia*

Specimens of *B. latifolia* and one specimen of *B. nana* (879) consistently grouped with *Be. wiesei* and *Be. semibarbata* in the individual and combined trees **(Figure 22- 26).** More sequences of *Kniphofia* and *Bulbine* spp. from Genebank for the individual genes, were added to the *Bulbinella* datasets (**Figure 27, 28, 29 and 30**). This was an attempt to determine if these *B. latifolia* and *B. nana* specimens resided in their own clade, or in the genus *Bulbine*. A combined analysis could not be drawn because the genes available for the newly added species were too inconsistent.

The groupings of four major groups were investigated based on the four individual gene datasets, namely group one representing *Bulbinella*, group two representing *Kniphofia*, group three representing *Bulbine* and the fourth group representing the *B. latifolia* and *B. nana* specimens grouping outside the *Bulbinella* group. The *MatK* (PP=1.00 BS=99%, PP=1.00 BS=98%, PP=1.00 BS=100%) and *ITS* (PP=1.00 BS=99%, PP=1.00 BS=100%, PP=0.99 BS=91%) phylogenetic trees (**Figures 27 and 30**) strongly supported the three generic groupings of *Bulbinella, Bulbine* and *Kniphofia*. They also showed that *B. latifolia* grouped individually in the ITS set (sequences not available for *matK*) and *B. nana* grouped separately in the *matK* dataset (sequence not available for *ITS*). However, in the *psbA-trnH* and *rbcL* phylogenetic trees, the distinction between the *B. latifolia* and *B. nana* specimens and *Kniphofia* and *Bulbine*, become less clear. This is because in the *psbA-trnH* tree **(Fig 29**), the *Kniphofia* species are not forming a *Kniphofia* grouping, while the one *Kniphofia praecox* sequence groups with the rogues *B. latifolia* and *B. nana* specimens (now also including *B. chartacea*). In the conserved *rbcL* dataset, the rogue *B. latifolia* and *B. nana* specimens group with the *Be. wiesei* sequence.

What was consistently showed was that there is a core *Bulbinella* grouping that is always separate from *Bulbine* and *Kniphofia*. This core *Bulbinella* group includes the New Zealand specimens. However, the rogues *B. latifolia* and *B. nana* specimens always grouped outside *Bulbinella*. The relationship of these rogues species with *Kniphofia* and *Bulbine* appear to be distinct based on m*atK* and *ITS*, but still overlapping based on *psbA-trnH* and *rbcL*. However, phylogenetic analyses were very limited based on available data.

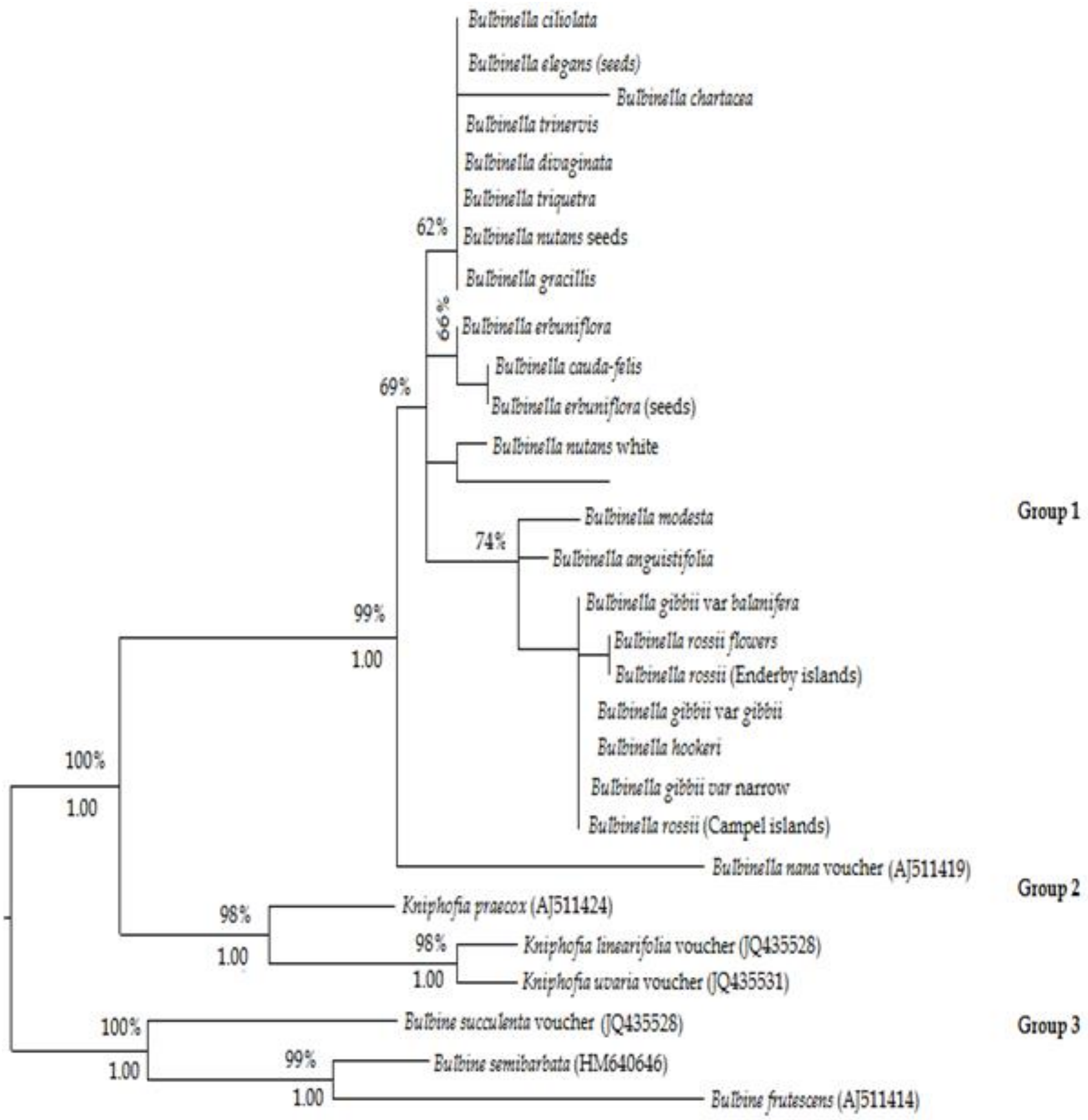

Figure 27: Reconstruction of a phylogenetic tree from *matK* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian probability values >0.5 are shown below the branches. (*depicts MLB and PB values <50%). *Kniphofia* species were presented as outgroup taxa.

Figure 28: Reconstruction of a phylogenetic tree from *rbcL* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (*depicts MLB and PB values <50%). *Kniphofia* species were presented as outgroup taxa.

Figure 29: Reconstruction of a phylogenetic tree from *psbA-trnH* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (* depicts MLB and PB values <50%). *Kniphofia* species were presented as outgroup taxa.

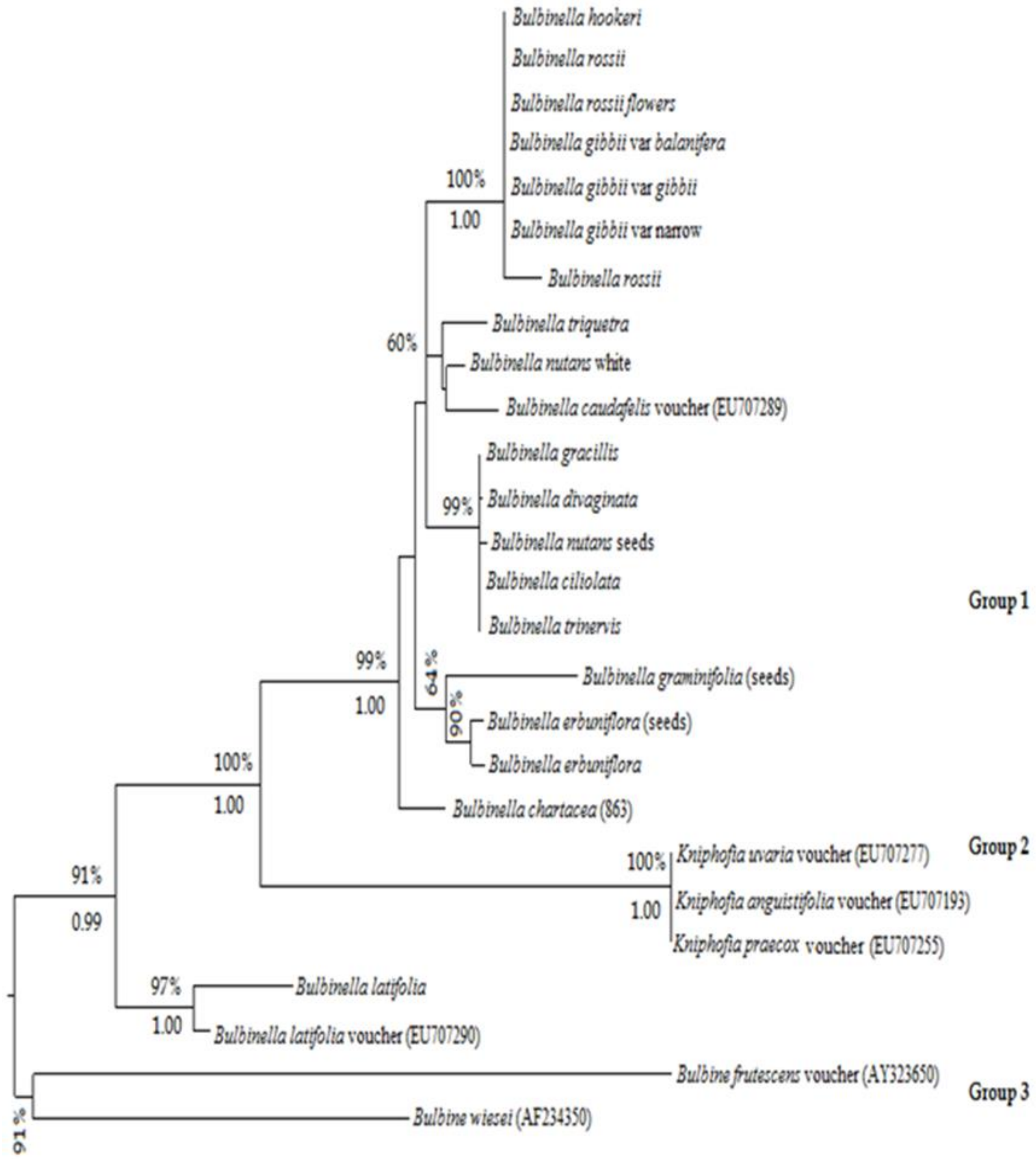

Figure 30: Reconstruction of a phylogenetic tree from *ITS* sequences dataset using Bayesian Inference. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (* depicts MLB and PB values <50%). *Kniphofia* species were presented as outgroup taxa.

CHAPTER 5: RESULTS AND DISCUSSION OF THE CHLOROPLAST GENOME DATA

5.1 Phylogenetic Analyses of Chloroplast genomes

Chloroplast genomes of several specimens were sequenced for South African species **(Table 5.1)**. The genome data will be submitted to https://submit.ncbi.nlm.nih.gov/subs/genome/.

Table 5.1: Chloroplast genomes were sequenced for the following specimens.

Species	Collection Number	Chloroplast accession number
B. latifolia [b,c]	Spies B002	
B. cauda-felis (seeds) [c]	Silverhill 9183	
B. cauda-felis [c]	Spies 9295	
B. cauda-felis [c]	Spies 9192	
B. ciliolata [c]	Stedge & Musara 872	
B. divaginata [c]	Stedge & Musara 877	
B. elata (seeds) [c]	Silverhill 9298	
B. elegans (seeds) [c]	Silverhill 9299	
B. erbuniflora (seeds) [c]	Silverhill 9297	

B. erbuniflora [c]	Spies 9184	
B. gracillis	Stedge & Musara 873	
B. graminifolia (seeds)	Silverhill 9185	
B. latifolia [c]	Stedge & Musara 860	
B. latifolia var. *granitus* [c]	Spies 9191	
B. trinervis [c]	Stedge & Musara 875	
K. praecox [b]	Spies 078	

[b]- Outgroups.

[c]- Specimen used for genome sequencing

5.2: Biodiversity assessment supplemented with Chloroplast genomes

The shortage of 'conventional' taxonomists in South Africa caused a major decrease in taxonomic revisions of these plants (Smith and Donoghue, 2008; Von Staden *et al*., 2013) and it calls for an urgent alternative method to identify species (Hebert *et al*., 2003). Also, conventional (non-molecular) taxonomy has several limitations in general, which pose threat in the genus *Bulbinella,* for example, species can be incorrectly identified due to variability in the characters used in species recognition (Hebert *et al*., 2003). On the other hand, morphological keys can often only be used effectively during certain developmental stages of the plants and these keys are often difficult to use, such that an inexperienced person may incorrectly identify a species (Hebert *et al*., 2003).

The diversity of life, as measured by numbers of species, is confounding and taxonomists could take decades to describe the estimated 10 million–15 million species and henceforth a major setback in taxonomic revisions (Von Staden et al., 2013). Nonetheless, the use of molecular techniques has been considered as a shortcut that would speed up species identifications and as a way to accelerate the discovery of new species (Rubinoff et al., 2006; Pires, 2010). As a result, the application of these DNA-assessment tools during diversity assessment would facilitate and complement descriptive taxonomic study and also assist in solving the crisis currently experienced with biodiversity assessments (DeSalle and Amato, 2004).

Bulbinella species have major gaps in their biodiversity assessments and very little molecular data is available for this genus. In this study, the aim was to evaluate the efficacy of different genes regions and individual genes prominently different from the genome as tools to augment morphological species discrimination within *Bulbinella* species. Specifically, the potential of four gene regions (*ITS, rbcL, matK* and *psbA-trnH*) were assessed, all of which have different mutation rates and which have a different number of mutation sites to discriminate South African species from New Zealand species. The objectives were to find a region with enough mutation sites to distinguish between the different species. The study will make a large contribution to the International Barcode of Life (iBOL) initiative and will provide a reference database for the identification of species in the genera under investigation.

5.2.1: Chloroplast Genome sequencing

Relying exclusively on descriptive taxonomy has problems of its own. Firstly, the productivity of taxonomist in South Africa has decreased, while the need for biodiversity assessment and conservation has increased at a greatly accelerated pace (von Staden *et al*., 2013). With the slow pace of current taxonomic efforts, taxonomic revisions may take centuries to complete (Wilson, 2003). Secondly, in many taxa, it is difficult to catalogue variation at lower taxonomic levels and diversity at levels below that of species is often neglected (Smith *et al*., 2005). Thirdly, it is now clear in many plant species that a single genome sequence does not certainly provide a better understanding of the phylogenetic relationships (Liu, 2015) and it is therefore, imperative to use more than one genome sequence to obtain a better inference.

Regarding the above, to enrich our sureness in the subsequent evolutionary hypotheses, the arrival of Illumina sequencing has significantly enhanced phylogenetic analyses (Givnish *et al*., 2010; Steele *et al*., 2012; 147 Xi *et al*., 2012). These advanced high throughput tools have the advantage of permitting faster (Smith *et al*., 2005) more detailed and accurate assessments of biodiversity (Bickford *et al*., 2007; Valentiniet *et al*., 2009; Young *et al*., 2013), and will also offer an alternative set of characters to contribute in deducing species boundaries throughout future taxonomic studies (DeSalle and Amato, 2004; Smith, 2005).

There was inadequate information in the use of multiple DNA fragments specially to deliver the high-resolution needed to discriminate closely related taxa, mainly some within-species taxa whose taxonomic relationships were unclear (Jansen, 2007). Nonetheless, with chloroplast genome analysis, sequences are valuable for decoding phylogenetic relationships amongst closely related taxa and for refining our understanding of the evolution of plant species (Jansen, 2007). The complete chloroplast genome has many applications such as assisting in phylogenetic studies at low taxonomic levels, population studies, phylogeographic studies (Stull *et al*., 2013). Since the current two loci chloroplast barcode for plants has 72% identification success at the species level, it is evident that whole chloroplast genome sequencing has the potential to be more efficient in discriminating between plants than DNA-barcodes (Parks *et al*., 2009; Singh, 2012).

Chloroplast DNA has been used extensively to infer plant phylogenies relationships at different taxonomic levels (Gielly and Taberlet, 1994; Small *et al*., 2004; Singh *et al*., 2012; Usama, 2015). Chloroplast DNA is an essential new tool for the reconstruction of plant phylogenies between closely related species (Small *et al*., 2004; Shaw *et al*., 2007; Dong *et al*., 2012). Even though South Africa and New Zealand has the richest flora, relatively few *Bulbinella* plant species have been sequenced, only the few sequence been determined for example *Bulbinella caudafelis* and *Bulbinella nana by* Chase *et al*., (2000); Treutlein *et al*., (2003) respectively. Chloroplast genome sequences were used to trace the phylogenetic relationships *Bulbinella* species.

According to Costa, (2010), the power of molecular data to elucidate this phenomenon has become particularly evident with the completion of whole-genome projects. Sequencing of the plastid genome is facilitated by rapid advances in Next-Generation Sequencing (NGS) technologies (Moore *et al*., 2006, 2007, 2010; Cronn *et al*., 2008, 2012; Stull, 2013). The advent of next-generation sequencing technologies has permitted the fast and efficient growth of new genomic resources for plant species (Claros, 2012; Goodstein *et al*., 2012). With its simple structure, highly conserved regions and being small, the plastid genome is consequently ideal for next-generation sequencing and assembly (Parks *et al*., 2009; Steele and Pires, 2011).

With the cost of whole chloroplast sequencing decreasing and with the improvement of bioinformatics programs, this field of research for biodiversity assessment has the potential to expand (Steele and Pires, 2011). According to Huang *et al*. (2013), the number of chloroplast genomes sequenced has increased rapidly, currently with 324 complete chloroplast genomes in the Complete Organelle Genome Sequences Database (http://amoebidia.bcm.umontreal.ca/pg-gobase/complete_genome/ogmp. html). This is as a result of improvement in next-generation technology (Nock *et al*., 2014).

The application of NGS and analyses of whole chloroplast genome data to assess biodiversity has not been extensively explored in the past. Stull *et al*. (2013), predicted that the high-throughput approach ought to advance large-scale plastid genome sequencing at any given level of phylogenetic diversity in angiosperms. Biodiversity assessment relies on an in-depth study of at least five samples per taxa. Analysing this number of whole chloroplast genomes per taxa in the genus under investigation (*Bulbinella*) was a daunting task and not feasible. Furthermore, the NGS facilities at the University of the Free State can accommodate 15 gigabase pairs per run. Correspondingly, for this study, it was not highly feasible to sequence whole chloroplast genomes of all species under investigation but we generated some draft genomes for *Bulbinella* species.

According to Claros *et al*., (2012) NGS is arguably becoming the new sequencing standard as it simplifies the sequencing process (no cloning), low cost (miniaturization) and good adaptation to a broad range of biological phenomena (genetic variation). The widespread espousal of NGS technology has facilitated the comprehensive analysis of genomes (Claros, 2012), and opens new research (Kumar *et al*., 2014). The boost up in plant sequence data has also incited the expansion of the plant family databases (repositories) for genome data (Wegrzyn *et al*., 2008 Kumar, 2014). At low cost without a weighty laboratory protocol, the NGS schemes permit a single template molecule to be directly used to generate millions of bases (Claros *et al*., 2012).

There are three pre-eminent technologies widely used nowadays which are the Genome Sequencer (FLX+/454), Genome Analyzer (Illumina), and SOLiD (Applied

Biosystems) for second-generation sequencing (Claros *et al*., 2012). The 454 sequencing is a pyrosequencing-based method that utilises emulsion PCR to achieve high throughput, parallel sequencing (Shulaev *et al*., 2011). On the other hand, the Solexa's sequencing-by-synthesis approach is based on a simplified library construction method (Mondini, 2009; Bento *et al*., 2011). Supported oligonucleotide ligation and detection (SOLiD) sequencing, contrasting the other two technologies, uses ligation-based sequencing technology (Heslop-Harrison, 2000; Mondini *et al*., 2009).

These three platforms arrange for the paired-end sequencing technique (Claros *et al*., 2012) and their approaches are well suitable to whole genome resequencing. Hence a novel genome sequence can be assembled and then compared to a reference sequence that is when the genome sequence of the species already exists (Claros *et al*., 2012). The paired-end sequencing technique enable large plant genomes to be sequenced on relatively inexpensive deep coverage with paired-end libraries from 1 to 5 kbp (Shendure and Ji, 2008; Mardis, 2008; Ansorge, 2009; Kircher and Kelso, 2010; Zhou, 2010; Niedringhaus *et al*., 2011; Pareek *et al*., 2011).

The short-read technologies recompense the shortness of the sequences with a high coverage so that bacteria can be successfully sequenced with 40 ×50 × coverage (Alkan *et al*., 2011; Barthelson, 2011; Claros, 2012; Finotello *et al*., 2012). The 454 sequencing, on the other hand, with longer read lengths can also be used for obtaining the first glimpse of a species' genome or transcriptome (Mondini *et al*., 2009; Sirokov, 2014; Lu and Xu, 2014). The long-read technologies do not need such deep coverage, with 20×30× being enough for a good compromise between costs and assembly quality (Finotello *et al*., 2012).

5.3: Bioinformatics Analyses of Genome Data

5.3.1: Data quality-trimming and filtering

Illumina sequencing produced a high number of paired-end reads that passed filtering and quality control for each taxon **(Table 5.3)**. After quality trim, the mean coverage of raw reads for each *Bulbinella* species in each alignment, the total number of raw reads for all taxa, reads filtered out against chloroplast database mean and coverage plastid protein-coding are listed **Table 5.3**. The sequencing data assessed using FastQC (http://bioinformatics.babraham.ac.uk/projects/fastqc/) showed a completely normal distribution of GC content indicating that there was no contamination in the library **(Fig 31)**. The Quality score distribution graph **(Fig 32)** shows high quality scoresfor all sequences and the average quality per read was 34, which is above the minimal standard score of 20 (refer to Chapter 3; 3.3.1). There were no uncalled bases in the library and this indicates there was no contaminant in the library **(Fig 33)**.

The quality score graph **(Fig 34)** displaying a summary of the range of quality values across all bases at each position in the FastQC file, indicated that the quality control we have performed to primarily check of the quantity and error rate of the sequencing data was within the acceptable range. In this study, the minimum score was 34 and thus well above the minimum required value of 20. The lower quartiles (yellow boxes) for all bases were higher than 5 and the median for all bases were more than 20. A low duplication level **(Fig 35)** was observed (sequence duplication levels are 0.2%) possibly indicating a very high level of coverage of the target sequence.

Table 5.2: South Africa Raw data information for each of the alignments used in phylogenetic analysis.

Taxa	Raw reads	Reads filtered out against chloroplast database	Mean coverage plastid protein-coding genes
1. ***Bulbine latifolia*** **(Spies B002)**	30485176	1314534	400
2. ***Bulbinella chartacea*** **(863)**	25812	358	NA
3. ***Bulbinella latifolia*** **(860)**	21528824	259782	52
4. ***Bulbinella caudafelis*** **(9183)**	4317279	56067	40
5. ***Bulbinella cauda-felis*** **(9295)**	9380534	104362	36
6. ***Bulbinella cauda-felis (white cats tails)*** **(9192)**	36122148	316406	101
7. ***Bulbinella ciliolata*** **(872)**	24460030	183454	61
8. ***Bulbinella divaginata*** **(877)**	13004998	109356	25
9. ***Bulbinella eburniflora*** **(9184)**	6460906	76360	22
10. ***Bulbinella elegans*** **(9299)**	18217636	205822	61
11. ***Bulbinella elata*** **(9298)**	7309616	81786	22
12. ***Bulbinella erbuniflora*** **(9297)**	19847824	151604	46
13. ***Bulbinella gracillis*** **(873)**	37939540	518196	200
14. ***Bulbinella graminifolia*** **(9185)**	12112360	161626	53

15. *Bulbinella latifolia* var *graniticus* (9191)	30710412	343260	119
16. *Bulbinella nana* (879)	10758430	101552	30
17. *Bulbinella trinervis* (875)	24960504	181138	52
***18.* *Kniphofia praecox* (Spies 078)**	17447506	209212	44

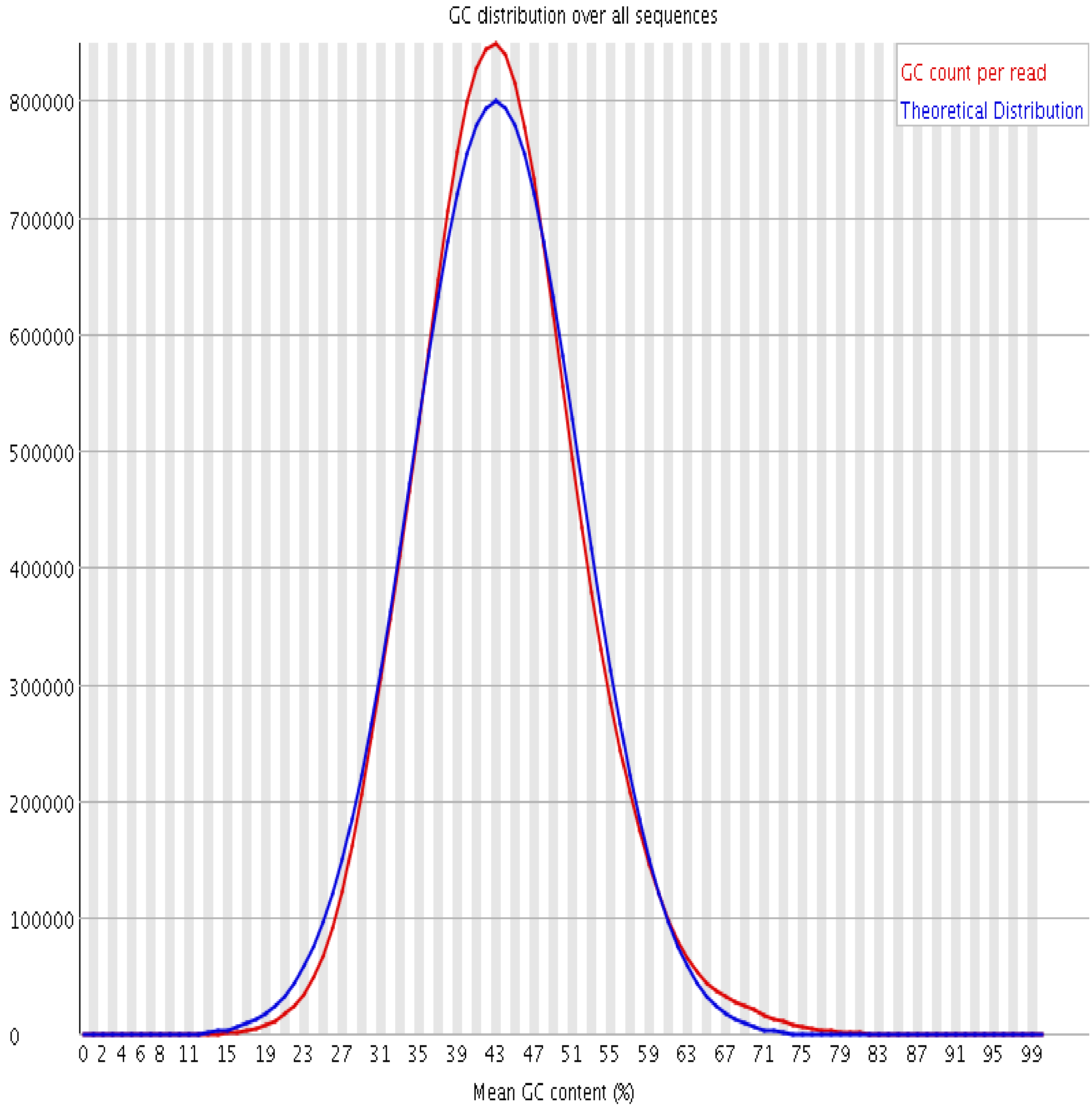

Figure 31: Quality graphs for all sequences: The red line is what the all the samples represent and the blue line is theoretical normal distribution.

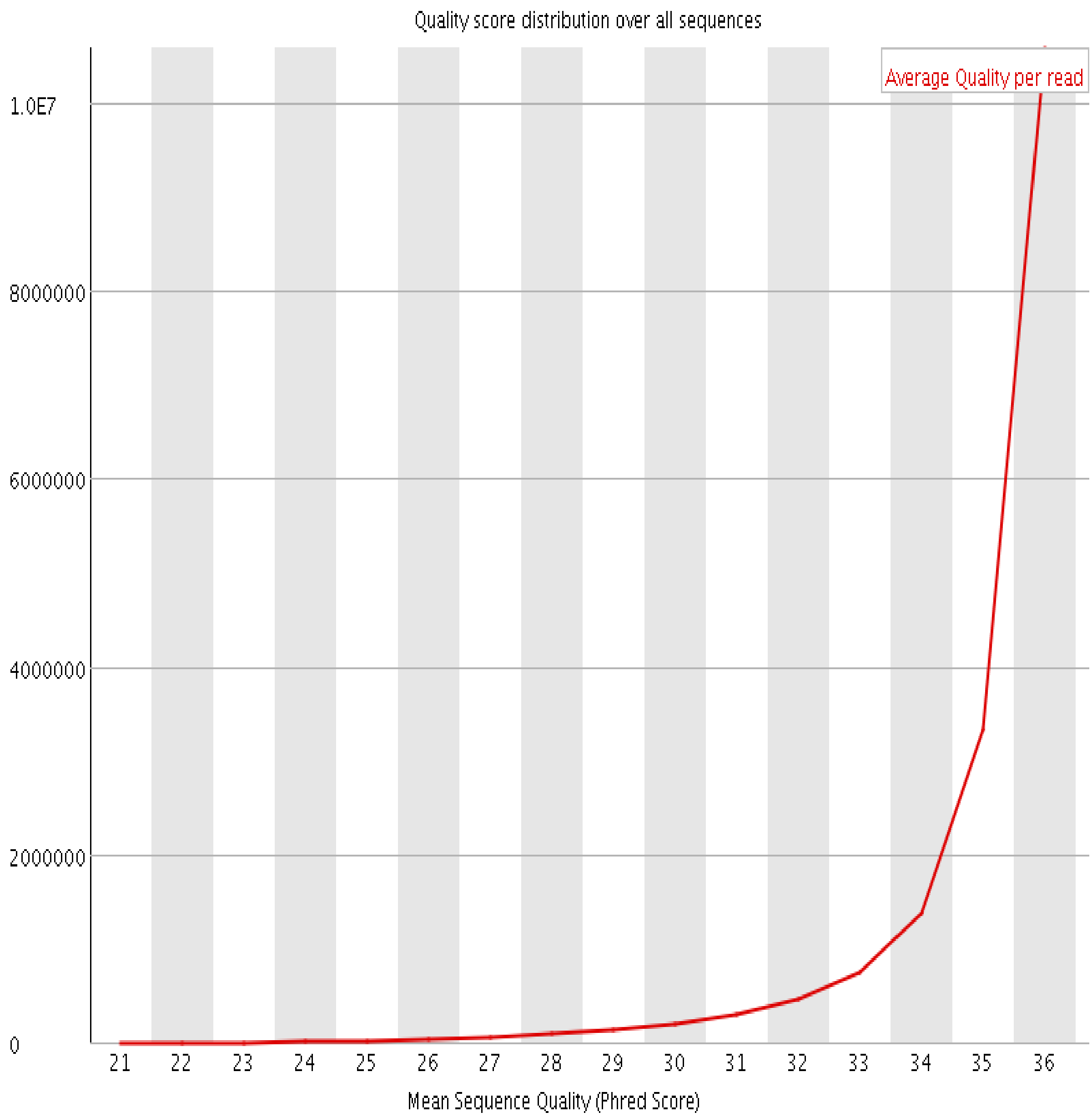

Figure 32: Quality Score distribution for all sequences: Plotting the distribution of this average quality where the y-axis shows the number of reads and the x-axis shows the mean quality score.

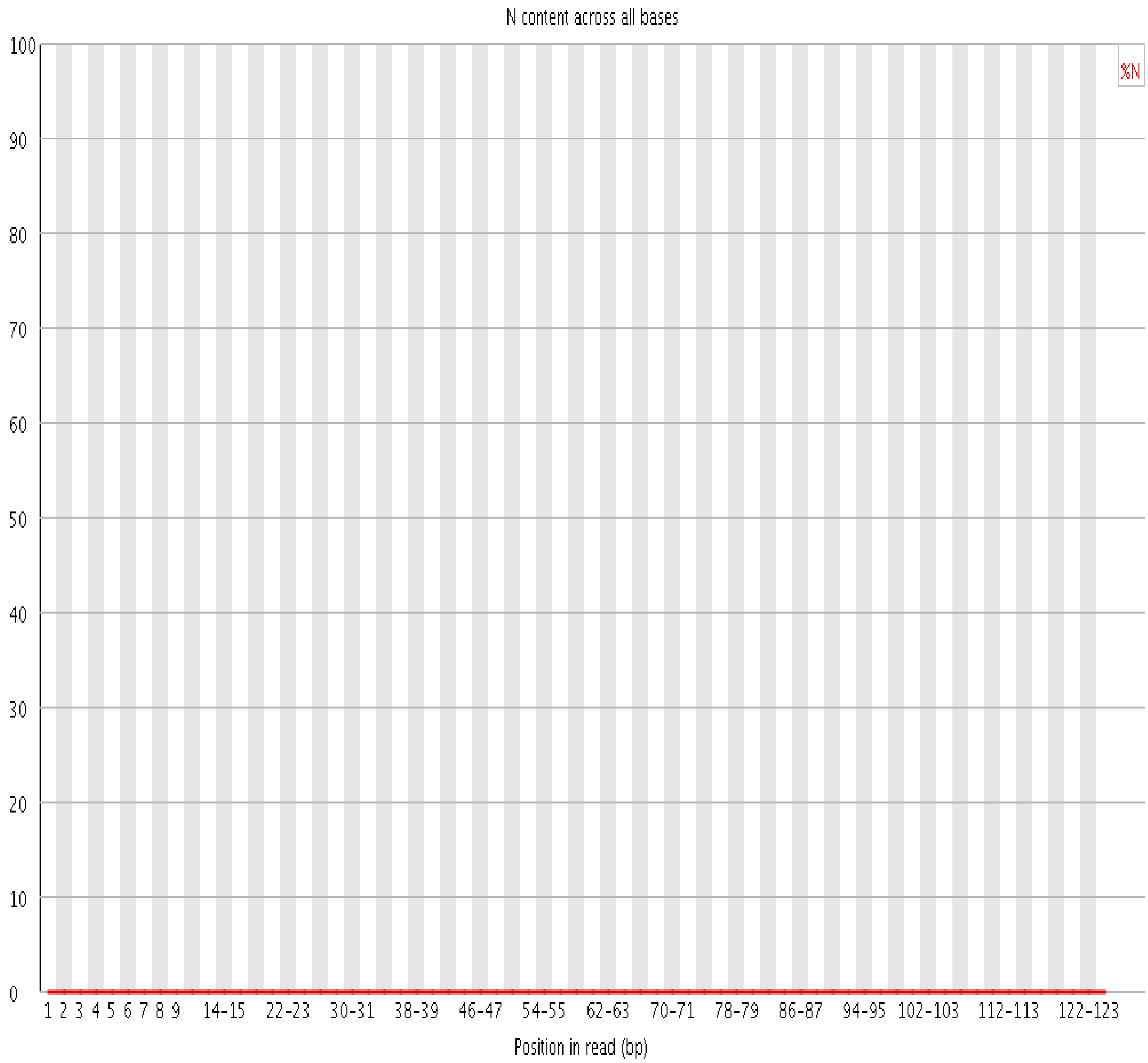

Figure 33: Percentage of base calls at each position for which an N was called.

If a sequencer is not capable to make a base call with sufficient confidence then it will routinely call an N rather than A, T, G or C. The y-axis displays percentage of Ns among all reads and the x-axis shows the read position. A very low percentage of Ns appearing near the end of a sequence is common and the percentage of Ns at each read position should be always lower than 20%.

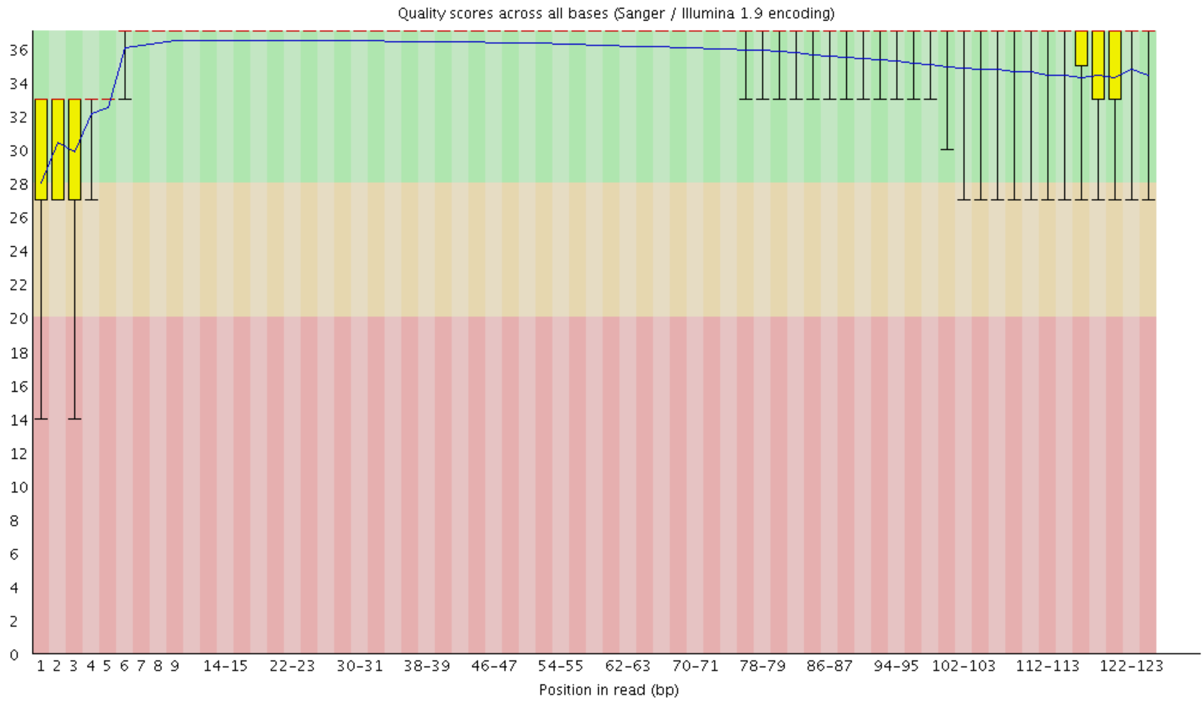

Figure 34: Quality scores across all sequences.

The Y-Axis on the graph shows the Quality Scores. The higher the score the better the base call. The central red line is the median value and the blue line is the mean quality score which should be generally high above 20 quality base score. The higher the score the better the base call. Mostly the quality of calls on most platforms will decrease as the run progresses. The yellow boxes represent quality scores for all bases within the inter-quartile range (25% - 75%). The colours used in the background of the graph divide the y axis into 3 quality groups, where green represents very good quality, orange represents reasonable quality, and red, poor quality.

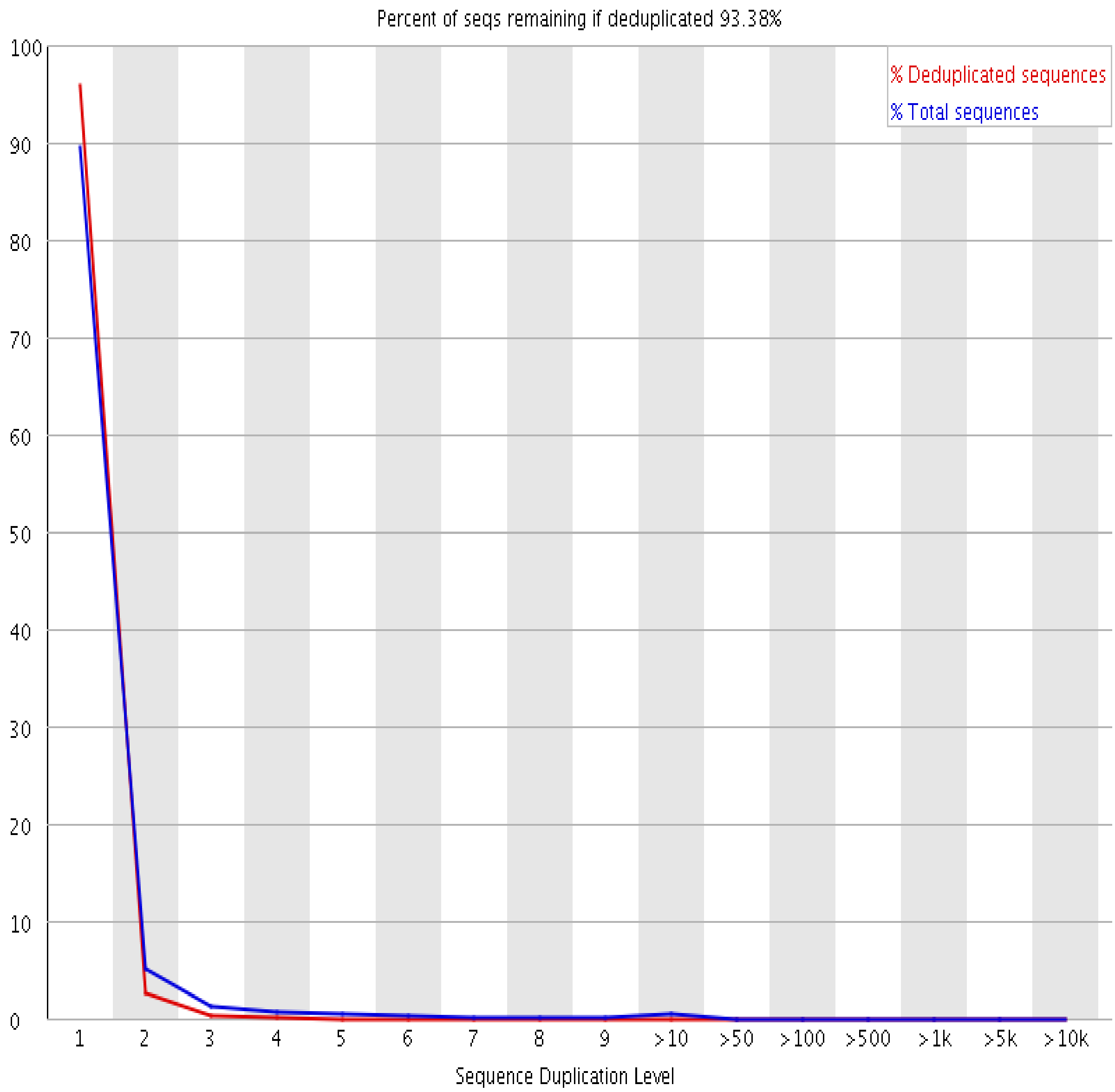

Figure 35: Sequence Duplication levels

The graph shows the number of sequences with different degrees of duplication (designated on the x-axis) relative to the number of unique sequences (which is set to 100%). The graph shows how many reads were represented once; the duplication level is 1 in the final set.

5.3.2: Chloroplast draft genome assembly and annotation.

The assemblies of samples for *Bulbinella graminifolia* (9185) and *Bulbinella gracillis* (873) had more than 120 genes. This number were close to the number of 120-130 for a chloroplast genome cited by Shaw *et al*. (2007) and were thus the most complete genomes out of the 34 samples. Based on their annotations, the genome data of the remaining samples were analysed, which were more incomplete. A number of protein coding genes were annotated and these were categorised into five groups according to functionality **(Table 5.3)**. The first group were associated with photosynthesis, and comprised of photosystem I and II, cytochrome b6/f complex, ATP synthase, the Calvin cycle and C-type cytochrome related genes. The second group encompassed all chlororespiration-associated genes for the synthesis of the NADH-dehydrogenase complex, while the third group involved transcription, splicing and translation. The fourth and fifth group included genes for metabolic pathway regulation and pseudogenes with unknown function, respectively.

Table 5.3. Gene composition of *Bulbinella* chloroplast genomes.

Groups	Functional system	Gene names
Photosynthesis	Photosystem I	*psaA, psaB, psaC, psaI, psaJ, ycf3, ycf4*
	Photosystem II	*psbA, psbB, psbC, psbD, psbE, psbF, psbG, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT*
	Cytochromeb6/f complex	*petA, petB, petD, petG, petL, petN*
	ATP synthase	*atpA, atpB, atpE, atpF, atpH, atpI*
	Calvin clycle	*rbcL*
	C-type cytochrome synthesis	*ccsA*
Chlororespiration	NADH oxidoreductase	*ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK*
Expression machinery	RNA polymerase	*rpoA, rpoB, rpoC1, rpoC2*
	Ribosomal large subunit	*rpl14, rpl16, rpl2, rpl20, rpl22, rpl23, rpl32, rpl33, rpl36*
	Ribosomal small subunit	*rps2, rps3, rps4, rps7, rps8, rps11, rps12, rps14, rps15, rps16, rps18, rps19*
	Maturase k	*matK*
Metabolic pathways	Acetyl-CoA carboxylase carboxyltransferase	*accD*
	Clp protease proteolytic subunit	*clpP*
	Chloroplast envelope membrane protein	*cemA*
Pseudogenes	Unknown functions	*ycf2, ycf15, ycf68, orf42, orf56, orf188.*

5.3.3: Phylogenetic Analyses

To aid with relationship definition of the South African *Bulbinella* species, phylogenomic analyses based on 34 protein-coding genes **(Table 5.4)** from 16 specimens were done. The corresponding genomic data for *Bulbine latifolia* (B002) and *Kniphofia praecox* (Spies 078) were used for outgroups. The 34 gene combined data matrix (*atpA, atpF, atpI, ndhI, psbI, ndhH, ndhF, rps16, rbcL, rpl2, rpl23, rpoC1, rpoC2, rps7, rps1.5, rps19, rps2, rps7, matK, ndhE, ndhB, ndhA, ccsA, atpH, orf42, orf56, psaC, rps12, ycf15, ycf68, psbA, rpoB* and *ndhD)* included 42 014 aligned nucleotide positions and the T92model was fitted to the analysis **(Table 5.4)**.

Analyses on the individual genes were also done and descriptions and phylogenetic trees can be found in Appendix (1 up to 34). Bayesian Inference analyses, using a best fit model for each gene **(Table 5.4)** and a partitioned analysis employing nine different models **(Table 5.4)** generated identical tree topologies with very similar posterior probabilities (PP) at each node. Each analysis resulted in one fully resolved tree **(Fig. 36)**. Overall, support for monophyly of most clades was strongly supported by both methods BI and ML.

TABLE 5.4: The Akaike Information Criterion (AIC) in JMODELTEST V.2.1

GENE	MODEL	Sub models finals
AtpA	T92+G+I	6- invgamma
NdhF	HKY+G	2-gamma
ndhH, rps16; rbcL	T92+G	6-gamma
atpF, atpI, ndhI; psbI; rpl23; rpoC1; rpoC2; rps7 rps1,5 rps19, rps2; rps7; matK; ndhE; ndhB; ndhA; ccsA	T92	6- invgamma
atpH; orf42; orf56; psaC; rps 12; ycf15; ycf68	JC	1- invgamma
PsbA	TN93+G	6- gamma
rpoB, ndhD	HKY	2- equal
rpl2	K2	6- invgamma
ycf 2	GTR+G	6- gamma
atpF, atpI, ndhI; psbI; rpl23; rpoC1; rpoC2; rps7 rps1,5 rps19, rps2; rps7; matK; ndhE; ndhB; ndhA; ccsA atpH; orf42; orf56; psaC; rps 12; ycf15; ycf68, PsbA, rpoB **and** ***ndhD, rpl2 ycf 2, ndhH, rps16; rbcL, ndhF, AtpA***	T92	6- invgamma

5.3.4: Combined analysis of all 34 genes

Three groupings could be seen in the phylogenetic tree. The first clade was based on strong support from Bayesian posterior probability of (PP=1.00) and a strong bootstrap support (BS=100%) respectively **(Fig 36)**, and include specimens *B. graminifolia* seeds (9185), *B. erbuniflora* (9297) and *B. caudafelis* (9295 & 9183). The second clade was based on strong statistical support (BS=95.9%; PP=1.00) and included *B. gracillis* (873), *B. trinervis* (875), *B. divaginata* (877), *B. erbuniflora* (9184), *B. ciliolata* (872), *B. latifolia* var. *granitus*, *B. caudafelis* (9192) specimen and *B. elegans* (9299). The third clade consisted of *B. latifolia* (860), *Be. latifolia* (Spies 002) and *K. praecox* (Spies 078) (BS=100%; PP=1.00) and is separated from the ingroup **(Fig 36)**. *Bulbinella elata* grouped on its own basally to clade 1. In the combined analyses, data were partitioned by the gene with model parameters unlinked across partitions.

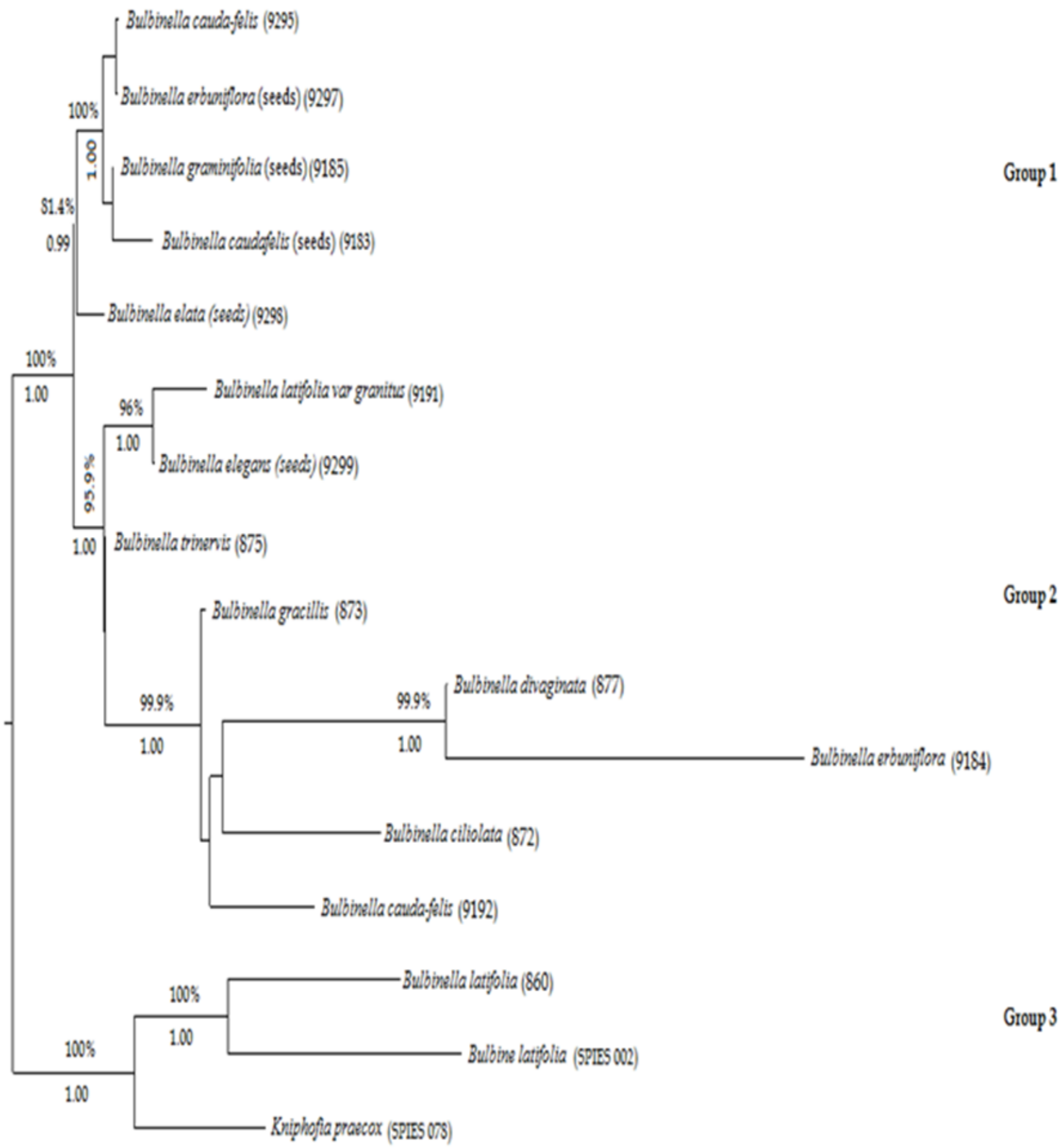

Figure 36: Phylogram based on sequence analysis of 34 chloroplast genes from 14 *Bulbinella* species. Maximum likelihood bootstrap (MLB) >50% are indicated above branches and Bayesian posterior probability values >0.5 are shown below the branches. (* depicts MLB and PB values <50%). *Kniphofia praecox* and *Bulbine latifolia* were presented as outgroups.

5.3.5: General conclusions from phylogenomic analyses

General trends observed in the previous phylogenetic analyses based on chloroplast and nuclear genes were confirmed in the phylogenomic approach. However, the phylogenomic tree had much better support for the branches than the four gene approach. The three general groups in which South African species of *Bulbinella* grouped **(Fig. 36)** could be observed again, with the same general species composition. The first strongly supported group consisted of *B. caudafelis* (9183; 9295), *B. graminifolia* (9185) and one specimen of *B. erbuniflora* (9297). However, contrary to the multigene approach, (9148) of *B. erbuniflora* specimen grouped in the other South African lineage. The second group consisted of *B. trinervis*, *B. gracillis*, *B. divaginata*, and *B. ciliolata*, which also grouped together in previous analyses. However, in the phylogenomic approach, these species were clearly distinguishable. The third group again consisted of the basal grouping of *B. latifolia* (860) with the outgroups *Bulbine* and *Kniphofia.*

A number of specimens for a species grouped separately, similar to what was found previously. Three specimens of *B. caudafelis* (9295; 9183; 9192) grouped distinctly, while a *B. latifolia* var. *granitus* specimen grouped inside *Bulbinella* separately from the basal grouping of *B. latifolia* (860). Different from previous analyses, the *B. erbuniflora* (9148) clade 2 grouped in the other group than specimen 9297 clade 1.

Based on the genome data, the results suggested that the following genes were complete and could be used to distinguish *Bulbinella* species. These included *atpA; atpF, atpI, rbcl, ndhI; ndhH, ndhF; rpl2; rpoC1; rpoC2; rps15, orf188, rps2; matK; ndhE; ndhG; ccsA, psaC; ycf2, psbA, rpoB* and *ndhD*. All the above genes can be used on their own or they may be combined. Based on the higher support values and distances observed, the closely related species in Group 2 that could not previously be distinguished were now easily differentiated.

CHAPTER 6: GENERAL CONCLUSIONS

Bulbinella is known for its horticultural importance and other applications by humans. Such uses, for instance, include livestock feed and herbal remedies for ailments caused by bacterial and fungal infections due to a range of phenylanthraquinones (Bringmann *et al.,* 2008; Richardson *et al.*, 2017; Musara *et al.,* 2017). In spite of its ethno medicinal value, the species relationships and complexes are poorly understood due to morphological homogeneity and not much scholarly attention has been given to these aspects (Von Staden *et al.,* 2013). Since then there has been no update on the systematics of the genus. This study was vital to address the urgent need for revision of *Bulbinella* in South Africa and New Zealand.

The study represents the first to extensively sequence species within *Bulbinella* with the purpose of characterizing the phylogenetic relationships within the genus and to develop additional tools to aid in their identification and conservation. It also investigated the relationship of New Zealand species with South African species, since there exists such a large biogeographic gap. A large number of gene data has been generated for the first time and revealed a number of useful genes that can be used to delimit and characterize species from the genus *Bulbinella*. It also showed that the New Zealand species are indeed *Bulbinella* and do not represent anything distinct.

Besides sequencing three plastid genes and one nuclear gene, a phylogenomic approach of the chloroplast genome was also followed to generate a large number of genes quickly. These genes were used to supplement phylogenetic analyses at a much larger scale. Thirty-four genes were used in the phylogenomic approach, and these genes significantly improved statistical support for the topology of the final phylogenetic tree. It aided in resolving the relationships of species that appeared to be synonymous based on the four gene analyses. However, in general the topology of the phylogenomic tree was similar to those obtained by the initial four genes sequenced, thus strengthening the species hypothesis obtained initially. It also aided in identifying additional genes besides the initial four that could individually be used in future phylogenetic studies, thus negating the need to generate genomic data every time.

The New Zealand *Bulbinella* species (*B. rossii, B. gibbii, B. hookerii, B. modesta, B. angustifolia*) represents a disjunct remnant lineage of *Bulbinella*. They are in no way connected geographically to the South African species. It is logical to assume that these isolated species could have possibly evolved into their own genus. Sequencing results from this study, however, unequivocally showed that the New Zealand species still are nested within *Bulbinella* although they form a constant and distinct group of their own. Further studies into the origin of these species compared to the South African species, that could possibly be related to ancient tectonic plate movement or other means of natural or possibly anthropogenic spread, would be interesting.

Results from the four gene analysis showed that the New Zealand species, especially *B. rossii, B. gibbii* and *B. hookeri*, are still very closely related despite morphological differences. The New Zealand species do not share geographic localities (Moore, 1964; Moore and Edgar, 1970; Milicich, 1993), while *B. hookeri* occur in both the Northern and Southern islands of New Zealand. The DNA sequence data could distinguish between *B. angustifolia* and *B. modesta*, but indicated that *B. gibbii* from different localities, and its variants, *B. rossii* and its variants, and *B. hookeri* are so closely related and appear to be conspecific. This is despite distinct morphological differences such as hermaphroditic and gynandromorphy, shorter and longer racemes, erect and flat leaves. Follow up studies using the additional genes developed in the phylogenomic study would proof useful to investigate this further.

A number of South African species appeared to be distinct based on current taxonomy while a small group were very closely related. These included *B. ciliolata, B. divaginata, B. gracillis, B. nutans* and *B. trinervis*. *B. elegans* groups closely while *B. triquetra* also occasionally grouped with these species. In fact, based on the four gene analysis *B. divaginata, B. gracillis, B. nutans* and *B. trinervis* appear to be closely related. These species were, however, distinct based on the phylogenomic analysis. Yet their close grouping based on genes routinely used for species delimitations in plants are curious. These four species also differ morphologically for example *B. nutans* have white flowers while the other species have yellow flowers, therefore differences in the shape of the inflorescences and the distinguishing feature of *B. gracillis* is the lack of sheathing fibres. This, together with the same close grouping of morphologically distinct species in the New Zealand group, indicates that these species most likely are still genetically very closely related based on the genes used, while morphological features appear to evolve more rapidly, and to occur with a measure of plasticity.

Comparisons of morphology with the phylogenetic groupings already showed that some of the clades do not have similar morphology in common. These include the New Zealand clade and the clade of *B. ciliolata, B. divaginata, B. gracillis, B. nutans* and *B. trinervis*. However, one other South African clade that consistently grouped together consisted of specimens of *B. caudafelis, B. graminifolia* and *B. eburniflora*. These species all have white stellate flowers, narrowly cylindrical inflorescence and they possess fibrous sheathing necks which are thin, loose, straight somewhat reticulate towards the inside (Perry, 1999).

A number of species represented by more than one sample, did not group into a single phylogenetic group. For instance, specimens of *B. caudafellis, B. eburnifolia, B. nutans* and *B. latifolia* grouped either individually or with other speceis. These could possibly be due to misidentifications because of the variable morphology of the species, or it could reflect that a number of species are paraphyletic. What was interesting to note was that the additional specimens of these species usually grouped on their own, indicating that the specimens were not mistaken as another species, but represents additional species that could possibly be new or cryptic. These cryptic species could also be indicative of hybridization occurring, giving rise to new morphotypes and genotypes. These multiple groupings of certain species should be taken into account in future surveys of *Bulbinella*, to ensure that these additional groupings can be accurately studied. Furthermore, it will have to be ascertained which of the sequences truly represents the species, and which does not. Careful morphological studies against type specimens will have to carried out towards this end.

The majority of specimens sequenced in this study originated from vouchered field collections. However, a number was obtained as seed. In some cases, the seed and plant samples did not correspond in the phylogenetic analyses e.g. *B. eburniflora, B. nutans, B. graminifolia*. This raises an important point in that the identity of seeds should be carefully verified by the collectors. Based on the polyphyletic grouping of some species observed in this study, it may be difficult and problematic since it will first have to be determined to which genotype the collection belongs to. This highlights the importance of this study that provided the foundation against which seed and field collections can be verified based on DNA sequence data. This should be invaluable to breeders, horticulturists and conservationists.

A specimen of *B. latifolia* and a *B. latifolia* sequence from GenBank (Ramdhani *et al*., 2006) consistently grouped outside the general clade representing *Bulbinella*. It often grouped with the *Bulbine* species used as outgroup, but the inclusion of more *Kniphofia* and *Bulbine* species in the phylogenetic analyses showed that the grouping varied, albeit always basal to *Bulbinella*. A specimen of another species, *B. nana*, also grouped in this manner. *Bulbinella latifolia* is the only *Bulbinella* species with orange flowers, its leaves are triangular-lanceolate, reduced or absent in outer leaves (squamae) and *B. nana* has erect leaves and narrow, broader inflorescences, with yellow flowers and lack sheathing fibres. Accordingly, there are no morphologically features that could suggest that these two species are not *Bulbinella* and there is also a likelihood that these species could have been mistakenly collected or wrongly identified but represent *Bulbine* species.

Bulbinella represents a distinct genus separate from *Bulbine* and *Kniphofia*. This has been confirmed in the phylogenetic analyses. *Kniphofia* also consistently grouped separately based on the limited number of sequences used in the study. However, the position of *Kniphofia* and *Bulbine* differed compared to the *B. latifolia* and *B. nana* sequences generated in our study. This may indicate that the generic positions of these genera still need to be solidified. It is also not clear if the specimens of *B. latifolia* and *B. nana* represent a previously unrecognized genus or are they possibly part of *Bulbine*. The use of more species, representative sequences and expansion of the gene set to those developed in the phylogenomic approach, should delimit the generic boundaries of these plants.

Results from this project provided an important indication of the complexity of the systematics of *Bulbinella* and related genera. This has been referred to in previous studies (Perry, 1999; Bringmann, 2008; Klopper *et al*., 2010). Our results were vital to indicate a number of aspects still awaiting elucidation. Whereas certain species appear to be solid, the polyphyletic grouping of others questions the position of each species. The very close relationship of some species that are nonetheless morphologically distinct will have to be investigated further by also ensuring multiple representatives of each species and cryptic grouping. Morphologically variable species will also have to be represented by all of the variations. By such thorough treatment a robust system of identification can be developed. Moreover, it will also aid in the verification of the generic positions of *Kniphofia* and *Bulbine*, and the basal grouping of *B. nana* and *B. latifolia*. The arsenal of phylogenetically informative genes developed in this study, would be invaluable.

CHAPTER 7: REFERENCES

Abegaz, B.M., Bezabih, M., Msuta, T., Brun, R., Menche, D., Mühlbacher, J. and Bringmann, G. (2002). Gaboroquinones A and B and 4′-O-demethylknipholone-4'-O-Beta-D-glucopyranoside, phenylanthraquinones from the roots of *Bulbine frutescens*. *Journal of Natural Products*. **65**:1117-1121.

Afolayan, A.J. and Yakubu, M.T. (2009). Effect of *Bulbine natalensis* Baker stem extract on the functional indices and histology of the liver and kidney of male Wistar rats. *Journal of Medicinal Food.* **12**(4): 814-820.

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*. **19**(6): 716–723.

Akerele, O. (1993). Summary of World Health Organization guidelines for the assessment of herbal medicines. *Herbalgram*. **28**:13-16.

Alam, A., Mudassar, I. and Sharad, V. (2013). Cultivation of some overlooked geophytes ornamentals- A review on its commercial viability. *Report and Opinion.* **5** (3): 9-34.

Alasbahi, R. and Melzig, M.F. (2007). Screening of some Yemeni medicinal plants for inhibitory activity against neutral endopeptidase. *Planta Medica*. 73: 451.

Albu, M., Nikbakht, H., Hajibabaei, M. and Hickey, D.A. (2011). The DNA Barcode Linker. *Molecular Ecology Resources.* **11**: 84–88.

Ali, M.A., Ga´ bor G., Norbert, H., Bala´zs, K., Fahad, M.A., Al, H., Arun, K.P. and Joongku L. (2014). The changing epitome of species identification - DNA barcoding. *Saudi Journal of Biological Sciences*. **21**: 204-231.

Alkan, C., Sajjadian, S. and Eichler, E.E. (2011). Limitations of next-generation genome sequence assembly. *Nature Methods*. **8**: 61-65.

Amos, W. and Balmford, A. (2001). When does conservation genetics matter? *Heredity.* **87**: 257-265. CrossRef, PubMed.

Ansorge, W.J. (2009). Next-generation DNA sequencing techniques. *New Biotechnology*. **25**(4): 195–203.

Antofie, M. (2011). "Current political commitments' challenges for ex situ conservation of plant genetic resources for food and agriculture," Analele Universit˘at¸ii din Oradea-*Fascicula Biologie.* **18**: 157–163.

Arca, M., Hinsinger, D.D., Cruaud, C., Tillier, A. and Bousquet, J. (2012). Deciduous Trees and the Application of Universal DNA Barcodes: A Case Study on the Circumpolar Fraxinus. *PLoS ONE.* **7**(3).

Avise, J.C. (1989). A role for molecular genetics in the recognition and conservation of endangered species. *Trends in Ecology and Evolution*. (4): 279–281.

Baker, J.G. (1876). Revision of the genera and species of Anthericaceae and Eriospermaceae. *Journal of the Linnean Society*. **15**: 253–363.

Baker, J.G. (1872). Revision of the nomenclature and arrangement of the Cape Species of Anthericum, *Journal of Botany*. **10**:137-140.

Baker, J.G. (1896). *Bulbinella kunth*. Flora Capensis, 6: 335-358. Reeve, London.

Bangert, R.K., Turek, R.J., Martinsen, G.D., Wimp, G.M., Bailey, J.K. and Whitham, T.G. (2005). Benefits of conservation of plant genetic diversity to arthropod diversity. *Conservation Biology*. **19:** 379– 390.

Bankevich, A., (2012). SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*. **19:** 455–477.

Barker, M.S. and Wolf, P.G. (2010). Unfurling Fern Biology in the Genomics Age. *BioScience* **60**(3): 177–185.

Barnes, J.E., Turton L.M and Kalake, E. (1994). A List of Flowering Plants of Botswana. *The Botswana Society and the National Museum*, Monuments and Art, Gaborone: 46–47.

Barnard R.O. and Newby, T.E. (1999). Sustainability of terrestrial ecosystems. In: National state of environment report. Pretoria: *Department of Environmental Affairs and Tourism.* Available at http://www.environment.gov.za/soer/issues/land/index.htm.2/2/2014

Barthelson, R. (2011). Plant agora: modelling whole genome sequencing and assembly of plant genomes. *PLoS One*. **6**: e28436.

Batugal, P., Ramanatha, R.V. and Oliver, J. (2005). Coconut Genetic Resources. *International Plant Genetic Resources Institute*- Regional Office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. and Courchamp, F. (2012). Impacts of Climate Change on the future of biodiversity. *Ecology Letters*. **15**(4):365-77.

Bento, M., Gustafson, J.P., Viegas, W. and Silva, M. (2011). Size matters in Triticeae polyploids: Larger genomes have higher remodelling. *Genome.* **54**: 175-183.

Berhanu, E., Fetene, M. and Dagne, E. (1986). Anthraquinones as taxonomic markers in Ethiopian *Kniphofia* species. *Phytochemistry.* **25**: 847–850.

Berjak P., Sershen., Varghese, B., Pammenter N.W. (2011). Cathodic amelioration of the adverse effects of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant-seeded species. *Seed Science Research Journal*. **21**(187).

Bezabih, M., Motlhagodi, S. and Abegaz, B.M. (1997). Isofuranonaphthoquinones and phenolic and knipholone derivatives from the roots of *Bulbine capitata*. *Phytochemistry.* **46**: 1063-1067.

Bhattarai, K., Bushman, B.S., Johnson, D.A. and Carman, J.G. (2010). Phenotypic and genetic characterization of western prairie clover collections from the western United States. *Rangeland Ecology and Management. BioOne*. **63:** 696–706.

Bickford, D., Lohman, D., Sodhi, NS. NG, PKL. Meier, R., Winker, K., Ingram, K. and Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*. **22**: 148–155.

Bindroo, B.B. and Moorthy, S.M. (2014). Genetic Divergence, Implication of Diversity, and Conservation of Silkworm, *Bombyx mori*. *International Journal of Biodiversity*. Article ID 564850: 15 pp.

Boatwright, S. and Manning, J. (2012). *Bulbinella* Kunth. Compton Herbarium, *Kirstenbosch Research Centre March*. National Botanical Garden in Rhodes.

Bodley, E., Duncan, G. and Du, Plessis. (1989). Geophytes plants of Southern Africa. A guide to their cultivation and propagation. Cape Town. *Tafelberg Publishers*, Capetown, South Africa.

Bora, L. (2010). Principles of Paleobotany. Hardcover: 286 pages. *Mittal Publications*. New Dehli.

Borokini, T.I., Okere, A.U., Giwa, A.O., Daramola, B.O. and Odofin, W.T. (2010). Biodiversity and conservation of plant genetics resources in Field Gene bank of the National Centre for Genetic Resources and Biotechnology, Ibadan Nigeria. *International Journal of Biodiversity and Conservation* **2**(3): 37-50.

Bousquet, J., Strauss, S.H. and LI., P. (1992b). Complete congruence between morphological and *rbcL*-based molecular phylogenies in birches and related species (Betulaceae). *Molecular Biology and Evolution*. **9**:1076-1088.

Bowman, D.D. (2005). What's in a name? *Trends in Parasitology*. **21**:267–269.

Bringmann G, Mutanyatta-Comara, J., Knauera, M. and Abegazb, B.M. (2008). Knipholone and related 4-phenylanthraquinones: structurally, pharmacologically, and biosynthetically remarkable natural products. *Natural Products Report*. **25**: 696-718.

Briskie, J.V. and Mackintosh, M. (2004). Hatching failure increases with severity of population bottlenecks in birds. *Proceedings of the National Academy of Sciences.* **101**: 558-561.

Brooks, T. and Kennedy, E. (2004). Biodiversity barometers. *Nature.* **431**: 1046–1047.

Brown, T.A. (2002). Genomes 2nd edition. *BIOS Scientific Publishers*. EScholar ID: 4c28.

Bruhn, T. (2011). Sequence and analysis of the mitochondrial DNA control region of nine Australian species of the genus Chrysomya (*Diptera: Calliphoride*). Master of Sciences thesis, University of Wollongong, School of Biological sciences. http://ro.uow.edu.au/theses/3236.

Brummitt, R.K. (1992). Vascular plant families and genera. Richmond: *Royal Botanic Gardens, Kew.*

Bryan, J. and Griffiths, M. (Eds) (1995). The new Royal Horticultural Society dictionary: *manual of bulbs. Timber Press, Portland*.

Burner, D. (1997). Chromosome transmission and meiotic behaviour in various sugarcane crosses. *Journal of the American Society of Sugar Cane Technologists*. **17**: 38-50.

Caruccio N. (2011). Preparation of next-generation sequencing libraries using Nextera™ technology: simultaneous DNA fragmentation and adaptor tagging by in vitro transposition. *Methods in Molecular Biology*. **733**:241-55.

CBOL Plant Working Group. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences*, USA. **106**: 12794–12797.

Chase, M.W. & Hills, H.H. (1991). Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon*. **40**: 215–220.

Chase, M.W, Salamin N, Wilkinson, M, Dunwell, J.M, Kesanakurthi, R.P (2005). Land plants and DNA barcodes: short-term and long-term goals. *Philosophical Transactions of the Royal Society Biological Sciences.* **360**:1889–1895.

Chase, M. W., Cowan, R. S., Hollingsworth, P. M., Van den Berg, C., Madriñán, S., Petersen, G., Seberg, O., Jørgsensen, T., Cameron, K. M., Carine, M., Pedersen, N., Hedderson., T. A. J., Conrad, F., Salazar, G. A., Richardson, J. E., Hollingsworth, M. L., Barraclough, T. G., Kelly, L. and Wilkinson, M. (2007). A proposal for a standardised protocol to barcode all land plants. *Taxon.* **56**: 295-299.

Chase, M.W., Michaels, H. J., Scott, K. M., Olmstead, R.G., Szaro, T., Jansen, R.K. and Palmer, J.D. (1993). Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene *rbcL*. *Annals of the Missouri Botanical Garden.* **80**(3): 528–580.

Chase, M.W., Annette, Y.D., Anthony, V., Cox, G.R., Rudall, P.J., Johnson, M.A.T. and Eguiarte, LE. (2000). Phylogenetics of Asphodelaceae (Asparagales): An Analysis of Plastid *rbcL* and *Trnl-F* DNA Sequences. *Annals of Botany*. **86**(5): 935-951.

Chase, M.W., Reveal, J.L., Fay, M.F., (2009). A subfamilial classification for the expanded Asparagalean families *Amaryllidaceae, Asparagaceae* and *Xanthorrhoeaceae*. *Botanical Journal of Linnean Society*. 161:132-136.

Chen, S. L, Yao, H., Han, J.P., Liu, C., Song, J.Y. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE*. **5**: e8613.

Christelova, P., Hribova, E. and Cizkova, J. (2011). The *ITS1-5, 8 S-ITS2* Sequence regions in the *Musaceae*: structure, diversity and use of molecular in molecular phylogeny. *PLoS One* **6** (3): 17863.

CITES, (2007). Convention on International Trade in endangered species of wild fauna and flora. www.cites.org. 11 /5/2015.

Claros, M.G., Bautista, R. and GuerreroFernández, D. (2012). Why assembling plant genome sequences is so challenging. *Biology.* **1**: 439-459.

Clegg, M.T. and Durbin, M.L. (1990). Molecular approaches to the study of plant biosystematics. *Australian Journal for Systematic Botany*. **3**: 1-8.

Codd, L.E. (1968). The South African Species of *Kniphofia*". *Bothalia*. **9**:363-513.

Coetzer, W.G, Downs, C.T., Perrin, M.R., Willows, M.S. (2015). Molecular Systematics of the Cape Parrot (*Poicephalus robustus*): Implications for Taxonomy and Conservation. *PLoS ONE*. **10**(8): e0133376.

Conrad, F., Reeves, G. and Rourke, J.P. (2003). Phylogenetic relationships of the recently discovered species- *Clivia mirabilis*. *South African Journal of Botany*. **69**: 204-206.

Costa, F.O. Gary, R.C. (2010). Theory Biosci. New insights into molecular evolution: prospects from the Barcode of Life Initiative (BOLI). **129**:149–157.

Coyne, J.A, Orr, H.A. (2004). Speciation. *Sinauer*.

Cracraft, J. (2005). Phylogeny and evo-devo: characters, homology, and the historical analysis of the evolution of development. *Zoology*. **108**: 345-356.

Crawford, D.J. (2000). Plant macromolecular systematics in the past 50 years: One view. *Taxon*. **49**: 479-501.

Crisci, J.V. (2006). One-dimensional systematist: Perils in a time of steady progress. *Systematic Botany*. **31**:217-221.

Cromwell, M.K. and Anthony J.A., (2015). "Preliminary Phytochemical Screening and Biological Activities of *Bulbine abyssinica* Used in the Folk Medicine in the Eastern Cape Province, South Africa," *Evidence-Based Complementary and Alternative Medicine*, Article ID 617607, 12 pp.

Cronn, R., Knaus, B. J., Liston, A., Maughan, P.J., Parks, M., Syring, J.V. and Udall, J. (2012). Targeted enrichment strategies for next-generation plant biology. *American Journal of Botany*. **99:** 291–311.

Cronn, R., Liston A., Parks, M., Gernandt, D.S., Shen, R. and Mockler, T. (2008). Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. *Nucleic Acids Research.* **36**: e122.

Cunningham, A.B. (1988). An investigation of the herbal medicine trade in Natal/Kwazulu. University of Natal, Institute of Natural Resources. Investigational **Report 29**, Pietermaritzburg.

Dagne, E. and Steglich, W. (1984). Knipholone: A unique anthraquinone derivative from *Kniphofia foliosa*. *Phytochemistry.* **23**: 1729-1731.

Dagne, E. and Yenesew, A. (1994). Anthraquinones and chemotaxonomy of the Asphodelaceae. *Pure Applied Chemistry*. **66**: 2395-2398.

Dahlgren, R.M.T. and Clifford H.T. (1982): The monocotyledons: A comparative study. London: *Academic Press*.

Dahlgren, R.M.T., Clifford, H.T. and Yeao, P. F. (1985). The families of monocotyledons: structure, evolution and taxonomy. Berlin: *Springer*.

Daly, D.C., Cameron, K.M. and Stevenson, D.W. (2001). Plant Systematics in the Age of Genomics. Scientific Correspondence. American Society of Plant Biologists. *Plant Physiology*. 127.

Daubin, V., Gouy, M., & Perrière, G. (2002). A Phylogenomic Approach to Bacterial Phylogeny: Evidence of a Core of Genes Sharing a Common History. *Genome Research.* **12**(7), 1080–1090.

Darriba, D, Taboada, G.L, Doallo, R., Posada, D. (2012). JModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*. **9**(8): 772.

Daru, B. H., Manning J.C., Boatwright, J.S., Maurin, O., Maclean, N., Schaefer, H., Kuzmina, M. and van der Bank, M. (2013). Molecular and morphological analysis of subfamily Alooideae (Asphodelaceae) and the inclusion of *Chortolirion* in Aloe. Phylogeny of Alooidae. *Taxon.* **62** (1): 62-76.

Dayrat, B. (2005). Towards integrative taxonomy. Society of London, *Biological Journal of the Linnean Society*. **85**:407–415.

Debela, H. (2007). "Human influence and threat to biodiversity and sustainable living," *Ethiopian Journal of Education and Sciences*. **3** (1): 85–95.

De Hertogh, A.A. and Lenard, M. (1993). World production and horticultural utilization of flower bulbs. In: The physiology of flower bulbs, eds. A.A. De Hertogh and M. Le Nard.: 21-28. *Elsevier, Amsterdam.*

de Klemm, C. and Shine, C. (1993). Biological Diversity Conservation and the Law, IUCN, Gland, Switzerland and Cambridge, UK. xix + 292.

de Meeûs, T., Durand, P. and Renaud, F. (2003). Species concepts: What for? *Trends Parasitol.* **19**:425–427.

DEAT (2005). Guideline 3: General Guide to the Environmental Impact Assessment Regulations, 2005, Integrated Environmental Management Guideline Series, *Department of Environmental Affairs and Tourism* (DEAT), Pretoria.

Degtjareva, G.V., Logacheva, M.D., Samigullin, T.H., Terentieva, E.I. and Valiejo-Roman, CM. (2012). Organization of chloroplast *psbA-trnH* intergenic spacer in dicotyledonous angiosperms of the family Umbelliferae. *Biochemistry*. **77**(9):1056-1064.

Delsuc, F., Brinkmann, H. and Philippe, H. (2005). Phylogenomics and the reconstruction of the tree of life. *Nature Review Genetics*. **6**:361–375.

Delsuc, F., Ranwez, V., Tilak, M.K, Ranwez, S. and Douzery, E.J.P. (2007). A database of candidate coding markers for mammalian phylogenomics. *Evolution*. **48** (7):241

DeSalle, R., and Amato, G. (2004). The expansion of conservation genetics. *Nature Review Genetics*. **5**: 702–712.

Devey, D.S., Leitch, I., Rudall, P.J., Pires, J.C., Pillon, Y., and Chase, M.W. (2006). Systematics of Xanthorrhoeaceae sensu lato, with an emphasis on *Bulbine*. Pp.

345-351, in Columbus, J. T., Friar, E. A., Porter, J. M., Prince, L. M., and Simpson, M. G. (Eds), *Monocots: Comparative Biology and Evolution. Excluding Poales*. Rancho Santa Ana Botanical Garden, Claremont, Ca. 22: 345-351.

Dong, W., Chao, X., Changhao, L., Jiahui, S., Yunjuan, Z., Shuo, S., Tao, C., Junjie, G. and Shiliang, Z. (2015). *ycf1*, the most promising plastid DNA barcode of land plants. *Scientific Reports* **5**: 8348.

Dong, W., Liu, J., Yu, J., Wang, L. and Zhou, S. (2012). Highly variable chloroplast markers for evaluating plant phylogeny at low taxonomic levels and for DNA barcoding. *PLOS ONE*. **7**: e35071.

Douglas, S.E. (1998). Plastid evolution: origins, diversity, trends. *Current Opinion in Genetics and Development*. **8**: 655–661.

Dowell, K. (2008). An introduction to computational methods and tools for analyzing evolutionary relationships. *Molecular Phylogenetics*. Technical report: 1-19. University of Maine, Orono, USA.

Duffy, A.M., Kelchner, S.A. and Wolf, P.G. (2009). Conservation of selection on *matK* following an ancient loss of its flanking intron. *Gene*. **438**: 17–25.

Dulloo, M, E., Hunter, D and Borelli, T. (2010). Ex situ and in situ conservation of agricultural biodiversity. Major advances and research needs. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **38**,123-135.

Dunham, J., Peacock, M., Tracy, C.R., Nielsen, J., and Vinyard, G. (1999). Assessing extinction risk: integrating genetic information. *Conservation Ecology*. **3** (1): 2.

Durand, T. and Schinz, H. (1894). *Liliacea*. Conspectus Florae, Africae, 5 (Monocotyledonae et gymnospermae): 277-418. Jardin botanique de 1 Etat, Brussels.

Drummond, A.J, Ho, S.Y.W, Phillips, M.J. and Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biol*ogy. **4** (5): e88.

Dyson, A. (1998). Discovering indigenous healing plants of the herb and fragrance gardens at *Kirstenbosch National Botanical Garden*. NBI, Cape Town.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research.* **32**(5), 1792–1797.

Eeley, H.A.C., Lawes, M. and Reyers, B. (2001). Priority areas for the conservation of subtropical indigenous forest in southern Africa: A case study from KwaZulu-Natal. *Biodiversity and Conservation*, **10**: 1221–1246.

Eliades, N.G. (2008). Fingerprinting of genetic diversity and patterns of spatial genetic variation in the endemic tree *Cedrus brevifolia* (Hook f.) Henry from Cyprus: implications for its conservation. *Optimus, Goettingen*.

Erkens, R.H.J. (2007). From morphological nightmare to molecular conundrum phylogenetic evolutionary and taxonomic studies on Guatteria (Annonaceae). Utrecht University.

Escudero, A., Iriondo, J.M. and Torres, M.E. (2003). Spatial analysis of genetic diversity as a tool for plant conservation. *Biological Conservation.* **113**(3): 351–365.

Esselstyn, J.A. (2007). Should universal guidelines be applied to taxonomic research? *Biological Journal of the Linnean Society*. **90**:761–764.

Evans, A. (1987). New Zealand in Flower. An illustrated guide to native flowering plants. *Reed Methuen Publishers Ltd*., Birkenhead, Auckland.

Faegri, K. and van der Pijl, L. (1979). The Principles of Pollination Ecology, *Pergamon.*

Fairbanks, D.J. and Anderson, W.R. (1999). Genetics: The continuity of life. Recombinant DNA and Molecular Analysis. *International Thomson Publishing Inc*., United States of America: 286-817.

Fazekas, A.J, Burgess K.S, Kesanakurti, P.R, Graham, S.W, Newmaster, S.G, (2008). Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS ONE.* **3**: e2802.

Febbraro, Di. M, Lurz, P.W., Genovesi, P., Maiorano, L., Girardello, M. and Bertolino, S. (2013). The use of climatic niches in screening procedures for introduced species to evaluate risk spread. A case with the American eastern grey squirrel. *PLoS One.* **8**(7): e56559.

Felsenstein, J. (1989). PHYLIP–Phylogeny Inference Package, Version 3.2. *Cladistics*. **5**: 164–166.

Felsenstein, J. (2009). PHYLIP–Phylogeny inference package, Version 3.69. *Epub* 3.69.

Feng J, Zhang, Z., Wu, X., Mao, A. and Chang, F. (2013). Discovery of Potential New Gene Variants and Inflammatory Cytokine Associations with Fibromyalgia Syndrome by Whole Exome Sequencing. *PLoS ONE.* **8**(6): e65033.

Fennell, C.W. and Van Staden, J. (2001). Crinum species in traditional and modern medicine. *Journal of Ethnopharmacology*. **78:** 15-26.

Ferreira, D.I. and Hancke, F.L. (1985). Indigenous flower bulbs of South Africa. A source of new genera and species for ornamental bulb cultivation. *Acta Horticulturae.* **177**: 405- 410.

Finotello, F., Lavezzo, E., Fontana, P., Peruzzo, D., Albiero, A., Barzon, L., Falda, M., di Camillo, B. and Toppo, S. (2012). Comparative analysis of algorithms for whole-genome assembly of pyrosequencing data. *Briefings in Bioinformatics*. **13**: 269-280.

Foden, W. and Potter, L. (2005). *Bulbinella barkerae* P.L. Perry. *National Assessment*: Red List of South African Plants version 2015.1.

Foster, P.G., Cox, C.J., and Embley, T.M. (2009). The primary divisions of life: a phylogenomic approach employing composition-heterogeneous methods. *Philosophical Transactions of the Royal Society B: Biological Sciences.* 364(1527), 2197–2207.

Fox M.G. and Sorhannusi, U.L.F.M. (2003). *RpoA*: A Useful Gene for Phylogenetic Analysis in Diatoms Journal of Eukcuyot Microbiology, **5006:**471-475. The Society of Prolozoologists

Francisco-Ortega, J., Santos-Guerra, A., Seung-Chul and Crawford, D.J. (2000). Plant Genetic Diversity in the Canary Islands: A Conservation Perspective, *American Journal of Botany.* **87**(7): 909-919.

Frankham, R. (2010). Challenges and opportunities of genetic approaches to biological conservation. *Biological conservation.* **143**.(9):1919-1927.

Fulekar, M.H (2010). Environmental biotechnology. *CRC Press publishers*. Textbook Frontiers of Energy and Environmental Engineering.

Furlan, E., Stoklosa, J., Griffiths, J., Gust, N., Ellis, R., Huggins, R.M., and Weeks, A.R. (2012). Small population size and extremely low levels of genetic diversity in island populations of the platypus, *Ornithorhynchus anatinus*. *Ecology and Evolution.* **2**(4), 844–857.

Gaafar, A.Z., Al-qurainy, F. and Khan, S. (2014). Assessment of genetic diversity in the endangered populations of *Breonadia salicina* (*Rubiaceae*) growing in The Kingdom of Saudi Arabia using inter-simple sequence repeat markers. *BMC Genetics*. **15**(1): 1–10.

Gao, L., Liu, N., Huang, B. and Hua, X. (2008). Phylogenetic analysis and genetic mapping of Chinese Hedychium using SRAP markers. *Scientia Horticulturae.* **117**(4): 369–377.

Gao, T., Yao, H., Song, J., Liu, C., Zhu, Y., Ma, X., Pang, X., Xu. H, and Chen, S. (2010). Identification of medicinal plants in the family Fabaceae using a potential DNA barcode *ITS2*. *Journal of Ethnopharmacology*. **130**:116–121.

Ge, S., Li, A., Lu, B.R., Zhang, S. Z. and Hong, D.Y. (2002). A phylogeny of the rice tribe Oryzeae (Poaceae) based on *matK* sequence data. *American Journal of Botany*. **89**: 1967–1972.

Gentili, R. Regazzoni, L., Vitalini S., Orsenigo S., Tomè F., Maffei F.R. (2011). Assessing extinction risk across borders: Integration of a biogeographical approach into regional IUCN assessment? *Journal for Nature Conservation.* **19**(2):69–71.

Gielly, L. and Taberlet, P. (1994). The use of chloroplast DNA to resolve plant phylogenies: noncoding versus *rbcL* sequences. *Molecular Biology and Evolution.* **11**(5): 769–777.

Given, D.R. (1981). Rare and Endangered Plant of New Zealand. A.H. and A.W. Reed.

Givnish, T.J., Ames, M., McNeal, J.R., McKain, M.R., Steele, P.R., dePamphilis, C.W., Graham, S.W., Pires, J.C., Stevenson, D.W., Zomlefer, W.B., Briggs, B.G., Duvall, M.R., Moore, M.J., Heaney, J.M., Soltis, D.E., Soltis, P.S., Thiele, K., Lebbens-Mack, J.H., (2010). Assembling the tree of the monocotyledons: plastome sequence phylogeny and evolution of Poales 1. *Annals of the Missouri Botanical Garden.* **97** (4), 584–616.

Glover, A.G., Sundberg, P. and Dahlgren, T.G. (2009). In Linnaeus' wake: 300 years of marine discovery. *Zoologica Scripta*. **38**:1–6.

Goldblatt, P. and Manning, J.C. (2000). Cape Plants: A conspectus of the Cape Flora of South Africa. *Strelitzia* 9. National Botanical Institute, Cape Town.

Goodstein, D.M., Rokhasar, D.S., Shu, S., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U. and Putman, N. (2012). Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Research.* **40**.

Goulding, J.H. (1971). Identification of Archaeological and Ethnological Specimens of Fibre-Plant Material Used by Maori. Records of the Auckland Institute and Museum **8**: 57-102.

Grassi, F., Imazio, S., Gomarasca, S., Citterio, S., Aina, R., Sgorbati, S., Sala, F., Patrignani, G. and Labra, M. (2004). Population structure and genetic variation within Valeriana wallrothii Kreyer in relation to different ecological locations. *Plant Science.* **166**: 1437-1441.

Gravendeel, B. (2000). Reorganising the orchid genus *Coelogyne*: A phylogenetic classification based on molecules and morphology. PhD Thesis, University of Leiden, Leiden.

Greenbaum, E. and Portillo, F. (2014). At the edge of a species boundary: A new and relatively young species of Leptopelis (Anura: Arthroleptidae) from the Itombwe Plateau, Democratic Republic of Congo. *Herpetological*. **70**:100-119.

Greene S.L., Kisha, T J., Yu. L. and Parra-Quijano M. (2014). Conserving plants in gene banks and nature: investigating complementarity with Trifolium thompsonii Morton. *PLOS ONE*. **9**(8): e105145.

Guerra-Garcia J.M., Espinosa F. and García-gómez J.C. (2008). Trends in Taxonomy today: an overview about the main topics in Taxonomy. *Zoologica Baetica.* **19**: 15-49.

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics.* **29**(8): 1072–1075.

Habtemariam, S. (2007). Antioxidant activity of knipholone anthrone. *Food Chemistry*. **102**: 1042–1047.

Hajibabaei, M., de Waard, J.R., Ivanova, N.V., Ratnasingham, S., Dooph, R.T., Kirk, S.L., Mackie, P.M. and Hebert, P.D.N. (2005). Critical factors for assembling a high volume of DNA barcodes. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences.* **360**:1959–1967.

Hajibabaei, M., Spall, J.L., Shokralla, S. and van Konynenburg, S. (2012). Assessing biodiversity of a freshwater benthic macroinvertebrate community through non-destructive environmental barcoding of DNA from preservative ethanol. *BioMedCentral Ecology*. **12:** 28.

Halevy, A.H. (1990). Recent advances in control of flowering and growth habit of geophytes. *Acta Hortic.* 266:35-42.

Hall, L.I. (1984). New Species of *Bulbine* Liliaceae from Vanrhynsdorp District Cape Province South Africa. *South African Journal of Botany.* **50:** 356–358.

Hallick, R.B (1984). Identification and partial DNA sequence of the gene for the alpha-subunit of the ATP synthase complex of *Chlamydomonas reinhardtii* chloroplasts. *Plant Physiology*. **177**: 374–376

Hammer, K. and Teklu, Y. (2008). Plant Genetic Resources: Selected Issues from Genetic Erosion to Genetic Engineering. *Journal of Agriculture and Rural Development in the Tropics and Subtropics.* **109**(1): 15–50.

Hamish C and Hutching, G. (2007). In Search of Ancient New Zealand. North Shore, New Zealand: Penguin Books. 121 pp.

Hao, D.A.C., Mu, J., Chen, S.L. and Xiao, P.G. (2010). Physicochemical evolution and positive selection of the gymnosperm *matK* proteins. *Journal of Genetics*. **89**(1): 81–89.

Harding, J., Singh, F. and Mol, J.N.M. (1991). Genetics and breeding of ornamental species. *Kluwer Academic Publishers*, Netherlands.

Hawksworth, D.L. (Ed) (1995). Biodiversity, Measurement and Estimation. *Chapman and Hall*, London.

Hayward, M.W. (2012). Time to agree on a conservation benchmark for Australia. Pacific *Conservation Biology.* **18** (2):69-76.

Hebert, P.D.N., Cywinska, A., Ball, SL. and deWaard, JR. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society London B.* **270**:313–321.

Hedrick, P.W. (2001). Conservation genetics: Where are we now? *Trends in Ecology and Evolution.* **16** (11): 629 – 636.

Hedrick, P.W. (2000). Application of population genetics and molecular techniques to conservation: 113-125. In Young and G. Clarke Eds Genetics, Demography and

Viability of fragmented populations Cambridge University Pres. Hedrick, P.W., 2004. *Recent Developments in Conservation Genetics*. **197**: 3–19.

Hendry, A.P., Lohmann, L., Conti, E., Cracraft, J., Crandall, K.A., Faith, D.P., Hauser, C. and Joly, C.A. (2010). Evolutionary biology in biodiversity science, conservation, and policy: a call to action. *Evolution*. **64**:1517–1528.

Heslop-Harrison, J.S. (2000). Comparative genome organization in plants: From sequence and markers to chromatin and chromosomes. *Plant Cell*. **12:** 617-636.

Hessayon, D.G. (1999). The Bulb expert. London. *Transworld Publishers*.

Hidayat, T. and Pancoro, A. (2006). Short Comunication: DNA Technology and Studies in Phylogenetic Relationship of tropical Plant: Prospect in Indonesia. International Conference on Mathematics and Natural Sciences (ICMNS) on 29-30 November 2006. Indonesia.

Hilton-Taylor, C. (1996). Red Data List of southern African plants. 1. Corrections and additions. *Bothalia.* **26** (2): 177-182.

Hinrikson, H.P, Hurst, S.F., Lott, T.J. (2005). Assessment of ribosomal large-subunit D1–D2, internal transcribed region spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important Aspergillus species. *Journal of Clinical Microbiology*. **43**:2092–2103.

Hodgkin, T., Roviglioni, R., de Vicente, M.C. and Dudnik, N. (2001). Molecular methods in the conservation and use of plant genetic resources. *Acta Horticulturae.* **546**: 107–118.

Holsinger, K. and Anon. (2005). Conservation of genetic resources. I Can: 1–10.

Hoot, S.B., Culham A. and Crane P.R. (1995). The utility of *atp B* gene sequences in resolving phylogenetic relationships: Comparison with *rbcL* and 18S ribosomal DNA sequences in the Lardizabalaceae. *Annals of the Missouri Botanical Garden*. **82**: 194 – 207.

Horn, W. (1962). Breeding research in South African plants 1. Fertility relationships in *Bulbinella* Kunth. *South African Journal of Agricultural Science.* **5**(1): 79-88.

Huang, W., Hang, S., Deng, T., Razafimandimbison, S.G., Nie, Z.L. and Wen, J. (2013). Molecular phylogenetics and biogeography of the eastern Asian-eastern North American disjunct Mitchella and its close relative Damnacanthus (Rubiaceae, Mitchelleae). *Botanical Journal of the Linnean Society.* **171:** 395–412.

Hutchings, A., Scott, A.H., Lewis, G. and Cunningham, A.B. (1996). Zulu Medicinal Plants: *An Inventory*. University of Natal Press, Pietermaritzburg. Iizuka, M., Warashina.

IUCN, (2001). IUCN Red List Categories and Criteria: version 3.1. IUCN Species Survival Commission, Gland, Switzerland, and Cambridge, UK.

IUCN, (2003). Guidelines for application of IUCN Red List Criteria at regional levels. Version 3.0. Gland, Switzerland and Cambridge, UK: *IUCN Species Survival Commission*.

IUCN, (2007). IUCN Red List of threatened Species- Extinction Crisis escalates. *Biodiversity.* **8**, (3).

Jansen, R.K, Cai Z., Raubeson, L.A., Daniell, H, Leebens-Mack, J. and Müller KF. (2007). Analysis of 81 genes from 64 plastid genomes resolves relationships in angiosperms and identifies genome-scale evolutionary patterns. *Proceedings of the National Academy of Sciences of the United States of America*. **104**:19369–74.

Jarvis, D.I. (1999). Strengthening the scientific basis of in situ conservation of agricultural biodiversity on-farm. *Botanica Lituanica Supply*. **2**: 79–90.

Jing, Yu., Xue, J.H. and Zhou, S.L. (2011). New universal *matK* primers for DNA barcoding angiosperms. *Journal of Systematics and Evolution.* **49** (3): 176–181.

Johnson, A.D. and Hall, R.W. (2005). Chapter 7 Morphological and Molecular Methods for Creating Phylogenetic Trees.

Jones, C.J., Karp, A., Castiglione, S. and Edwards, K.J. (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding.* **3**: 381–390.

Judd, W.S., Campbell, C.S., Kellogg, E.A. and Stevens, P.F. (1999). Plant systematics. A phylogenetic approach. *Sinauer Associates*, USA.

Kamenetsky, R. and Miller, B. (2010). The global trade in Ornamental geophytes. *Chronica Horticulturae*. **50**(4): 27-30.

Kamenetsky, R.; Okubo, H. (2013). Ornamental Geophytes: From Basic Science to Sustainable Production; *CRC Press: Boca Raton*, FL, USA, p. 578.

Karp, A. and Edwards, K.J. (1995). Molecular techniques in the analysis of the extent and distribution of genetic diversity. IPGRI Workshop on Molecular Genetic Tools in Plant *Genetic Resources*, 9–11 October, Rome, IPGRI.

Kasso, M. and Mundanthra, B. (2013). Ex Situ Conservation of Biodiversity with Particular Emphasis to Ethiopia. Hindawi Publishing Corporation. *ISRN Biodiversity.* Article ID 985037, 11 pages.

Keneni G, Endashaw B., Muhammad I., Kifle D. (2012). Genetic Vulnerability of Modern Crop Cultivars: Causes, Mechanism and Remedies. *International Journal of Plant Research.* **2**(3): 69-79.

Kevan, P.G. and Phillips, T.P. (2001). The economic impacts of pollinator declines: an approach to assessing the consequences. *Conservation Ecology.* **5**(1): 8.

Khodorova, N.V. (2011). A study of adaptation to cold in a geophyte species (Corydalis bracteata (Steph.) Pers, Fumariaceae DC.) and an approach of secondary metabolism during plant development. PhD Thesis, Jules Verne University of Picardy, Amiens, France. 181 pp.

Kircher, M. and Kelso, J. (2010). Methods, Models and Techniques. High-throughput DNA Sequencing-Concepts and limitations. *Article in Bioessays.*

Kjaer, E. D., Graudal, L. and Nathan, I. (2001). Ex situ conservation of commercial tropical trees: Strategies, options and constraints; A paper presented at ITTO International Conference on ex situ and in situ Conservation of Commercial Tropical Trees, Yogyakarta, Indonesia.

Kleynhans, R. and Spies, J.J. (2011). Requirements for the development and breeding of new flower bulb crops. *Philosophical Transitions in Genetics*. **1**: 80-101.

Klich, M.A. (2002). Identification of Common Aspergillus Species. Centraalbureau voor Schimmelcultures, Utrecht.

Klopper, R.R., van Wyk, A, E. and Smith, G.F. (2010). Phylogenetic relationship in the family Asphodelaceae (Asparagales). *Biodiversity and Ecology* 3. South Africa.

Koetle M.J., Finnie, J.F., Balázs, E., Van Staden, J. (2015). A review on factors affecting the Agrobacterium-mediated genetic transformation in ornamental monocotyledonous geophytes. *South African Journal of Botany*. **98** (2015) 37–44.

Kohn, M.H., Murphy, W.J., Ostrander, E.A. and Wayne, R.K. (2006). Genomics and conservation genetics. *Trends in Ecology and Evolution.* **21**(11): 629–637.

Koonin, E.V. (2003). Comparative genomics, minimal gene-sets and last universal common ancestor. *Nature Reviews Microbiology*. **1** (2) 127-36.

Kornienko, A. and Evidente, A. (2008). Chemistry, biology and medicinal potential of narciclasine and its congeners. *Chemical Reviews.* **108**: 1982–2014.

Kreivi, M., Rautiainen, P., Aspi, J. and Hyva¨rinen, M. (2005). Genetic structure and gene flow in an endangered perennial grass, *Arctophila fulva var. pendulina*. *Conservation Genetics* **6**:683–696.

Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A. and Janzen, D.H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of the United States of America*. **102**(23): 8369–8374.

Kress, W.J. and Erickson, D.L. (2007). A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. *PLoS ONE.* **2**: e508.

Krishnankutty, N. and Chandrasekaran, S. (2007). Linnaeus 300: Tips for tinkering morphological taxonomy. *Current Science.* **94**: 565-567.

Kumar, S., Govil, S., Sadana, S. and Pathak, A.N. (2014). Algorithms and databases: A key to solve genetic equation in Next Generation Sequencing. *International Journal of Advances in Pharmacy, Biology and Chemistry*. Review Article. IJAPBC **3**(3).

Kunth, C.S. (1843). " ·Enumeratio Plantarum Omnium Hucusque Cognitarum, etc." 4. Stutgardiae et Tubingae, Sumtibus J. G. Cottae. 752 pp.

Kuroda, M., Mimaki, Y., Sakagami, H. and Sashida, Y. (2003). Bulbinelonesides A-E, Phenylanthraquinone glycosides from the roots of *Bulbinella floribunda*. *Journal of Natural Products*. **66**: 894-897.

Kyiashchenko, I. and Berlin, A. (2011). Taxonomic and phylogenetic study of rust fungi forming aecia on Berberis speciesS. in Sweden. Master's thesis. Ecology.

Lahaye, R., Van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, S., Barraclough, T.G. and Savolainen, V. (2008). DNA barcoding the floras of biodiversity hotspots. *Proceedings of the National Academy of Sciences USA.* **105**: 2923–2928.

Lamble S, Batty, E., Attar, M, Buck, D., Bowden, R., Lunter, G., Crook, D., El-Fahmawi B and Piazza, P (2013). Improved workflows for high throughput library preparation using the transposome-based nextera system. *BMC Biotechnology*. **13**:104.

Lamoreux, J., Akc¸ Akaya, H.R., Bennun, L., Collar, N.J., Boitani, L. and Brackett, D. (2003). Value of the IUCN Red List. *Trends in Ecology and Evolution.* **18**: 214-215.

Lankau, R.A. and Strauss, S.Y. (2007). Mutual feedbacks mantain both Genetic and species diversity in a plant community. *Science*. **317** (5844):1561-1563.

Lee, S.W., Ledig, F.T. and Johnson, D.R. (2002). Genetic variation at allozyme and RAPD markers in Pinus longaeva (Pinaceae) of the White Mountains, California. *American Journal of Botany*. **89**: 566–577.

Lewin, R. (1999). Patterns in evolution: The new molecular view. New York: *Scientific American Library*.

Liang, H.P. and Hilu, K.W. (1997). Application of the *matK* gene sequences to grass systematics. *Canadian Journal of Botany*. **74**: 125-134.

Lin, J.J., Kuo, J., Ma, J., Saunders, J.A., Beard, H.S., MacDonald, M.H., Kenworthy, W., Ude G.N. and Matthews B.F. (1996). Identification of molecular markers in soybean comparing RFLP, RAPD and AFLP DNA mapping techniques. *Plant Molecular Biology*. Reporter **14**:156-169.

Linder, C.R., Goertzen, L.R., Heuvel, B.V., Francisco-Ortega, J. and Jansen, R. K. (2000). The complete external transcribed spacer of 18S-26S rDNA: amplification and phylogenetic utility at low taxonomic levels in Asteraceae and closely allied families. Molecular phylogenetics and evolution, *Molecular Phylogenetics and Evolution*: 285–303.

Linnaeus, C. (1753). Species Plantarum 1. Stockholm: Impensis Laurentii Salvii.

Liu L, Xi Z, Wu S, Davis C, Edwards SV, (2015). Estimating phylogenetic trees from genome-scale data. *Annals of the New York Academy of Sciences*. DOI: 10.1111/nyas.12747.

Louw, C.A.M., Regnier, T.J.C. and Korsten, L. (2002). Medicinal geophytes plants of South Africa and their traditional relevance in the control of infectious diseases. *Journal of Ethnopharmacology*. **82**(2-3): 147-154.

Lu, L. and Xu, Y.P. (2014). Genomic full-length sequence of two HLA-A alleles, A*01:01:01:01 and A*01:03, identified by cloning and sequencing. *Tissue Antigens*. **83**(6): 423–424.

Mace, G.M., Collar, N.J., Gaston, K.J., Hilton-Taylor, C., Akcakaya, H.R. and Leader- Williams, N. (2008). Quantification of extinction risk: IUCN's system for classifying threatened species. *Conservation Biology*. **22**:1424–1442.

Majinda, R.R.T., Abegaz, B.M., Bezah, M., Ngadjui, B.T., Wanjala, C.C., Mdee, W. and Bojase, L.K. (2001). Recent Results from Natural Product Research at University of Botswana, *Pure and Applied Chemistry.* **73**(7): 1197-1208.

Maleka, M.F., Albertyn, J. and Spies, J.J. (2013). The Floriculture Industry and Flower Pigmentation- A Review. *Philosophical Transactions in Genetics*: 55-110.

Mallet, J. and Willmott, K. (2003). Taxonomy: Renaissance or Tower of Babel? *Trends in Ecology and Evolution.* **18**:57–59.

Mallet, J. (2006). Species concepts. In Fox, C. and Wolf, J.: Evolutionary Genetics: Concepts and Case Studies. OUP, *Oxford*: 367-373.

Mallet, J. (2008). Hybridization, ecological races, and species: empirical evidence for the ease of speciation. *Philosophical Transactions of the Royal Society B.* **363**: 2971–2986.

Manning, J.C. and Goldblatt, P. (2010). *Bulbinella calcicola*, a new species from Saldanha Bay, Western Cape. *Bothalia*. **40**: 197-199.

Mardis, E.R. (2008). The impact of next-generation sequencing technology on genetics. *Trends in Genetics.* **24**(3): 133-141.

Maria von Cra¨utlein, Korpelainen, H., Pietila¨inen, M. and Rikkinen, J. (2011). DNA barcoding: A tool for improved taxon identification and detection of species diversity. *Biodiversity and Conservation.* **20**:373-389.

Matsuki T, Watanabe K, Fujimoto J. (2002). Development of 16S rRNA-Gene-Targeted Group-Specific Primers for the Detection and Identification of

Predominant Bacteria in Human Feces. *Applied and Environmental Microbiology*. **68**(11): 5445-5451.

Maxted, N., Dulloo, M.E., Ford-Lloyd, B.V., Frese, L., Iriondo, J.M. and Pinheiro de Carvalho, M.A.A. (2011). *Agrobiodiversity Conservation: Securing the Diversity of Crop Wild Relatives and Landraces*. CAB International, Wallingford.

May, R. (1990). Taxonomy as destiny. *Nature*. **347**: 129-130.

Mayo, S.J., Allkin, R., Baker, W., Blagoderov, V., Brake, I., Clark, B., Govaerts, R., Godfray, C., Haigh, A., Hand, R., Harman, K., Jackson, M., Kilian, N., Kirkup, D.W., Kitching, I., Knapp, S., Lewis, G.P., Malcolm, P., Von Raab-Straube, E., Roberts, D.M., Scoble, M., Simpson, D.A., Smith, C., Smith, V., Villalba, S., Walley, L. and Wilkin, P. (2008). Alpha e-taxonomy: Responses from the systematics community to the biodiversity crisis. *Kew Bulletin*. **63**:1–16.

Mayr, E. and Bock, W.J. (2002). Classifications and other ordering systems. *Journal of Zoological Systematics and Evolutionary Research*. **40**: 169-194.

Mayr, G., Manegold, A. and Johansson, U.S. (2003). Monophyletic groups within "higher land birds"- comparison of morphological and molecular data. *Journal of Zoological Systematics and Evolutionary Research*. **41**: 233–248.

McCartan, S.A. and Van Staden, J. (1999). Micro propagation of members of the Hyacinthaceae with medicinal and ornamental potential – A review. *South African Journal of Botany*. **65**: 361-369.

McClean, C.J., Lovett, J.C., Ku¨ per, W., Hannah, L., Henning Sommer, J., Barthlott, W., Termansen, M., Smith, G.F., Tokumine, S., Taplin, J.R.D., (2005). African plant diversity and climate change. *Annals of the Missouri Botanical Garden*. **92:** 139–152.

McNeely, J.A., Miller, K.R., Reid, W.V., Mittermeier, R.A. and Werner, T.B. (1990). Conserving the world's biological diversity. World Conservation Union, World Resources Institute, Conservation International, World Wildlife Fund–US, and the World Bank, Washington, D.C.

Milicich, L.D. (1993). Allozyme and other aspects of variation in the Genus *Bulbinella* in New Zealand. nzresearch.org.nz.

Millennium Ecosystem Assessment, (2005). Ecosystems and Human Well-Being: Biodiversity Synthesis. Washington, Dc: *World Resource Institute*.

Moar N.T, Wilmshurst, J.M and McGlone, M.S (2011). Standardizing names applied to pollen and spores in New Zealand Quaternary palynology, *New Zealand Journal of Botany*. **49**:(2): 201-229

Mondini, L., Noorani, A. and Pagnotta, M.A. (2009). Assessing plant genetic diversity by molecular tools. *Diversity*. **1**(1): 19–35.

Moore, L.B. (1964). The New Zealand Species of *Bulbinella* (Liliaceae), *New Zealand Journal of Botany*. **2**(3): 286-304.

Moore, L.B.; Irwin, J.B. (1978). The Oxford book of New Zealand plants. Oxford, *Oxford University Press.*

Moore, Lucy B.; Edgar, Elizabeth (1970). "Flora of New Zealand". Vol. II, Government Printer, Wellington. 354 pp.

Moore, M. J., Bell, C. D., Soltis, P.S. and Soltis, D.E. (2007). Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. *Proceedings of the National Academy of Sciences, USA* **104**: 19363 – 19368.

Moore, M. J., Soltis, P. S., Bell, C.D., Burleigh, J. G. and Soltis, D E. (2010). Phylogenetic analysis of 83 plastid genes further resolves the early diversification of eudicots. *Proceedings of the National Academy of Sciences. USA* **107**: 4623 – 4628.

Moore, M.J., Dhingra, A., Soltis, P.S., Shaw, R., Farmerie, W.G., Folta, K.M. and. Soltis, D.E. (2006). Rapid and accurate pyrosequencing of angiosperm plastid genomes. *BMC Plant Biology*. **6**: 17- 30.

Moritz, C. (2002). Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic Biology*. **51**: 238–254.

Mueller, U.G. and Wolfenbarger, L.L. (1999). AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution*. **14**(10): 389–394.

Musara, C, Spies, P., Spies, J and Stedge, B. (2017). A review of *Bulbinella* (Asphodelaceae): distribution, conservation status, and economic importance. Botanical Sciences, [S.l.], v. 95, n. 2, p. 155-168. ISSN 2007-4476.

Naeem, A., Khan, A.A., Cheema, H.M.N., Khan, I.A. and Buerkert, A. (2014). DNA barcoding for species identification in the Palmae family. *Genetics and Molecular Research*. **13** (4): 10341-10348.

Naderi Safar, K., Kazempour Osaloo, S., Assadi, M., Zarrei, M., and Khoshsokhan Mozaffar, M. (2014). Phylogenetic analysis of *Eremurus*, *Asphodelus*, and *Asphodeline* (Xanthorrhoeaceae-Asphodeloideae) inferred from plastid *trnL-F* and nrDNA ITS sequences. *Biochemical Systematics and Ecology.* **56**: 32-39.

Naughton, T.L., Kammen, D.M. and Hapman C. (2007). Burning biodiversity: woody biomass uses by commercial and subsistence groups in western Uganda's forests. *Biological Conservation*. **134**: 232-240.

Nesbitt, K.A., Potts, B.M., Vaillancourt, R.E., West, A.K. and Reid, J.B. (1995). Partitioning and distribution of RAPD variation in a forest tree species, *Eucalyptus globulus* (Myrtaceae). *Heredity*. **74**: 628-637.

Ness, R.W., Wright, S.I. and Barrett, S.C.H. (2010). Mating-System Variation, Demographic History and Patterns of Nucleotide Diversity in the Tristylous Plant *Eichhornia paniculata*. *Genetics*. **184**(2):381-92.

Nevo, E. (1998). Genetic diversity in wild cereals: Regional and local studies and their bearing on conservation ex situ and in situ. *Genetic Resources and Crop Evolution.* **45**(4): 355-370.

Newmaster, S.G., Fazekas, A.J. and Ragupathy, S. (2006). DNA barcoding in land plants: evaluation of *rbcL* in a multigene tiered approach *Canadian Journal of Botany.* **84**:335–341.

Niedringhaus, T.P., Milanova, D., Kerby, M.B., Snyder, M.P. and Barron, A.E. (2011). Landscape of next-generation sequencing technologies. *Analytica Chimica Acta*. **83**: 4327-4341.

Niino, T. (2006). Developments in PGR reservation technologies. In: Kangi, J.H. (ed.) Effective gene bank management for an integrated system on sustainable conservation and utilization of plant genetic resources, RDA Korea: 149–158.

Nock, C.J., Baten, A. and King, G.J. (2014). Complete chloroplast genome of Macadamia integrifolia confirms the position of the Gondwanan early-diverging eudicot family Proteaceae. *BMC Genomics*, **15** (Suppl 9), S13 pp.

Noor, M.A.F., Garfield, D.A., Schaeffer, S.W. and Machado, CA. (2007). Divergence between the Drosophila pseudoobscura and D. persimilis Genome Sequences in Relation to Chromosomal Inversions. *Genetics*. **177**(3): 1417–1428.

Ntie-kang, (2014). A chemotaxonomy and cheminformatics analysis of natural products from African flora with anti-cancer like activities. *Journal of Chemical Information and Modelling.* **54**: 2433-2450.

Obici, S., Otobone, F.J., da Silva-Sela, V.R., Ishida, K., da Silva, J.C., Nakamura, C.V., Cortez, D.A.G. and Audi, E.A. (2008). Preliminary toxicity study of dichloromethane extract of *Kielmeyera coriacea* stems in mice and rats. *Journal of Ethnopharmacol.* **115**:131-139.

Oliveira, E.J., Pádua, J.G., Zucchi, M.I., Vencovsky, R. and Carneiro Vieira, M.L. (2006). Origin, evolution and genome distribution of microsatellites. *Genetics and Molecular Biology.* **29**(2): 294-307.

Oyler-McCance, S.J. and Leberg, P.L. (2005). Conservation genetics in wildlife management. In: Braun, C.E. (Ed.). Techniques for wildlife investigations and management, 6th Edition. *The Wildlife Society, Bethesda*: 632 – 657.

Özhatay, N., Koçyiğit, M., Yüzbaşıoğlu, S. & Gürdal, B. (2013). Mediterranean flora and its conservation in Turkey: with special reference to Monocot geophytes. *Flora Mediterranea*. **23**: 195-208.

Pacific Bulb Society (2017). *Bulbinella*. https://www.pacificbulbsociety.org/pbswiki/index.php/*Bulbinella*. (Date accessed 12 June 2017).

Pareek, C.S., Smoczynski, R. and Tretyn A. (2011). Sequencing technologies and genome sequencing. *Journal of Applied Genetics*. **52**:413-435.

Parks, M., Cronn, R. and Liston, A. (2009). Increasing phylogenetic resolution at low taxonomic levels using massively parallel sequencing of chloroplast genomes. *BMC Biology* **7**: 84.

Parnell, J. (1993). Plant taxonomic research, with special reference to the tropics: problems and potential solutions. *Conservation Biology*. **7**(4): 809–814.

Patel S., Shah, D.B. and Hetalkumar, J.P. (2014). Evolutionary studies in sub-families of Leguminosae family based on *matK* gene. *Plant Gene and Trait*. **5**(7):1-9.

Patwardhan, A., Ray, S. and Roy, A. (2014). Molecular Markers in Phylogenetic Studies-A Review. *Phylogenetics and Evolutionary Biology.* **2**(2).

Perry, P.L. (1987). 'A synoptic review of the genus *Bulbinella* (Asphodelaceae) in South Africa". *Jourrnal of South African Botany*. **53**(6): 431-444.

Perry, P.L. (1999). *Bulbinella* in South Africa-*Strelitzia.* **8**:1-77.

Petitjean, C., Deschamps, P. and López-García, P. (2014). Rooting the Domain Archaea by Phylogenomic Analysis Supports the Foundation of the New Kingdom Proteoarchaeota. *Genome Biology and Evolution*. **7**(1):191–204.

Pires, A.C. and Marinoni, L. (2010). DNA barcoding and traditional taxonomy unified through Integrative Taxonomy: A view that challenges the debate questioning both methodologies Revitalizing Taxonomy through DNA Barcoding. *Biota Neotropica*. **10**(2): 339-346.

Plassmann, G. (2004). Rete ecologica transfrontaliera. Studio mandato Convenzione delle alpi: "aree protette transfrontaliere e rete ecologica delle Alpi". Innsbruck: Segretariato permanente della Convenzione delle Alpi.

Prance, G.T. (1997). The conservation of botanical diversity. In: Maxted, N., B.V. FordLloyd and J.G. Hawkes (Eds), Plant Genetic Conservation. The in situ Approach: 1-4, Chapman and Hall.

Prasad, P.K, Tandon, V., Biswal, D.K., Goswami, L.M., Chatterjee, A. (2009). Phylogenetic reconstruction using secondary structures and sequence motifs of ITS2 rDNA of Paragonimus westermani (Kerbert, 1878) Braun, 1899 (Digenea: Paragonimidae) and related species. *BMC Genomics.* **10**: 3-10.

Primack, R.B. (2006). Essentials of conservation Biology, 4thEd. *Sinauer Associates.*

Proost, S., van Bel, M., Sterck, L., Billiau, K., van Parys, T., van de Peer, Y. and Vandepoele, K. (2009). PLAZA: A comparative genomics resource to study gene and genome evolution in plants. *Plant Cell.* **21**: 3718-3731.

Procheş, Ş, Cowling R.M., Goldblatt, P, Manning J.C., Snijman, D.A. (2006). An overview of the Cape geophytes, *Biological Journal of the Linnean Society.* **87**(1) 1: 27–43.

Pujol, J. (1990). Naturafrica- The Herbalist Handbook: African Flora, Medicinal Plants. Jean Pujol Natural Healers' Foundation, Durban, South Africa: 25-28.

Qhotsokoane-Lusunzi, M.A. and Karuso, P.J. (2001). Secondary metabolites from Basotho medicinal plants. I. *Bulbine narcissifolia*. *Journal of Natural Products*. **64**:1368-1372.

Raimondo, D., von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. and Manyama, P.A. (2009). Red List of South African Plants. Strelitzia 25. South African National Biodiversity Institute, Pretoria.

Rainbow, P.S. (2009). Marine biological collections in the 21st century. *Zoologica Scripta*. **38**:33-40.

Rajapakse, S., Iddamalgoda, P., Ratnayake, R. and Wijesundara, D.S.A. (2012). Evaluation of species limits of Hortonia by DNA barcoding. *Journal of the National Science Foundation of Sri Lanka* **40** (4): 345-349.

Rambaut A, Drummond, A.J. (2007). Molecular evolution, phylogenetics and epidemiology, Tracer v.1.5. Available from: http://tree.bio.ed.ac.uk/software/tracer/.13/09/2017

Rambaut, A. (2012). FigTree v1.4.0. Available from: http://tree.bio.ed.ac.uk/software/figtree/.13/09/2017/

Ramdhani, S., Barker, N.P. and Baijnath, H. (2006). Phylogenetics of the genus *Kniphofia Moench* (Asphodelaceae). In: Taxonomy and Ecology of African Plants: their conservation and sustainable use (Proceedings of the 17th AETFAT Congress), eds. Ghazanfar, S.A. and Beentje, H.J. 559–573. *Royal Botanic Gardens, Kew.*

Rao, V.R. and Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell Tissue Organ Culture*. **68**: 1–19.

Rao, K.V., Latha, A.M. and Reddy, V.D. (2005). Production of transgenic plants resistant to leaf blast disease in finger millet (*Eleusine coracena* (L) Craertn) *Plant science.* **169** (4): 657-667.

Ratnasingham, S., & Hebert, P.D.N. (2007). BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes.* **7**(3), 355–364.

Reid, C. and Glen, H.F. (1993). Asphodelaceae (Part B). In: T.H. Arnold and B.C. de Wet (eds), Plants of Southern Africa: 133. *National Botanical Institute, Pretoria.*

Renau-Morata B.1., Nebauer, S.G., Sales, E., Allainguillaume, J., Caligari, P., Segura, J. (2005). Genetic diversity and structure of natural and managed populations of Cedrus atlantica (Pinaceae) assessed using random amplified polymorphic DNA. *American Journal of Botany*. **92**(5):875-84.

René L.W., Chen, Yang., Benjamin, P., Vandervalk, B.B., Lagman, A., Steven, J.M.J. and Inanç Birol. (2015). LINKS: Scalable, alignment-free scaffolding of draft genomes with long reads. *Giga Science*. **4**:35

Reumers, J., Schymkowitz, J., Ferkinghoff-Borg, J., Stricher, F., Serrano, L. and Rousseau, F. (2005). SNP effect: a database mapping molecular phenotypic effects of human non-synonymous coding SNPs. *Nucleic Acids Research*. **33:** D527-D532.

Richardson, A.T.B., Lord, J.M., Perry, N.B. (2017). Phenylanthraquinones and Flavone-C-glucosides from the disjunct *Bulbinella* in New Zealand. *Phytochemistry*. 134: 64-70

Riley, M., (1994). Maori Healing and Herbal. Viking Sevenseas N.Z. Ltd, Paraparaumu, New Zealand.

Robbirt, K.M., Roberts, D.L. and Hawkins, J.A. (2006). Comparing IUCN and probabilistic assessments of threat: Do IUCN Red List criteria conflate rarity and threat? *Biodiversity and Conservation*. **15**: 1903-1912.

Ronquist F, Teslenko, M., van der Mark P, Ayres, D.L., Darling, A. and Höhna, S. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*. **61**(3): 539–542.

Rossi, R. (1990). Guía de Bulbos (in Spanish). Barcelona: Grijalbo.

Rubinoff, D., Cameron, S. and Will, K. (2006). Are plant DNA barcodes a search for the Holy Grail? *Trends in Ecology and Evolution*. **21**(1):1-2.

Rudd, K.E (2000). EcoGene: a genome sequence database for Escherichia coli K-12. *Nucleic Acids Res*earch. **28**:60-4.

Russel G, Reid G.E, Van rooy C.J. and Smook, L. (1985). List of species of southern African plants, edn 2. Memoirs of the Botanical Survey of South Africa no.**51**. *Bothalia*. 29(1):91-104.

Saggu, S., Divekar, H.M., Gupta, V., Sawhney, R.C., Banerjee, P.K. and Kumar, R. (2007). Adaptogenic and safety evaluation of seabuckthorn (Hippophae rhamnoides) leaf extract: a dose dependent study. *Food and Chemical Toxicology*. **45**: 609-617.

Salmon, J. T. (1985). Collins guide to the alpine plants of New Zealand. Auckland, Collins.

SANBI, (2009). List of SA red data listed species. Available online: http://www.sanbi.org/index.php?option+com_docmanandtask=documentdetailsandid+43.

SANBI, (2014). Red List of South African Plants. SANBI: Biodiversity of life. SANBI. Statistics: Red List of South African Plants version 2014.1. (http://redlist.sanbi.org/). 5/10/2017

Sanderson, E.W., Redford, K.H., Vedder, A., C Oppolillo, P.B. and Ward, S.E. (2002). A conceptual model for conservation planning based on landscape species requirements. *Landscape and Urban Planning*. **58**:41-56.

Sang, T., Crawford, D.J. and Stuessy, T.F (1997). Chloroplast DNA phylogeny, reticulate evolution and biogeography of Paeonia (Paeoniaceae). *American Journal of Botany*. **84**: 1120–1136.

Scherr, S.J. and McNeely, J.A. (2008). Biodiversity conservation and agricultural sustainability: towards a new paradigm of 'Eco agriculture' landscapes. *Philosophical of Transaction of Royal Society. B*. **363**: 477-494.

Schultz, T. (2013). The *Bulbinella* herb plant and its medicinal uses. Knoji Consumer knowledge. *Knoji Consumer Knowledge*. (https:// natural-herbal-remedies.knoji.com/the-*Bulbinella*-herb-plant-and-its-medicinal-uses/).

Schmieder, R, and R Edwards (2011) Quality control and preprocessing of metagenomic datasets. *Bioinformatics*. **27:** 863–864.

Seberg. O., Humphries, C.J., Knapp, S., Stevenson, D.W., Petersen, G., Scharff, N. and Andersen, N.M. (2003). Shortcuts in systematics? A commentary on DNA-based taxonomy. *Trends in Ecology and Evolution*. **18**:63-65.

Selvaraj, D., Sarma, R.K. and Sathishkumar, R. (2008). Phylogenetic analysis of chloroplast matK gene from Zingiberaceae for plant DNA barcoding. *Bioinformation*. (1):24-27.

Selvaraj, S., Dixon, J.R., Bansal, Y. and Bing, R.B.C. (2013). Whole genome haplotype reconstruction using proximity-ligation and shotgun sequencing. *Nature Biotechnology*. **31**:1111-1118.

Serino, G., & Maliga, P. **(1998)**. RNA Polymerase Subunits Encoded by the Plastid *rpo* Genes Are Not Shared with the Nucleus-Encoded Plastid Enzyme. *Plant Physiology*, *117*(4), 1165–1170.

Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W., Miller, J., Siripun, K.C., Winder, C.T., Schilling, E.E. and Small, R. (2005). The tortoise and Hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany*. **92**: 142-166.

Shaw, J., Lickey, E.B., Schilling, E.E. and Small, R.L. (2007). Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany.* **94**:275-288.

Shendure, J. and Ji, H. (2008). Next-generation DNA sequencing. *Nature biotechnology.* **26**(10): 1135-1145.

Shulaev, V., Sargent, D.J., Crowhurst, R.N., Mockler, T.C., Folkerts, O., Delcher, A.L., Jaiswal, P., Mockaitis, K., Liston, A. and Mane, S.P. (2011). The genome of woodland strawberry (*Fragaria vesca*). *Nature Genetics*. **43**:109-116.

Singh, H.K., Parveen, I., Raghuvanshi, S. and Babbar, S.B. (2012). Loci recommended as universal barcode for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of Dendrobium species. *BMC Research Notes*. **5**:42.

Sirokov, R. (2014). Towards faster RNA Sequencing analysis. Permalink. http://hdl.handle.net/10138/136467.

Sivarajan, V.V. (1991). Introduction to Principles of Plant Taxonomy. 2nd Ed. Editor N.K.P Robson.

Small, R.L., Cronn, R.C. and Wendel, J.F. (2004). Use of nuclear genes for phylogeny reconstruction in plants. *Australian Systematic Botany*. **17**:145-170.

Smith, S.A, Donoghue, M.J. (2008). Rates of molecular evolution are linked to life history in flowering plants. *Science.* **322**: 86–89.

Smith, M.A., Fisher, B.L., and Hebert, P.D.N. (2005). DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transaction of Royal Society. B Biological Science*. **360**: 1825-1834.

Smith, G. F. and Steyn, E.M.A. (2004). Taxonomy of Aloaceae. In: T. Reynolds (ed.): Aloes: the genus Aloe: 13-36. London: *CRC Press*.

Smith, G. F. and Van Wyk, B.-E. (1998). Asphodelaceae. In: K. Kubitzki (Ed.): The families and genera of flowering plants **3**: 130-140. *Springer*. Berlin and Heidelberg.

Solomon, E. P., Berg, L. R. and Martin, D. W. (2002). Biology. (6th Ed.). Thompson Learning, Inc. London, UK: 1233: G9- G29.

Soltis, P.S. and Gitzendanner, M.A. (1999). Molecular systematics and the conservation of rare Species. *Conservation Biology*. **13**: 471-483.

Spies, P. (2004). Phylogenetic relationships of the genus *Lachenalia* with other related liliaceous taxa. M. Sc thesis. University of the Free State.

Staub, J.E., Box, J., Meglic, V., Horejsi, T. and McCreight, J.D. (1997). Comparison of isozyme and random amplified polymorphic DNA data for determining intraspecific variation in *Cucumis*. *Genetic Resources and Crop Evolution*. **44**: 257-269.

Steele, P.R., Hertweck, K.L., Mayfield, D., McKain, M.R., Leebens-Mack, J., Pires, J.C., (2012). Quality and quantity of data recovered from massively parallel sequencing: examples in Asparagales and Poaceae. *American Journal of Botany*. **99** (2): 330–348.

Steele, P.R. and Pires, J.C. (2011). Biodiversity assessment: State-of-the-art techniques in phylogenomics and species identification. *American Journal of Botany*. **98**: 415-425.

Steele, P.R., Friar, L.M., Gilbert, L.E. and Jansen, R.K. (2010). Molecular systematics of the Neotropical genus Psiguria (Cucurbitaceae): Implications for phylogeny and species identification. *American Journal of Botany*. **97**:156-173.

Steyn, E.M.A. and Smith, G. F. (2001). Are ovules and seeds in Lomatophyllum Willd? (Aloe Sect. Lomatophyllum sensu auct.) Anatropous and exarillate? *Bothalia.* **31**: 237–240.

Stock, J. T. (2008). Are humans still evolving? Technological advances and unique biological characteristics allow us to adapt to environmental stress. Has this stopped genetic evolution? *European Molecular Biology Organization* EMBO reports Volume **9**.

"Studies in Conservation", (1998). Studies in Conservation, volume. **43**.

Stull, G.W., Moore M.J., Mandala V.S., Douglas N.A., Kates H.-R., Qi X. and Brockington S. F. (2013). A targeted enrichment strategy for massively parallel sequencing of angiosperm plastid genomes. *Applications in Plant Sciences.* **1**: 1200497.

Sunyaev, S., Warren, Lathe. and Peer, B. (2001). Integration of genome data and protein structures: prediction of protein folds, protein interactions and ‘molecular phenotypes’ of single nucleotide polymorphisms. *Current Opinion in Structural Biology.* **11**:125-130.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C. and Willerslev, E. (2007). Power and limitations of the chloroplast trn L (UAA) intron for plant DNA barcoding. *Nucleic Acids Research*. **35**(3): e1–e8.

Tachida, H. and Yoshimaru, H. (1996). Genetic diversity in partially selfing populations with the stepping-stone structure. *Heredity.* **77**(5): 469-475.

Takundwa M, Nepolo E, Mogotsi K, Kandawa-Schulz MA, Cullis AC, Kunert K, Jackson-Malete JJ, Chiwona-Karltun L, Chimwamurombe PM (2012). Development and use of microsatellite markers in Marama bean. *African Crop Science Journal.* **20** (2):95 – 105.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary genetic analysis version 6.0. *Molecular Biology and Evolution*. **30**: 2725-2729.

Tassy, P. (1986). Construction systématique et soumission au test: Une forme de connaissance objective. In: Tassy P. editors. L'ordre et la diversité du vivant. Paris: Fayard. 83-89.

Tassy, P. (1991). L'arbre à remonter le temps. Paris: Christian Bourgeois (in French).

Tate, J.A, Simpson, B.B. (2003). Paraphyly of Tarasa (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany.* **28**: 723–737.

Tatineni, V., Cantrell, R.G. and Davis, D.D. (1996). Genetic Diversity in Elite Cotton Germplasm Determined by Morphological Characteristics and RAPDs. *Crop Science.* **36**: 186-192.

Templeton, A.R. (2001). Using phylogeographic analyses of gene trees to test species status and processes. *Molecular Ecology Resources*. **10**(3):779-91.

Thomas, J.A., Telfer, M.G. and Roy, D.B. (2004). Comparative losses of British butterflies, birds and plants and the global extinction crisis. *Science*. **303**: 1879-1881.

Tisdell, CA. (2011). Core issues in the economics of biodiversity conservation in “Ecological Economics Reviews.” Robert Costanza, Karin Limburg and Ida Kubiszewski, Eds. *Annals of the New York Academy of Sciences.* **1219**: 99–112.

Timme, R.E., Bachvaroff, T.R. and Delwiche, C.F (2012). Broad Phylogenomics Sampling and the Sister Lineage of Land Plants. *PLoS ONE* 7(1): e29696.

Torres, E., Iriondo, J.M. Escudero, A.N. and Pe´ Rez, C. (2003). Analysis of Within-Population Spatial Genetic Structure in *Antirrhinum Microphyllum* (*Scrophulariaceae*). *American Journal of Botany*. **90**(12): 1688-1695.

Treutlein, J., Smith G. F., Van Wyk B.-E. and Wink M. (2003a). Phylogenetic relationships in Asphodelaceae (subfamily Alooideae) inferred from chloroplast DNA sequences (*rbcL, matK*) and from genomic fingerprinting (ISSR). *Taxon* **52**: 193-207.

Tsukamoto, Y. (1989). Exec. Ed. The Grand Dictionary of Horticulture; Shogakukan: Tokyo, **4**: 293.

Ulukan, H. (2011). The use of plant genetic resources and biodiversity in classical plant breeding. *Acta Agriculturae Scandinavica Section B Soil and Plant Science*. **61**: 97-104.

Unda, F. (2006). Evaluating the Role of the Raffinose Family of Oligosaccharides in Hybrid Poplar (Populus Alba × Grandidentata). The University of British Columbia (Vancouver) © Faride Unda, 2012.

Uribe-Convers, S., Settles, M.L. and Tank, D.C. (2016). A Phylogenomic Approach Based on PCR Target Enrichment and High Throughput Sequencing: Resolving the Diversity within the South American Species of Bartsia L. (Orobanchaceae). *PLoS ONE.* 11(2): e0148203.

Usama, K.A. (2015). Molecular Phylogenetics of Moringaceae Martinov with Emphasis on Ethnomedicinal Plant *Moringa oleifera Lam*. Grown in Egypt. School *of Academy Journal of Biosciences.* **3**(2A):139-142.

Utelli, A., Roy, B.A. and Baltisberger, M. (1999). History can be more important than ` pollination syndrome' in determining the genetic structure of plant populations: the case of *Aconitum lycoctonum* (*Ranunculaceae*). *Heredity* (Edinb). **82** (5):574-84.

Valentiniet, A., Pompanon, F. and Taberlet, P. (2009). DNA barcoding for ecologist. *Trend in ecology and evolution*. **24**: 110-117.

Van der Westhuizen, H.M., Spies, P. and Spies, J.J. (2010). Genetic variation between and within *Clivia nobilis* and *Clivia mirabilis* modified from Taxon.

Van Staden, L.F. and Drewes, S.E. (1994). Knipholone from Bulbine latifolia and *Bulbine frutescens*. *Phytochemistry*. **35**: 685-686.

Van Uffelen, R., de Groot L.M. and Nico S.P. (2005). Floriculture worldwide; production, trade and consumption patterns show market opportunities and challenges. *Agriculture Economics Research Institute.*

Van Wyk, B-E, Van Oudtshoorn, B. and Gericke, N. (1997). Medicinal Plants of South Africa. *Briza Publications*, Pretoria, South Africa: 64-65.

Van Wyk, B.-E., Yenesew, A. and Dagne, E. (1995). Chemotaxonomic significance of anthraquinones in the roots of Asphodeloideae (Asphodelaceae). *Biochemical Systematics and Ecology*. **23**: 277-281.

Van Wyk, B.E., Whitehead, C.S., Glen, H.F., Hardy, D.S., Van Jaarsveld, E.J. and Smith, G.F. (1993). Nectar sugar composition in the subfamily Alooideae (Asphodelaceae). *Biochemical Systematics and Ecology*. **21**:249-253.

Vernooy, R., Haribabu, E., Muller, M.R., Vogel, J.H. and Schindel, D.E. (2010). Barcoding Life to Conserve Biological Diversity: Beyond the Taxonomic Imperative. *PLoS Biology.* **8**(7): e1000417.

Veteläinen, M., Negri, V. and Maxted E, N. (2009). European landraces: management and use. *Biodiversity International*. Via dei Tre Denari, 472/a 00057 Maccarese, Rome, Italy.

Vié, J.-C., Hilton-Taylor, C., Pollock, C., Ragle, J., Smart, J., Stuart, S.N. and Tong, R. (2008). The IUCN Red List: a key conservation tool. In: J.-C. Vié, C. Hilton-Taylor and S.N. Stuart (Eds). The 2008 Review of the IUCN Red List of Threatened Species. IUCN Gland, Switzerland.

Vigueira, C.C., Olsen, K.M. and Caicedo, A.L. (2013). The red queen in the corn: agricultural weeds as models of rapid adaptive evolution. *Heredity*. **110**(4): 303–311.

Von Staden, L., Ebrahim, I. and Claassens, J.G. (2011). *Bulbinella* calcicola J.C. Manning and Goldblatt. *National Assessment*: Red List of South African Plants version 2015.1.

Von Staden, L., Raimondo, D., and Dayaram, A. (2013). Taxonomic research priorities for the conservation of the South African flora. *South African Journal of Science*. **109**, Art. #1182, 10 pp.

Wahlberg, N., Braby, M.F., Brower, A.V.Z., Jong, R., Ming-Min, L., Nylin, S., Pierce, N.E., Sperling, F.A.H., Vila, R., Warren, A.D. and Zakharov, E. (2005). Synergistic effects of combining morphological and molecular data in resolving the phylogeny of butterflies and skippers. *Proceedings of Biological Science*. **272**(1572): 1577–1586.

Wakasugi K, (1998) Genetic code in evolution: switching species-specific aminoacylation with a peptide transplant. *The EMBO Journal*. **17**(1):297-305

Wang X, Augusto S. Auler, R. L. Edwards, Hai Cheng, Emi Ito, Yongjin Wang, Xinggong Kong, and Maniko Solheid. (2007). Millennial-scale precipitation changes in southern Brazil over the past 90,000 years. *Geophysical Research Letters*. **34:** L23701.

Warren RL, Yang C, Vandervalk BP, Behsaz B, Lagman A, Jones SJM, (2015**).** Software and supporting material for "LINKS: Scalable, alignment-free scaffolding of draft genomes with long reads". GigaScience Database.

Watt, J.M. and Breyer-Brandwijk, M.G. (1962). The Medicinal and Poisonous Plants of Southern and Eastern Africa, Livingstone, Edinburgh, 2nd Edition: 695-696.

Webb, C.J., Johnson, P.N., Sykes, W.R. (1990). Flowering plants of New Zealand, Christchurch, Botany Division, Department of Scientific and Industrial Research.

Weaver, N. (2002). Molecular Systematics. Animal Sciences. Encyclopedia.com. 8 Mar. 2016 <http://www.encyclopedia.com.

Wegrzyn, J.L., Lee, J.M., Tearse, B.R. and Neale, D.B. (2008). Tree Genes: A forest tree genome database. *International Journal of Plant Genomics.* 412875.

Wheeler, Q. (2005). Losing the plot: DNA "barcodes" and taxonomy. *Cladistics*. **21**:405–407.

White, T.J., Bruns T, Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications, pp. 315–322. *Academic Press*, Orlando, Florida.

Wiley, E.O., Siegel-Causey, D., Brooks, D.R. and Funk, V.A. (1991). The compleat cladist: A primer of phylogenetic procedures. *University of Kansas Museum of Natural History Special Publication* No. **19**.

Will, K.W. and Rubinoff, D. (2004). Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics*. **20**:47-55.

Wilson, E. O. (2003). The encyclopaedia of life. *Trends in Ecology and Evolution*. **18**: 77-80.

Winston, J.E. (1999). Describing species: Practical taxonomic procedures for biologists. New York: *Columbia University Press*.

Woodruff, D. (2001). Populations, Species and Conservation Genetics. University of California, San Diego. *Encylopedia of Biodiversity*: **4**.

Wyman, S.K., Jansen, R.K., Boore J.L. (2004). Automatic annotation of Organellar genomes with DOGMA. *Bioinformatics.* **20** (17): 3252-3255.

Xi, Z., Ruhfel, B.R., Schaefer, H., Amorim, A.M., Sugumaran, M., Wurdack, K.J., Endress, P.K., Matthews, M.L., Stevens, P.F., Mathews, S., Davis, C.C., (2012). Phylogenomics and a posteriori data partitioning resolve the Cretaceous angiosperm radiation Malpighiales. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (43), 17519–17524.

Yadav, S. and Srivastava, J. (2014). Of Recent Scientific Research Article Genetic Diversity Analysis On *Moringa Oliefera* by Using Different Molecular Markers: *A Review.* **2278** (5): 2277–2282.

Young, M.K., Mckelvey, K.S., Pilgrim, K.L. and Schwartz, M.K. (2013). DNA barcoding at riverscape scales: assessing biodiversity among fishes of the genus *Cottus (Teleostei*) in northern Rocky Mountain streams. *Molecular Ecology Resources.*

Yu Song, Dong, W., Liu, B., Chao, Xu C., Yao, X., Gao, J. and Corlett, R.T. (2015). Comparative analysis of complete chloroplast genome sequences of two tropical trees *Machilus yunnanensis* and *Machilus balansae* in the family Lauraceae. *Frontiers in Plant Science.* 6:662.

Young, N.D. and dePamphilis, C.W. (2000). Purifying selection detected in the plastid gene *matK* and flanking ribozyme regions within a group II intron of nonphotosynthetic plants. *Molecular Biology and Evolution*. **17**: 1933–1941.

Zahlbruckner, A. (1990). *Bulbinella punctualata*. *Annalen des k.k. Naturhistorischen Hofmuseums*. **15**(1): 16, 17.

Zhou, Y. (2010). Genetic diversity of *Rehmannia glutinosa* cultivars based on sequence-related amplified polymorphism markers. *Scientia Horticulturae.* **125**(4):789–794.

Zoschke, R. (2009). Analysis of the regulation of *matK* gene expression: *Endocytobiosis Cell Research*. 19: 127-135.

Zwickl D.J. (2006). Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation. The University of Texas at Austin.

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Table 8.1: Morphological variations of *Bulbinella* in South Africa and New Zealand

Species	Leaves	Diagnostic Feature	Flowers	Seeds	Plant Height	Habitat
Bulbinella chartacea	3-5 per plant, erect barely developed at flowering time	The basal sheathing fibres being very loose, straight and papery clearly distinguish *Bulbinella chartacea* from all other species. Both the *Bulbinella chartacea* and *Bulbinella trinervis* flowers at the same time of year often in similar areas, but *Bulbinella trinervis* has white flowers (Perry, 1999).	20-40 flowers, stellate to recurved.	Dullish black, up to 4.5mm long and 3mm in diameter	0.4m high	700- 1100m in rocky areas
Bulbinella ciliolata	12-40 per plant, Erect to sub erect	Is easily distinguished from *Bulbine elegans* by the fibrous sheath which is loose and straight whereas in *Bulbine elegans* sheaths are compactly reticulate (Perry, 1999).	Stellate flowers with +/-125	Black with flat brown hyaline extension, 2.75mm wide & 4.75mm long	Lower than 0.6m in height	Sandy loamy soils

Bulbinella gracillis	Erect to sub erect, 4-8 per plant	The absence of dead leaf remains forming a fibrous sheath around the stem and leaf bases, is not encountered in any other *Bulbinella species* in South Africa, except in *Bulbinella gracilis* (Perry, 1999).	20-80 flowers, stellate	Black with amber-colour hyaline extension, 2mm long and 1mm wide	Up to 0.3m high	Dampish areas
Bulbinella trinervis	narrow leaves, 5-7 per plant,	Owing to the narrow leaves *Bulbinella trinervis* may be mystified with *Bulbinella triquetra;* but the presence of the non-sheathing leaf bases, small bracts and also the smaller seeds distinguishes the two.	30-60 flowers, Stellate white flowers	Black smaller seeds, 3.5mm long, 2mm wide	Up to 0.4m high	Rocky, clay soils
Bulbinella divaginata	Up to 40 per plant, filiform, semiterete, dark green	The membranous white cataphylls surrounding the base of the leaves, which show beyond the fibrous remains, is a crucial diagnostic characteristic (Perry, 1999).	20-150 flowers, stellate and green	4.0m long and 2mm wide	Up to 0.45m high	Fine clays to sandy soils

Bulbinella nana	10-20 per plant, equal and erect.	The species have close resemblance with *B. gracilis*, but it is the smallest (0.25m tall) of the *Bulbinella* species forming dainty, delicate-looking plants	15-30 flowers, stellate.	Black, 2.25m long	0.25m high	
Bulbinella latifolia	5-10 per plant	The main difference between *Bulbinella nutans* and *Bulbinella latifolia* is in the plant size, with *Bulbinella latifolia* being taller (up to 1m) (Perry, 1999). There are also differences in their leaves. The leaves of *Bulbinella latifolia* are significantly broader, arched, and more spreading than those of *Bulbinella nutans,* which are erect and narrow	Up to 500 flowers, stellate.	6.5mm long, 3.75m wide	Up to 1m high	Granitic soils, sandy and peaty soils.
Bulbinella cauda-felis (white cats tails)	5-11 per plant, cream coloured.	The large dull black seeds and thin walled, pale fawn capsule are considered as significant	50-150 flowers, stellate.	5mm long and 3mm wide	04-0.8m high	Sandy, flats on clayey soils.

		diagnostic characters for the species				
Bulbinella barkerae	6-13 per plant, sub erect to spreading.	Is separated from the other species with ciliate margins *(B ciliolata),* on locality and also on the broader and few leaves. The strong-smelling flowers are characteristics of *B barkerae* which separates it from *B cauda-felis*, which is a similar species	60-100 flowers, stellate.	4mm long and 1mm wide.	Up to 0.6m high	Stony, sandy soils.
Bulbinella calcicola	broader, channelled leaves	Is most similar to *B. triquetra* but differs in its broader, channelled leaves with narrowly cylindrical racemes and flowers that are orange-tipped (Manning and Goldblatt 2010).	flowers are orange-tipped			Saldanha Limestone Strandveld
Bulbinella erbuniflora	3-7 per plant.	The characteristic that makes *B. erbuniflora* distinct is the ivory-white flowers which habitually have a strong musty odour.	50-200 flowers, stellate.	3.5m long and 2.5mm wide.	0.75m high	Silty loamy soils

Bulbinella triquetra	narrow leaves, 10-40 per plant.	*Bulbinella triquetra* have yellow flowers *as B. divaginata* but the two are separated by the sheathing leaf bases in *B. triquetra*, whereas in *B. divaginata* the fibrous sheath is formed from separate cataphylls (Perry, 1999).	50- 80 flowers, stellate	3.5mm long and 1.75mm wide	Up to 0.35m high	Damp depression, organic rich sandy soils.
Bulbinella graminifolia	4-9 per plant,	It is closely related to *B. cauda-felis*, but is distinguished from that species by its considerably finer, reticulate fibrous sheath. Furthermore, the fruit and the seeds of *B graminifolia* are just about half the size of those of *B cauda-felis*. The inflorescence of *B graminifolia* is shorter than the one of *B. cauda-felis*	70-100 flowers, stellate.	2.5mm long.	Up to 0.65m high	Stony, clayey or loamy soils.

Bulbinella elata	6- 8 per plant	it has close resemblance to *B. latifolia* and *B. nutans* it differs in having leaves that are flat, spreading, coriaceous, non-canaliculate, which are thinner and more delicate when pressed than those of *B. latifolia*	200-500 flowers, stellate	4.5mm long	Up to 1m tall	Clayey soils, granitic sandy soil.
Bulbinella elagans	3- 25 per plant, erect and sub equal.	It possesses the dense reticulate fibrous sheath which separates it from *B ciliolata* which has a loose straight fibrous sheath (Perry, 1999). It seems to be most morphologically similar to *B. triquetra*, but is taller (Perry, 1999).	70-100 flowers, stellate	4.5mm long	Up to 0.6m tall	Sandy or shale derived soils, clayey soils.
Bulbinella nutans	Rosete forming, erect, 5-13 per plant	*Bulbinella nutans* can be distinguished from *B. latifolia* by its broader and shorter inflorescence (Perry, 1999). However, they can be hard to	100-250 flowers, stellate	7mm long and 3.25mm wide.	0.3-0.8m high	Clayey or peaty soils

		identify when pressed. The main difference between *B. nutans* and *B. latifolia* is in the plant size, with *B. latifolia* being taller (up to 1m) (Perry, 1999). There are also differences in their leaves. The leaves of *B. latifolia* are significantly broader, arched, and more spreading than those of *B. nutans,* which are erect and narrow				
Bulbinella punctualata	2-4 per plant	Is a very unique species because of its little number of leaves, which are comparatively longer and narrower than of *B. latifolia* and is also distinguished from the rest of other *Bulbinella* species by its long and narrow inflorescences with yellow flowers (Perry, 1999). Also its	75- 150 flowers, stellate.	5.5mm long and 3.5mm wide.	0.5- 1.0m tall	Sandy soils

		loose net-like veins sheath, with the inner cataphyll extending for some distance up the leaves				
Bulbinella potbengensis	Not known	It has close resemblance to *B. punctualata* (Perry, 1999) but it has a single long leaf and neatly reticulate sheath that makes the species unique.	40-50 flowers, stellate	Not known	Medium sized, actual size not known	Clayey soils
Bulbinella anguistifolia		The species is hermaphroditic and its plants are smaller in size than that of *Bulbinella hookeri*.	50 flowers		smaller than that of *Bulbinella hookeri.*	
Bulbinella gibbi balanifera		Is closer to *Bulbinella rossii* than to *Bulbinella hookeri* but altogether smaller plant with much slenderer shape and very much shorter and more open raceme both varieties of *Bulbinella gibbsii* are gynodioecious	40 or fewer flowers per raceme			

Bulbinella modesta		*Bulbinella modesta* is hermaphroditic and it differs from all described New Zealand species in its short lax raceme (Moore, 1964).	*10-20 flowers.*			
Bulbinella rossi	Erect, 60 x 8cm	It is only *Bulbinella rossii* that possesses fibrous leaf bases and it is therefore considered to bear the closest physical resemblance to plants of the South African genus (Perry, 1987).	50 flowers with short pedicels	6mm long, dark and narrow winged.	height of more than 1m	Swampy areas
Bulbinella hookeri	Leaf 20-75cm long	*Bulbinella hookeri* is hermaphroditic, with a columnar habit	contain more than 50 flowers	5-6mm long	height of 0.4m	Seepages and wet areas

8.2: Genes removed from the alignment of plastid protein- coding sequences (*Bulbinella* species)

atpE, atpF, atpH, atpI, ccsA, cemA, clpP, infA, matK, ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK, orf188, orf42, orf56, petA, petB, petD, petG, petL, petN, psaA, psaB, psaC, psaI, psaJ, psbA, psbB, psbC, psbD, psbE, psbF, psbG, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ, psi_psbT, rbcL, rpl14, rpl16, rpl2, rpl2, rpl20, rpl22, rpl23, rpl33, rpl36, rpoA, rpoB, rpoC1, rpoC2, rps11, rps12,rps14, rps15, rps16, rps18, rps19, rps2, rps3, rps4, rps7, rps8, rrn16, rrn23, rrn4.5, rrn5, trnA-UGC, trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnfM-CAU, trnG-GCC, trnG-UCC, trnH-GUG, trnI-CAU, trnI-GAU, trnK-UUU

Table 8.2: Thirty-Four (34) Protein-Coding Genes From 21 *Bulbinella* And Their Functions

GENE	FUNCTION
rpl2	Actin-binding and Alpha-amylase inhibitor.
rps19	Proteins conjugated with ribonucleic acid (RNA).
ycf1	Is essential for plant viability and encodes Tic214, a vital component of the Arabidopsis TIC complex.
rps16	Ribosomes, the organelles that catalyse protein synthesis, consist of a small 40S subunit and a large 60S subunit.
accD	Essential for leaf development and might be required to maintain the plastid compartment.
atpA	The atpA gene encodes the a-subunit of the chloroplast ATP synthase.
atpB	atpB genes encode beta subunits, of chloroplast ATP synthase.

atpE	atpE genes encode epsilon subunits, of chloroplast ATP synthase.
atpF	Component of the F_0 channel, it forms part of the peripheral stalk, linking F_1 to F_0.
atpH	Responsible for the expression of the ATP synthase III subunit
atpI	Produces synthase IV subunit.
cemA	Involved in proton extrusion and indirectly promotes efficient inorganic carbon uptake into chloroplasts.
clpP	Provides instructions for making the ClpP subunit protein.
infA	Acts as transcription antiterminator and has RNA chaperone activity in vivo and in vitro.
ndhC	NDH-1 shuttles electrons from NADH, via FMN and iron-sulfur (Fe-S) centers, to quinones in the respiratory chain.
ndhJ	quinone binding
ndhK	Metal ion binding and NADH dehydrogenase (ubiquinone) activity
petA	Apocytochrome F precursor.
petB	Encoding the cytochrome B6 subunit.
petD	Encode for the cytochrome b6/f complex subunit 4.
petG	Is required for either the stability or assembly of the cytochrome b6-f complex.
petL	Is important for photoautotrophic growth as well as for electron transfer efficiency and stability of the cytochrome b6-f complex.

petN	Mediates electron transfer between photosystem II (PSII) and photosystem I (PSI), cyclic electron flow around PSI, and state transitions.
psaA	RNAs function as cis-regulatory elements of these genes
psaB	Encode proteins that form subunits in the photosystem I structure used for photosynthesis.
psaI	Photosystem I reaction center subunit VIII.
psaJ	photosystem I reaction center subunit IX
psbB	It binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII.
psbC	photosystem II CP43 protein
psbD	photosystem II protein D2
psbE	Tightly associated with the reaction center of photosystem II (PSII) and With its partner (PsbF) binds heme.
psbF	With its partner (PsbE) binds heme.
psbG	Component of NADH/NADPH dehydrogenase which acts to reduce plastoquinone.
psbH	Codes for the so called 9k Da or 10k Da phosphoprotein
psbJ	Encode a low molecular weight polypeptide of PSII.
psbL	Codes for a gene product of 37 residues after removal of the initiating N-formyl methionine residue.
psbM	One of the components of PSII and it binds multiple chlorophylls, carotenoids and specific lipids.
psbN	May play a role in photosystem I and II biogenesis.
psbT	Seems to play a role in the dimerization of PSII

psi_psbT	It binds chlorophyll and helps catalyze the primary light-induced photochemical processes of PSII
psbZ	Controls the interaction of photosystem II (PSII) cores with the light-harvesting antenna.
rpl14	cadherin binding, RNA binding and structural constituent of ribosome
rpl16	chloroplast gene encoding for the ribosomal protein L16
rpl20	Binds directly to 23S ribosomal RNA and is necessary for the in vitro assembly process of the 50S ribosomal subunit. It is not involved in the protein synthesizing functions of that subunit.
rpl22	Among its related pathways are Metabolismand Viral mRNA Translation.
rpl23	RNA binding, structural constituent of ribosome, transcription coactivator binding and ubiquitin protein ligase binding
rpl33	Structural constituent of ribosome and translation.
rpl36	Structural constituent of ribosome and cytoplasmic translation.
rpoA	Encoding the alpha subunit of RNA polymerase
rpoB	Encodes the β subunit part of RNA polymerase.
rpoC1	Codes for the RNA polymerase (β) beta′ subunit.
rpoC2	DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates.
rps2	Encodes for proteins
rps3	a DNA repair endonuclease and ribosomal protein, is involved in apoptosis
rps4	protein resistant to P. Syringae 4

rps8	Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S and Metabolism.
rps11	Encodes a member of the S17P family of ribosomal proteins that is a component of the 40S subunit.
rps12	RNA binding source and structural constituent of ribosome.
rps14	Encodes for proteins
rps18	Is involved in the binding of fMet-tRNA, and thus, in the initiation of translation.
ycf2	ATP binding and protein import into chloroplast stroma
ycf3	is essential for the accumulation of the photosystem I (PSI) complex and acts at a post-translational level
ycf4	Required for the assembly of the photosystem I complex.
ycf68	Play a role in photosynthesis.
ycf15	probably not a protein-coding gene because the protein in these species has premature stop codons
ndhB	2 iron, 2 sulfur cluster binding and electron carrier activity.
orf42	DNA packaging
orf56	nucleic acid binding and RNA-DNA hybrid ribonuclease activity
rpl2	transferase activity, structural constituent of ribosome and RNA binding
rpl23	Encodes a ribosomal protein that is a component of the 60S subunit.
rps7	provides instructions for making one of approximately 80 different ribosomal proteins

[Source: (Hallick, 1984), http://www.uniprot.org/uniprot/Q8KPP7; (Wakasugi *et al*, 1998); (Fox, 2003); (Farchaus & Dilley, 1986); (Nixon et al., 1989); (Gudynaite-Savitch *et al*, 2006) and Rudd, (2000)]

APPENDIX II

This appendix includes all the genomes used and the results of 34 genome trees for *Bulbinella* species of South Africa

8.7: Gene Trees

The ***atpA*** region have a complete alignment included **1519** nucleotide positions. This gene region some potential to identify *Bulbinella* based on a monophyletic grouping with a weakly posterior probability support of (PP= 0.59) in the first group; the second group is weakly supported with only the posterior probability of (PP=0.59) in the BI cladogram and the third group had a weakly supported posterior probability and bootstrap support of (PP=0.73 & BS=54.5%) respectively. Group 4 has a weakly bootstrap support of (BS= 55.5%) (Appendix A1).

The ***atpF*** had a complete alignment included **409** nucleotide positions and the gene had both weak statistical support of posterior probability and bootstrap support of (PP=0.67 and 64.8%) in the first grouping; the second group is moderately supported with a posterior probability of (PP=0.78) & a weak bootstrap support of (BS=55.8%); lastly the third group has both weak statistical support of posterior probability and bootstrap support of (PP=0.64 & BS=60.5%) for the support for monophyletic groupings of *Bulbinella* species in South Africa (Appendix A2).

The ***atpH*** gene region had a complete alignment included **244** nucleotide positions. The gene is unable to clearly distinguish amongst *Bulbinella* species except for one monophyletic grouping with only a weak bootstrap support of (BS=59.3%) in the ML and BI cladogram (Appendix A3).

Sequences of the ***atpI***, region were obtained for 15 *Bulbinella* South African species and the complete alignment included **741** nucleotide positions. The **atpI**, groups the genus *Bulbinella* in 3 groups with the first group having a weakly supported posterior probability and bootstrap support of (PP= 0.74 & BS=58%); second group has a weak posterior probability and a moderate bootstrap support of (PP= 0.72 & BS=84.4%) respectively; the third group has a weakly supported posterior probability and a weak bootstrap support of (PP= 0.56 & BS=60.2%) in the BI and ML cladogram (Appendix A4).

The ***ccsA***, has a complete alignment included **989** nucleotide positions. This gene region some potential to identify *Bulbinella* based on a 4 monophyletic groupings with a very strong bootstrap support and posterior probability (BS=99% & PP = 0.97) in the ML cladogram for the first group. The second group is strongly supported with a posterior probability and bootstrap support of (PP= 0.98 & BS=94%); third group a very strong posterior probability of (PP= 0.96) & a weak bootstrap support of (BS=62%); the fourth group is strongly supported with a posterior probability and bootstrap support of (PP= 0.85 & BS=98%) respectively in the BI and ML cladogram (Appendix A5).

The ***matK*** gene has its complete alignment included **1588** nucleotide positions. The gene groups species into 5 monophyletic groupings (Appendix A6) had only a weak posterior probability support in the first grouping (PP=0.68); with high posterior probability and weak bootstrap support in the second group (PP=0.9 & BS=68%); third group is strongly supported with both posterior probability and bootstrap support of (PP=0.97 & BS=81%); the fourth group has a weak bootstrap support of (BS=61%) and a very strong posterior probability of (PP=0.93) and lastly group five is strongly supported with a posterior probability support for the monophyletic grouping of (PP=0.87).

The complete alignment of ***ndhA*** gene included **538** nucleotide positions. The ***ndhA***; groups the genus *Bulbinella* into 3 monophyletic groups with both strong posterior probability in the first group and strong bootstrap support of (PP= 1.00 & BS=94.9%); and the second group has strong posterior probability and a strong bootstrap support of (PP= 0.98 & BS=87%) in the BI and ML cladogram (Appendix A7).

The ***ndhB*** gene has a complete alignment which included **754** nucleotide positions. The gene was unable to clearly distinguish amongst *Bulbinella* species but groups the genus as one group except a monophyletic group with a moderate posterior probability support of (PP=0.79) and a weak bootstrap support of (BS=56%) in the ML and BI cladogram (Appendix A8).

The ***ndhD*** has a complete alignment included **1504** nucleotide positions. The gene region have some potential to identify *Bulbinella* based on 4 monophyletic groupings (Appendix A9). The first group is strongly supported with both posterior probability and bootstrap support of (PP=1.00 & BS=93.4%) respectively; the second group have very high posterior probabilities and weak bootstrap support of (PP=0.99 & BS=63.3%); third group have very high posterior probability and a weak bootstrap support of (PP=0.96 & BS=64.4%) respectively; fourth group has a very strong bootstrap support of (BS=100%) and a very strong posterior probability of (PP=1.00).

The ***ndhE*** gene region had a complete alignment included **304** nucleotide positions and groups the genus *Bulbinella* with a high posterior probability and a moderate bootstrap support of (PP=0.99 & BS=75.7%) for the first group and the second group is strongly supported only with a moderate posterior probability of (PP=0.77) for the monophyletic grouping in *Bulbinella* species (Appendix A10).

The ***ndhF*** had a complete alignment included **2071** nucleotide positions. The gene groups species into 5 monophyletic groupings (Appendix A11) based on a monophyletic grouping with the first grouping of high posterior probability and a weak bootstrap support (PP=0.95 & BS= 63.8%) respectively, the second group has a very high posterior probability and a strong bootstrap support (PP=0.97 & BS=90.5%); third group has a strong posterior probability support of (PP=0.96) and a weak bootstrap support of (BS=59.2%); fourth group has a moderate support of (BS=75.6%) and a strong posterior probability of (PP=0.87) and group five has a weak posterior probability support of (PP=0.74) and a strong bootstrap support of (BS=87%) for the monophyletic grouping.

The ***ndhG*** gene region had a complete alignment included **529** nucleotide positions and the gene region groups the genus *Bulbinella* based on 4 monophyletic groupings (Appendix A12). The first group had a very high posterior probabilities and weak bootstrap support of (PP=0.98 & BS=62.8%) respectively; second group had a very high posterior probabilities and weak bootstrap support of (PP=0.95 & 61.1%); third group had a very high posterior probabilities and weak bootstrap support of (PP=0.98 & BS=65.3%) respectively, and lastly the fourth group has a very strong bootstrap support of (BS=95.3%) and a very strong posterior probability of (PP=1.00).

The ***ndhH*** gene region had the complete alignment included **1180** nucleotide positions and the gene groups the genus *Bulbinella* with a very high posterior probability support and a bootstrap support of (PP=0.99 and BS=87%) in the first grouping, followed by second group with a very high posterior probability support of (PP=0.99) and a weak bootstrap support of (BS=65%); third group has a very high posterior probability and bootstrap support of (PP=1.00 & BS=100%); and lastly the fourth group has a very high posterior probability and bootstrap support of (PP=0.99 & BS=82.1%) respectively for the support for monophyletic groupings of *Bulbinella* species in South Africa (Appendix A13).

The ***ndhI*** gene region had a complete alignment included **541** nucleotide positions. The gene groups species into 4 monophyletic groupings (Appendix A14) based on a monophyletic grouping with the first grouping with weak posterior probability and a weak bootstrap support (PP=1.00 & BS=89.8%) respectively, the second group has a strong posterior probability support of (PP=0.95) and a weak bootstrap support of (BS=56.2%); third group has a weak bootstrap support of (BS=60%) and a strong posterior probability of (PP=0.89) and lastly the fourth grouping with weak posterior probability and a weak bootstrap support (PP=0.71 & BS= 62%).

The ***orf42*** gene region had a complete alignment included **117** nucleotide positions and groups the genus *Bulbinella* into one monophyletic group (Appendix A15) with only a high bootstrap support of (BS=91.4%).

The ***orf56*** gene region had a complete alignment included **165** nucleotide positions. The gene is unable to clearly distinguish amongst *Bulbinella* species with only one monophyletic (Appendix A16) with a very weak bootstrap support of (BS=54.4%) for the first group.

The ***orf188*** gene region had a complete alignment included **565** nucleotide positions. The *orf188*; groups the genus *Bulbinella* into 3 monophyletic groups (Appendix A17) with both high posterior probability and a strong bootstrap support of (PP=0.99 & BS =88.9%) in the first group, followed by second group with only a weak posterior probability of (PP= 0.65) and lastly the third group has both high posterior probability and bootstrap support of (PP=0.99 & BS=92.8%).

The ***psaC*** gene had a complete alignment included **244** nucleotide positions. The ***psaC***; groups the genus *Bulbinella* into 2 monophyletic groups with a highest posterior probability of (PP=0.96) & a weak bootstrap support of (BS =62.8%) in the first group, followed by second group with a very strong posterior probability of (PP= 0.97) & a weak bootstrap support of (BS=61.3%) in the BI and ML cladogram (Appendix A18).

The ***psbA***, had a complete alignment included **1060** nucleotide positions. The ***psbA***; groups the genus *Bulbinella* into 3 monophyletic groups with both weak posterior probability and bootstrap support of (PP=0.50 & BS=71.5%) in the first group, followed by second group with a moderate posterior probability of (PP= 0.83) & a weak bootstrap support of (BS=62%) and lastly the third group has strong posterior probability and a weak bootstrap support of (PP= 0.91 & BS=74.4%) in the BI and ML cladogram (Appendix A19).

The ***rbcL*** gene region had a complete alignment included **1453** nucleotide positions and the gene groups the genus *Bulbinella* with a very high posterior probability support and a weak bootstrap support of (PP=0.99) and (BS=60%) in the first grouping, followed by second group with a very high posterior probability support of (PP=0.99) & a moderate bootstrap support of (BS=81%); the third group has a very high posterior probability and bootstrap support of (PP=0.99) & a weak bootstrap support (BS=64%); and the fourth group has highest posterior probability and strong bootstrap support (PP=1.00 & BS=96.3%) respectively for the support for monophyletic groupings of *Bulbinella* species in South Africa (Appendix A20).

The ***rpl2*** gene had a complete alignment included **447** nucleotide positions and groups the genus *Bulbinella* with both highest posterior probability of (PP=1.00) and very strong bootstrap support of (BS=100%) for the first group, followed by high posterior probability of (PP=0.89) and a weak bootstrap support of (BS=61%) in the second group (Appendix A21).

The ***rpl23*** gene had a complete alignment included **280** nucleotide positions. This gene region was unable to distinguish *Bulbinella* species with only 1 monophyletic grouping with a weak bootstrap support (BS=51.6%) and a weak posterior probability of (PP=0.72) in the ML cladogram (Appendix A22).

The ***rpoB*** had a complete alignment included **3208** nucleotide positions. The gene groups species into 4 monophyletic groupings (Appendix A23) based on a monophyletic grouping with the first grouping of a moderate posterior probability and a weak bootstrap support (PP=0.83 & BS= 62.3%) respectively, the second group has both weak support from posterior probability and bootstrap support (PP=0.70 & BS=54.8%); third group has only a strong posterior probability support of (PP=0.86);

and lastly the fourth group has a strong bootstrap support of (BS=86%) and a weak posterior probability of (PP=0.72).

The ***rpoC1*** had a complete alignment included **1618** nucleotide positions. The ***rpoC1****;* groups the genus *Bulbinella* into 3 monophyletic groups (Appendix A24) with both weak posterior probability and bootstrap support of (PP=0.72 & BS =61.4%) in the first group, followed by second group with a weak posterior probability and a weak bootstrap support of (PP= 0.72 & BS=61.4%) and lastly the third group has only a weak bootstrap support of (BS=62.8%).

The ***rpoC2****;* had a complete alignment included **4119** nucleotide positions. The gene groups species into 4 monophyletic groupings (Appendix A25) based on a monophyletic grouping with the first grouping of a moderate posterior probability and a weak bootstrap support (PP=0.79 & BS= 62.8%) respectively, the second group has a strong support from posterior probability and a weak bootstrap support (PP=0.87 & BS=72.3%); third group has both strong posterior probability support and strong bootstrap support of (PP=0.89 & BS=87.8%); and lastly the fourth group has a moderate bootstrap support of (BS=80.1%) and a weak posterior probability of (PP=0.52).

The ***rps2****;* has a complete alignment included **709** nucleotide positions. The gene groups species into 4 monophyletic groupings (Appendix A26) based on a monophyletic grouping with the first grouping with weak posterior probability and a weak bootstrap support (PP=0.69 & BS= 66.7%) respectively, the second group has a weak posterior probability and a weak bootstrap support (PP=0.63 & BS=55.9%); third group has a moderate posterior probability support of (PP=0.84) and a weak bootstrap support of (BS=85%); fourth group has a weak bootstrap support of (BS=56.5%) and a strong posterior probability of (PP=0.87)

The ***rps7*** has a complete alignment included **466** nucleotide positions and groups the genus *Bulbinella* with a high posterior probability and a weak bootstrap support of (PP=0.94 & BS=63.7%) for the first group and the second group is strongly supported with a very strong posterior probability and a weak bootstrap support of (PP=0.99 & BS=62.7%) for the monophyletic grouping in *Bulbinella* species (Appendix A27).

The ***rps12***; has a complete alignment included **255** nucleotide positions. The gene was unable to clearly distinguish amongst *Bulbinella* species and there was no monophyletic grouping in the ML and BI cladogram (Appendix A28).

The ***rps15*** has a complete alignment included 303 nucleotide positions. The ***rps15***; groups the genus *Bulbinella* into 3 monophyletic groups (Appendix A29) with both weak posterior probability and bootstrap support of (PP=0.52 & BS =57.8%) in the first group, followed by second group with a weak posterior probability and a moderate bootstrap support of (PP= 0.63 & BS=78.9%) and lastly the third group has a strong posterior probability and a weak bootstrap support of (PP= 0.87 & BS=62.7%).

The ***rps16***; had a complete alignment included **216** nucleotide positions. This gene region was unable to distinguish *Bulbinella* species with only one monophyletic grouping with only a weak bootstrap support (BS=58.9%) in the ML cladogram (Appendix A30).

The ***rps19***, had a complete alignment included **281** nucleotide positions. This gene region was unable to distinguish *Bulbinella* species with only 1 monophyletic grouping with a moderate bootstrap support (BS=83.2%) and a weak posterior probability of (PP=0.72) in the ML cladogram (Appendix A31).

The ***ycf2***, had a complete alignment included **6901** nucleotide positions. The gene is unable to clearly distinguish amongst *Bulbinella* species. The gene groups species into 5 monophyletic groupings (Appendix A32) had a strong posterior probability and very strong bootstrap support in the first grouping (PP=1.00) & BS=100%); the second group have high posterior probability and a moderate bootstrap support of (PP=1.00 & BS=80.3%); third group is strongly supported with posterior probability and a weak bootstrap support of (PP=0.99 & BS=62.5%); the fourth group has a strong bootstrap support of (BS=85.1%) and a very strong posterior probability of (PP=1.00) and group five is strongly supported with both a posterior probability and bootstrap support for the monophyletic grouping of (PP=0.63; BS=51.5%). Lastly group 6 is weakly supported with posterior probability of (PP=0.6).

The ***ycf15***; had a complete alignment included **186** nucleotide positions. The gene is unable to clearly distinguish amongst *Bulbinella* species except for one monophyletic grouping with very posterior probability and a strong bootstrap support of (PP=1.00 & BS=100%) of in the ML and BI cladogram (Appendix A33).

The ***ycf68***, had a complete alignment included **268** nucleotide positions. The gene was unable to clearly distinguish amongst *Bulbinella* species and there was no monophyletic grouping with both in the ML and BI cladogram (Appendix A34). The gene has very low bootstrap support for the *Bulbinella* clade and cannot be used as a barcoding region on an intergeneric level.

SUMMARY

The taxonomy of *Bulbinella* has been poorly studied. Yet these plants are important geophytes in South African and New Zealand, of which some species are threatened or endangered. This research thesis conducted phylogenetic comparisons paired to morphological characteristics to address this deficit. Phylogenetic comparisons were based on four genes, including barcoding genes, namely the *matk*, *rbcl*, *psbA-trnH* and *ITS*. The chloroplast genomes of South African species were also obtained in order to conduct a phylogenomic study and to identify additionaly genes suitable in distinguishing different *Bulbinella* species. The first research question aimed to assess if *Bulbinella* species from South Africa and New Zealand were monophyletic or if they belonged to different genera. The results showed that the New Zealand species did indeed group in *Bulbinella* and do not represent anything distinct. The second research question was to study the species status of species in more detail and identify potential problems in the biosystematics of the genus. Some species were shown to be potentially synonymous, while others were potentially paraphyletic. Some species also grouped basally to the other *Bulbinella* species and it was uncertain if these species represent *Bulbinella*. The last research question was whether tools could be developed to identify the various species. The *matk*, *psbA-trnH* and *ITS* genes were shown to have the most resolution for species description, while the addition of the thirty-four genes used in the phylogenomic approach on representatives of the *Bulbinella* species from South Africa, significantly improved statistical support for the topology of the final phylogenetic tree. A number of additional genes for species identification were also identified. Our studies thus established DNA sequences that can be used as DNA

barcodes and multigene phylogenies for the genus for the first time which will strengthen the taxonomy and future studies of the genus. These will also aid identifications by users of the plants for medical applications, the ornamental industry, as well as facilitate biodiversity and conservation efforts to protect the diversity of this genus. However, our results showed that there is a great need for increased sampling and morphological supported studies for these species, and a number of taxonomic issues to be resolved.