In Vitro Propagation of *Haemanthus pumilio* and *H. albiflos* (Amaryllidaceae) and the population genetics of *H. pumilio*

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Pectora roborant cultus recti

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DECLARATION

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ENGLISH - ABSTRACT

Haemanthus albiflos and *H. pumilio* are members of the Amaryllidaceae. *H. albiflos* is a widespread evergreen plant, while *H. pumilio* is an endangered narrow endemic species with only two known populations remaining. These populations, remnants of the one recently transferred from Wellington to the Stellenbosch University Botanical gardens and another in the Duthie Reserve in Stellenbosch, present with vastly different morphologies. It is therefore vital to understand the phylogenetics and population genetics of *H. pumilio* as well as to design a method of *in vitro* propagation to increase the numbers of these plants.

This study analysed the phylogenetics of *H. pumilio* using non-coding nuclear (Internal transcribed spacer), plastid (*trnL-trnF* intergenic spacer) and mitochondrial (*nadi1477*) gene regions and the population genetics using inter-simple sequence repeats (ISSR) and start codon targeted (SCoT) polymorphisms. The resulting phylogenetic trees revealed that *H. pumilio* is closely related to *H. canaliculatus* and *H. sanguineus.* The dendrograms from the DNA fingerprinting separated the two populations into distinct groups, while also revealing higher within population diversity than between them. While this is unexpected in rare narrow endemics, it might be explained by recent divergence from a more widespread species, *H. sanguineus*. These levels of genetic diversity are encouraging for species conservation as they suggest that intrapopulation breeding will not be detrimental to population survival. However, the two populations may become more homogenous as there exists a high level of gene flow between them.

A micropropagation protocol designed using *H. albiflos* plants was able to successfully multiply individuals from leaf explants within 8 weeks, likely due to its ability to readily multiply by offsets. This protocol proved difficult to replicate on *H. pumilio* and only a single treatment with 0.1 mg/L BAP and 2 mg/L NAA differentiated after 13 weeks. However, the study was able to identify two fungal species contaminating *H. pumilio* cultures that could be endophytes.

Future population genetics studies should aim to ascertain whether the translocation of plants from the Wellington population has increased the gene flow, thus subsequently reducing the interpopulation diversity. Additionally, this study provides crucial preliminary results on an optimum *H. pumilio* tissue culture method and is the first to identify possible endophytes of this species. Future micropropagation research should be conducted into providing an understanding of the plant-symbiont relationship to ascertain whether the symbionts confer necessary advantages to the plant that can be exploited to improve *H. pumilio* tissue culture.

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AFRIKAANS - OPSOMMING

Haemanthus albiflos en *H. pumilio* is lede van die Amaryllidaceae. *H. albiflos* is 'n wydverspreide, immergroen plant, terwyl *H. pumilio* 'n bedreigde, nou-endemiese spesie is waarvan daar slegs twee populasies oor is waarvan ons weet. Hierdie populasies, waarvan oorblywende lede van een onlangs vanaf Wellington oorgeplaas is na die Stellenbosch Universiteit botaniese tuin en die ander in die Duthie reservaat in Stellenbosch, toon sterk morfologiese verskille. Dit is daarom van kardinale belang om die filogenie en populasie genetika van *H. pumilio* te verstaan, en om 'n metode van *in vitro* vermeerdering te ontwikkel om die aantal individue van hierdie plante te vermeerder.

Hierdie studie het die filogenie van *H. pumilio* bestudeer deur van die nie-koderende kern (interne transkriberende plekhouer (spacer)) en plastiede (*trnL-trnF* intergeen plekhouer en nadi1477) geenareas gebruik te maak, en die populasie genetika deur van inter-eenvoudige basisvolgorde herhalings (ISSR) en beginkodon geteikende polimorfismes (SCoT) gebruik te maak. Die resulterende filogenetiese boom het getoon dat *H. pumilio* naverwant is aan *H. canaliculatus* en *H. sanguineus.* Die dendrogramme van die DNS vingerafdrukke het die twee populasies as twee aparte groepe geskei, en ook aangedui dat daar groter interne variasie is as variasie tussen die twee. Alhoewel dit onverwag is vir skaars, nou-endemiese spesies, mag dit verduidelik word as onlangse diversifikasie vanaf 'n eens meer wydverspreide spesie, *H. sanguineus*. Hierdie vlakke van genetiese diversiteit is bemoedigend vir die bewaring van die spesie, aangesien dit suggereer dat intra-populasie teling nie nadelig sal wees vir populasie oorlewing nie. Die twee populasies mag egter meer homogeen raak, aangesien daar 'n hoë vlak van geenvloei tussen hulle is.

ʼn Mikrovermeerdering protokol is ontwerp deur van *H. albiflos* plante gebruik te maak, en kon suksesvol binne 8 weke individue deur blaar-eksplante vermeerder, waarskynlik weens die vermoë van die plant om geredelik deur plantdele te vermeerder. Dit was moeilik om hierdie protokol op *H. pumilio* te herhaal, en slegs ʼn enkele behandeling met 0.1 mg/L BAP en 2 mg/L NAA het eers na 13 weke gedifferensieer. Hierdie studie kon egter twee fungi spesies identifiseer wat *H. pumilio* kulture kontamineer, en hulle mag endofiete verteenwoordig.

Toekomstige populasie genetiese studies moet mik om te bepaal of die translokasie van die Wellington populasie geenvloei vermeerder het, en dus daarna die interpopulasie diversiteit verminder het. Verder verskaf hierdie studie noodsaaklike voorlopige resultate van ʼn optimum weefselkultuur metode, en dit is die eerste studie wat moontlike endofiete binne hierdie spesie geïdentifiseer het. Toekomstige verdere mikro-vermeerdering navorsing moet mik om ʼn beter begrip te verkry van die plant-simbiont verwantskap, om te bepaal of die simbiont nodige voordele aan die plant verskaf wat gebruik kan word om weefselkutuur tegnieke vir *H. pumilio* te verbeter.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	2-Isopentenyladenine
aBayes	A Bayesian like transformation of the approximate likelihood ratio test
AC	Activated charcoal
AFLP	Amplified Fragment Length Polymorphisms
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BAP	6-Benzylaminopurine
CCR	Core Cape Subregion
cpDNA	Chloroplast DNA
CPPU	N-(2-chloro-4-pyridyl)-N′-phenylurea
dicamba	3,6-Dichloro-2-methoxybenzoic acid
DPU	1,3-Diphenylurea
EMR	Effective multiplex ratio
Gst	Genetic differentiation
H	Nei's gene diversity
$HgCl_2$	Mercuric chloride
Hs	Genetic diversity within populations
Ht	Total genetic diversity
I	Shannon's information index
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter-Simple Sequence Repeat
ITS	Internal transcribed spacer
LS	Linsmaier and Skoog
MI	Marker index
MS	Murashige and Skoog
mT	Metatopolin
mtDNA	Mitochondrial DNA
NAA	α-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
nDNA	Nuclear DNA
Ne	Effective number of alleles
Nm	Gene flow
NN	Nitsch and Nitsch

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PIC	Polymorphism information content
RAPD	Random Amplified Polymorphic DNA
SANBI	South African National Biodiversity Institute
SCoT	Start Codon Targeted
SUBG	Stellenbosch University Botanical Gardens
TDZ	Thidiazuron
trnL-F	trnL-trnF intergenic spacer
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

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CHAPTER 1: INTRODUCTION

1.1 Background

The Core Cape Subregion (CCR), previously known as the Cape Floristic Region or Cape Floristic Kingdom, is located at the south western tip of Africa and covers an area of about 90 000 km^2 (Linder, 2003; Manning and Goldblatt, 2012). This is about 0.5% of Africa's area, yet it contains approximately 20% of the total number of known plant species on the continent (Manning and Goldblatt, 2012). This region contains around 9 380 known vascular plant species in 997 genera, which represents about half of all genera in southern Africa and 20% of the species found on the continent (Manning and Goldblatt, 2012). About 150 of these genera, several being from six endemic or near-endemic families, are endemic to the CCR. These elevated numbers of overall and endemic flora are highly unusual, even considering the increased species diversity expected in Mediterranean regions, compared to the rest of southern Africa (Linder, 2003; Manning and Goldblatt, 2012).

Peculiarly, only 220 (2%) of these 9 380 Cape species are trees, with almost two thirds of the total vegetation being shrubs and perennial herbs, and a distinctly large proportion, 17.2%, being geophytes (Manning and Goldblatt, 2012; Bergh et al., 2014). The region has a proportionally low number of annual plants compared to other semi-arid climates (Manning and Goldblatt, 2012). This unique composition of vegetation in the Cape is partly due to the low levels of soil nutrients measured in most Cape soils. Additionally, the large number of geophytes has been attributed to the predictable seasonal rainfall patterns, with the summer-dry phytogeographic centres of the region possessing the largest proportion of geophytic plants (Procheş et al., 2006; Manning and Goldblatt, 2012) . These plants remain dormant throughout the dry season by making use of subterranean bulbs, corms or rhizomes, thus allowing them to avoid the effects of drought and wildfires that are common in the area (Kruger and Bigalke, 1984; Manning and Goldblatt, 2012). Although the dicotyledonous genera *Oxalis* L., *Pelargonium* L'Hér. and *Othonna* L. represent a large percentage of the total number of geophytes in the CCR (Procheş et al., 2006; Snijman, 2013), most of the geophytes are monocotyledonous belonging to several families (Manning and Goldblatt, 2012), including the family Amaryllidaceae (Procheş et al., 2006).

The Amaryllidaceae is divided into several subfamilies, including the subfamily Amaryllidoideae, within which the genus *Haemanthus* L. is included in the tribe Haemantheae (Müller-Doblies and Müller-Doblies, 1986; Meerow and Clayton, 2004). In 1984 (Snijman, 1984), this mostly southern African genus was known to be comprised of 22 species, separated between the summer and winter rainfall regions of southern Africa. The winter rainfall region being home to 15 *Haemanthus* species, while six species are restricted to the summer rainfall region and one species occurs in both regions (Snijman, 1984). However, since then the species *H. avasmontanus* was thought to be extinct until a possible rediscovery in 2010 (Craib, 2010). As the South African National Biodiversity Institute's

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(SANBI) Plants of Southern Africa (POSA) website lists 21 accepted species (South African National Biodiversity Institute, 2020a), this will be the number used throughout this thesis. The taxonomic revision of the genus by Snijman (1984) described the morphology, habitat and reproductive habits of *Haemanthus* plants in detail. During the rainfall season, *Haemanthus* plants develop flowers that range in colour from white to dark pink or red (Snijman, 1984). They bear globular or elliptical fruit, which become pulpy, soft and aromatic as they ripen. Their enclosed seeds are recalcitrant, soft and succulent, and thus unable to survive desiccation or animal digestion. Therefore, although it was previously thought that these seeds are animal vectored, this view has been discarded based on the delicate nature of the seeds and the lack of evidence of animals feeding on the fruit (Snijman, 1984). Snijman (1984) instead concluded that seeds of *Haemanthus* species most likely are dispersed by water flow following rain, or through the elongation and subsequent collapse of the peduncles near the mother plant (Figure 1.1).

Figure 1.1: An inflorescence of *Haemanthus pumilio* leaning as the fruit weighs it down. *Photograph: Dr PN Hills*

Following pollination and subsequent wilting of the flowers, *Haemanthus* plants generally develop two leaves, which persist throughout the rainy season and wither after the rains cease as the plant returns to its dormant state. Most species become dormant in order to survive, storing nutrients in tunicated subterranean bulbs (Snijman, 1984). Interestingly, the two evergreen species, *H. albiflos* Jacq. and *H. deformis* Hook.f., do not lose their leaves annually and may display up to six leaves at any given time (Snijman, 1984). Although not evergreen, the winter rainfall species *H. canaliculatus* Levyns can also retain leaves for more than a year (Snijman, 1984). The leaves of *Haemanthus* species are mostly suberect, with evidence of prostrate leaves evolving independently eight times within the genus (Snijman, 1984).

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Haemanthus albiflos Jacq. is the only species that occurs in both the winter and summer rainfall regions. It is endemic to the Western Cape, Eastern Cape and KwaZulu-Natal regions of South Africa and is listed as least concern on the South African National Biodiversity Institute's (SANBI) Red List for endangered species (Duncan, 2002; South African National Biodiversity Institute, 2020b). *H. albiflos* readily forms bulblets and is the only species within *Haemanthus* that can reproduce clonally (Snijman, 1984; Rabe and van Staden, 1999). Rabe and van Staden (1999) also showed that *H. albiflos* readily formed shoots on leaf explants in hormone-free media in tissue culture. This ability to easily multiply both in the wild and in tissue culture, as well as the availability of leaf material year-round, makes *H. albiflos* ideal for use in establishing and optimising a tissue culture protocol for other *Haemanthus* species. Unfortunately, this same ease of replication also means that *H. albiflos* may not be a perfect model for *Haemanthus* species that are more difficult to culture. However, as other *Haemanthus* species were not as freely available for tissue culture optimisation, this study used *H. albiflos* as a template for the tissue culture of the rarer *H. pumilio.*

Haemanthus pumilio Jacq., a winter rainfall species, produces pink flowers and prefers habitats that are marshy and waterlogged during the winter months and dry during the warmer summer months (Summerfield, 1990). These very specific habitat requirement makes it difficult to re-establish populations of *H. pumilio* in new habitats when its habitat is repurposed as farming or residential land. Its largest known locality is within the Duthie Reserve in Stellenbosch, where Summerfield (1990) recorded 1046 living plants. This population had shrunk to 37 flowering plants in a recent count in 2017 (West, 2017). Thus, this species has been listed as endangered in the SANBI Red List (South African National Biodiversity Institute, 2020b). Summerfield (1990) also noted two other populations, one in Klapmuts and another on the Meerlust farm in the Stellenbosch region, with a total of 62 individuals. By 2020, neither of these populations remained, as the land had been repurposed for agriculture.

Another population not mentioned by Summerfield (1990) was identified at the Newton Commonage in Wellington. Eleven individuals from this population were subsequently relocated to the Stellenbosch University Botanical Gardens (SUBG) as housing was developed on their native site (West, 2017). The individuals from these two remaining populations, the Duthie Reserve and SUBG, display consistent morphological differences, such as the Duthie Reserve plants typically having smaller leaves (Figure 1.2) and producing a sap that stains skin orange. Additionally, plants from the SUBG generally possess an increased number of flowers compared to those from the Duthie Reserve, though there are a few exceptions (Figure 1.3), which may either be attributable to genetic differences or phenotypic responses to variation in their environments. The genetic diversity within different populations of *H. pumilio* has, however, never been studied, with previous studies restricted to morphological and ecological assessments only (Summerfield, 1990). Previous phylogenetic

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studies focused on the genetic relationships between different species within the tribe Haemantheae (Meerow and Clayton, 2004; Conrad, 2008).

Figure 1.2: Differences in plant sizes of *Haemanthus pumilio*. Plants from the Duthie Reserve (left) produce leaves around 3 times smaller than those from the SUBG (right). *Photograph: Dr GI Stafford*

Figure 1.3: Differences in *Haemanthus pumilio* inflorescences. Most of the Inflorescences from the Duthie Reserve typically have less than nine flowers per stem (A) with a few deviations from this norm possessing a greater number of flowers per stem (B) similar to the SUBG plants (C) which can produce more than 13 flowers per stem. *Photograph: Dr PN Hills*

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1.2 Outline of this study

This project had three core aims:

1. To analyse the phylogenetic relationship between the two *H. pumilio* populations (Duthie Reserve and Wellington population now housed in the SUBG) to determine whether the observed phenotypic differences are due to genetic variability or environmental differences.
2. To assess the population genetics among and within clumps of plants at the Duthie Reserve to evaluate the levels of clonality and inform better decisions regarding individuals used for micropropagation. Plants produced through micropropagation will, in future, be used to repopulate extinct populations, therefore a high genetic diversity will be required to maintain the viability of such populations.
3. To develop a tissue culture protocol that enables micropropagation of *H. pumilio* to bulk up the number of individuals for distribution to botanic gardens and other collections to safeguard the species, as well as for possible repopulation of existing and extinct populations.

The first experimental chapter (Chapter 3) covers the first two aims of this project and was achieved using a combination of DNA fingerprinting and genetic marker analyses. Firstly, organellar and nuclear DNA markers were used to evaluate the genetic variation among individuals of the *H. pumilio* populations. Since the individuals from the Duthie Reserve are notably smaller than those currently in the SUBG, the ploidy levels of plants from the two populations were also analysed to determine whether differing DNA content could underpin some of the observed morphological differences. This chapter also assessed the population genetics of *H. pumilio* using microsatellite analysis of the individuals within clumps at the Duthie reserve, using inter-simple sequence repeat (ISSR) markers and start codon targeted (SCoT) polymorphism.

The second experimental chapter (Chapter 4) focused on the development and optimisation of a tissue culture protocol using *H. albiflos*, which can be used for the micropropagation of *H. pumilio* to increase wild populations. For this, plant leaf and bulb material of *H. albiflos* was first cultured *in vitro* to produce plantlets, as this species is not endangered and leaf material was available throughout the year. The optimal protocol was then used for the attempted multiplication of *H. pumilio* from the limited material available for the species. Additionally, the flowers of *H. pumilio* plants were artificially pollinated, the fruit harvested, and the seeds germinated *in vitro.*

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CHAPTER 2: PHYLOGENETICS, POPULATION GENETICS AND *IN VITRO* PROPAGATION OF AMARYLLIDACEAE SPECIES: A REVIEW

2.1 Introduction

The Amaryllidaceae is a family of mostly geophytic, perennial plants distributed throughout the Southern Hemisphere. These plants are of considerable scientific and conservational interest due to the bioactive, family-specific compounds they produce, their horticultural value and the high numbers of threatened species within this family. In order to produce large quantities of Amaryllidaceae species for conservation, horticulture and the analyses of their alkaloid content, *in vitro* propagation methods can be most useful. For conservation purposes, it is also critical that the genetic composition of Amaryllidaceae plants be investigated to preserve levels of genetic diversity in plants used to repopulate habitats. The aim of this review is to provide an overview of the methods of phylogenetic and population genetic analysis as well as tissue culture techniques that have been applied to members of the Amaryllidaceae, and to present an overview of the current conservation statuses and uses of a number of Amaryllidaceae species.

2.2 Amaryllidaceae

The Amaryllidaceae, a family in the order Asparagales, was thoroughly revised by Meerow and Snijman (1998). Members of this family are typically bulbous, perennial geophytes, consisting of mostly terrestrial plants, but also including a few epiphytes and aquatics. The family is diagnosed by producing one to many large, sessile or pedicellate flowers in umbellate inflorescences, protected by involucral bracts that tear along multiple fissure lines once flowers are mature (Stevens, 2017). The infra-familial divisions of Amaryllidaceae have been edited and reorganised several times (Hutchinson, 1934; Dahlgren et al., 1935; Hutchinson, 1959; Stevens, 2017). The most recent revision by Meerow et al. (1999) circumscribed the family into a monophyletic clade, including three subfamilies; the Agapanthoideae, Allioideae, and Amaryllidoideae. These subfamilies were previously recognized as the distinct families, Agapanthaceae, Alliaceae and Amaryllidaceae, respectively (Meerow et al., 1999). The distribution of Amaryllidaceae shows high endemism in specific geographic regions, occurring mostly in Africa, South America and the Mediterranean (Meerow and Snijman, 1998).

Several plants within the Amaryllidaceae are commercially cultivated and sold for their horticultural or nutritive value. Due to high demand, there is a need for faster production rates, but seed cultivation is not always a viable option. As a result, research has been conducted to create and optimise *in vitro* propagation of these economically important plants. Several ornamental and food species, including members of the *Allium* L., *Crinum* L., *Cyrtanthus* Schreb., *Narcissus* L., and *Hippeastrum*

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Herb. genera have been used in tissue culture research to determine the feasibility of micropropagation of these plants (Ayabe and Sumi, 1998; Smith et al., 1999; Fennell et al., 2003; Ncube et al., 2015; Tubic et al., 2016). Several South African species within the Amaryllidaceae are listed as endangered, vulnerable or near threatened, which may be attributed to overharvesting for ornamental or medicinal purposes, habitat loss and/or a decline in pollinators (South African National Biodiversity Institute, 2020b).

Members of the Amaryllidaceae produce several family-specific, biogenetically-related alkaloids, resulting in several genera of this family being used in traditional medicine to treat various ailments (Meerow and Snijman, 1998), with reported use in Africa (Crouch et al., 2005; Bay-Smidt et al., 2011) and Asia (Takos and Rook, 2013). In Africa, these plants are traditionally used to treat a wide range of ailments from broken bones, chronic coughs and inflammation (Louw et al., 2002; Crouch et al., 2005) to mental illnesses (Bay-Smidt et al., 2011), including Alzheimer's disease (Orhan and Şener, 2005; Bay-Smidt et al., 2011), and have been in use since before the invention of modern medicine. Due to their importance in traditional healing, which often leads to over-harvesting, several populations of Amaryllidaceae species are declining in the wild. More than half of South Africa's threatened plants species are available in the traditional medicine markets (Williams et al., 2013), including the Amaryllidaceae members *Amaryllis paradisicola* Snijman, *Brunsvigia josephinae* Ker-Gawl., *Brunsvigia litoralis* R.A.Dyer*, Haemanthus deformis* and several *Clivia* Lindl. species (Snijman et al., 2007a; Williams et al., 2008b; Williams and Crouch, 2011; Snijman et al., 2015) as well as other species that may be mistaken for these due to similar morphological characteristics. Over-harvesting is also, in part, attributable to collection of plants for sale in non-medicinal markets as ornamentals. Horticultural trade contributes to the threat on several Amaryllidaceae species including *Haemanthus canaliculatus* Levyns*, Haemanthus graniticus* Snijman*, Haemanthus pumilio, Brunsvigia radula* W.T.Aiton*, Clivia gardenii* Hook. and *Clivia miniata* Regel (Snijman, Raimondo, van Staden *et al.*, 2013; Snijman, Raimondo & Victor, 2007; Snijman & Victor, 2004b,c; Williams, Raimondo, Crouch, Cunningham, Scott-Shaw, Lotter *et al.*, 2008; Williams, Raimondo, Crouch, Cunningham, Scott-Shaw, Lötter *et al.*, 2008).

Other than the harvesting of wild plants, populations are also reduced by the destruction or fragmentation of habitats. As a result of an increasing human population and industrialisation, natural habitats of many plants are being cleared to provide room for housing, farming and other human (Brooks et al., 2002; Heywood and Iriondo, 2003). Areas such as the CCR, with a great number of endemic species, are termed biodiversity hotspots (Manning and Goldblatt, 2012). These regions cover 1.4% of Earth's land area, but are home to 44% of known plant species (Brooks et al., 2002). Worldwide, these regions are disproportionately affected by anthropogenic activity. The CCR alone has over 5 600 threatened or extinct endemic plant species, and the region's plant taxa are the most threatened in southern Africa, with 71% of all of South Africa's vulnerable flora located in the CCR

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(Manning and Goldblatt, 2012). An additional 1749 species in the CCR are predicted to become extinct should the currently undisturbed area of the region be reduced by just another 1 000km^2 (Brooks et al., 2002).

Inasmuch as no correlation was found in temperate climates between habitat loss due to changes in land use and species endangerment (Giam et al., 2010), several Amaryllidaceae species populations are declining due to agricultural, industrial and urban development of their habitats. For instance, *Haemanthus canaliculatus, Haemanthus graniticus, Haemanthus lanceifolius* Jacq*, Brunsvigia elandsmontana* Snijman*, Clivia gardenii* and *Clivia miniata* populations are declining due to increasing grazing pressure and clearing of land for crop farming (Snijman and Victor, 2004c; Snijman et al., 2007b; Snijman and Raimondo, 2007a; Snijman and Raimondo, 2007b; Williams et al., 2008a; Williams et al., 2008b). *Apodolirion macowanii* Baker*, Haemanthus nortieri* Isaac and *Haemanthus pumilio* populations have been lost or greatly reduced due to urban development (Summerfield and van der Walt, 1992; Snijman and Victor, 2004d; Dold et al., 2007; West, 2017), while mining has caused a reduction in habitable land for *Brunsvigia gariepensis* Snijman and *Brunsvigia radula* (Snijman et al., 2013; von Staden, 2013).

In addition to habitat clearing for human activity, climate change, an increasingly critical global concern, can also lead to a loss in plant habitat. Climate change can intensify the susceptibility of significant areas around the world to habitat loss and fragmentation by altering land-cover, reducing the ability of species to cope with these changes, thereby leading to an increased risk of extinction (Travis, 2003; Mantyka-Pringle et al., 2015). The correlation between habitat loss due to climate change and plant endangerment in biodiversity hotspots is positive (Giam et al., 2010), as species endemic to these regions require specialised habitats and therefore have a relatively reduced ability to adapt to changes (Travis, 2003). Climate change does not only impact plant biodiversity directly. Alterations to pollinator or seed disperser populations also impacts plant populations, as symbioses are integral to a well-functioning ecosystem. Changes in the climate were predicted to compound the risks of extinction of land mammals and birds to changes in land-cover (Mantyka-Pringle et al., 2015).

Plant pollinators and seed dispersers are also threatened by modifications to natural habitats, pesticide use in agriculture as well as invasion by alien species (Bond, 1994). Although plants appear to be more resilient to habitat fragmentation than other taxa, the true extent of habitat loss on plant populations may be masked by perennial plants that can survive for lengthy periods of time (Donaldson et al., 2002). This was evidenced by the significant reduction in the number of plant-pollinator interactions observed by Geslin et al. (2013) in urban landscapes as opposed to semi-natural landscapes where more than half of the evaluated land area was covered by forest and grassland. However, even where there are still considerable plant-pollinator interactions, pollination

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may not always occur, as has been observed in a study of *Brunsvigia* Heist species. *Brunsvigia litoralis, B. orientalis* Ait. ex Eckl. and *B. josephinae* are all sunbird-pollinated plants (Meerow and Snijman, 1998). However, in rural environments, *B. litoralis* is pollinated by the long billed Malachite Sunbird, and in more urban settings its only visitor is the Greater Double-collared Sunbird, which is short-billed and resorts to tearing the flower petals if unable to reach the nectar, resulting in no pollination (Geerts and Pauw, 2012).

Habitat alterations also affect the populations of seed dispersers and plant pollinators. The reduction of plant reproduction resulting from declines in these population sizes has a potentially critical effect of habitat alteration on plant species diversity (Donaldson et al., 2002). Pollinator extinction leads to a rapid decline in plant species diversity (Memmott et al., 2004) and plants that require specialised pollinators have a decreased probability of surviving pollinator extinction (Donaldson et al., 2002). Some plants therefore compensate for this increased extinction risk by being self-compatible, capable of vegetative reproduction or having extended lifespans (Bond, 1994). However, many plants in the Amaryllidaceae do not exhibit these compensatory characteristics. For instance, a single butterfly species, *Aeropetes tulbaghia* L.*,* is the sole pollinator of *Nerine sarniensis* Herb*, Brunsvigia marginata* (Jacq.) W.T.Aiton*, Cyrtanthus elatus* (Jacq.) Traub*, Cyrtanthus guthrieae* L.Bolus and *Cyrtanthus montanus* R.A.Dyer*,* yet the only Amaryllidaceae genus known to be fully self-compatible is *Cyrtanthus* (Meerow and Snijman, 1998). Although it is possible for a few species in a genus to be self-compatible, none of the other exclusively *A. tulbaghia*-pollinated species are (Meerow and Snijman, 1998).

To remedy the population declines of Amaryllidaceae plants due to overharvesting, habitat loss, climate change or variations in mutualistic interactions, *in vitro* propagation techniques may be applied as a method of species conservation. The term conservation is ambiguous, as it can have two meanings, one which expresses that biological resources should be used in such a way as to prevent depletion or extinction, and another, more well-known definition is concerned with the loss of species and the destruction of their natural environment (Heywood and Iriondo, 2003). The use of *in vitro* propagation, that is propagation of plants in a controlled laboratory environment, to increase populations falls into the second category of these definitions. This method of multiplication is an essential tool for the re-population of these endangered plants, both for conservation purposes as well as to increase the numbers of economically valuable plants. It is, however, critical to understand the population genetics of plants conserved through tissue culture to ensure that the plants re-introduced into the wild are both as genetically diverse as possible, and compatible to the genetic profile of the population into which it is planted back.

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2.3 Genetic diversity

It is important to understand the genetic diversity within populations of endangered species to aid in informed cross-breeding of propagated lines, as increased homozygosity could lead to population or species declines (Heywood and Iriondo, 2003; Laikre et al., 2010). Determination of diversity is usually conducted through the reconstruction of a phylogenetic tree and analysis of population genetics, which gives a graphical representation of the phylogenetic relationships between considered taxa (Niranjan Reddy, 2011). When using DNA or protein sequences, a multiple sequence alignment must first be performed on available sequence data from the species being considered, followed by the selection of an evolutionary model and finally phylogenetic tree reconstruction (Niranjan Reddy, 2011). In addition, DNA fingerprinting methods can be used to discern relationships at the lower taxonomic levels even when no DNA sequence information is available (Lacy, 1988).

Reduction in genetic diversity within a population potentially leads to a decrease in the ability of that population to respond to selective pressures within the environment. As explant tissue culture produces clonal plants, thereby reducing genetic diversity, using cross-pollination for seed production is a method of mitigating this loss of diversity. It is vital to understand the phylogenetic relationships between and within the populations when cross-pollinating and subsequently using *in vitro* tissue culture of seeds as a method of increasing population sizes. This understanding allows researchers to cross distantly related organisms, thereby reducing the probability of inbreeding. Several studies have therefore been conducted seeking to understand the genetic diversity of plant species, specifically for the sake of conservation.

Numerous plastid and nuclear marker genes have been used for species-level phylogenetic reconstruction of members of the Amaryllidaceae (Ito et al., 1999; Lledó et al., 2004; Conrad, 2008; Zhang et al., 2012; Shaw et al., 2014). The non-coding regions are the chloroplast genome's most variable regions, as these tend to accumulate genetic variation faster than protein-coding regions (Shaw et al., 2014). An in-depth taxonomic study of the Amaryllidaceae tribe Haemantheae, conducted by Conrad (2008), made use of four plastid regions, *rpoB-trnC* intergenic spacer, *psbA-trnH* spacer, *rps16* intron and the *trnL-trnF* intergenic spacer. The individual trees obtained using each of these regions were of low resolution with high levels of polytomy, that is, numerous sections of these phylogenetic trees could not be confidently resolved into dichotomous clades. Additionally, clade support was weak, as numerous clades had lower than 70% bootstrap support. Moreover, when the regions were combined into a single plastid tree, polytomies and poor support remained problematic, but most genera (except for *Gethyllis* L. and *Apodolirion* Baker) resolved as monophyletic clades, that is all species within the same genus are descendants of one common ancestor not shared with other genera, with 89% bootstrap support.

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The *trnL-trnF* (trnL-F) intergenic spacer has been used in other studies to determine relationships between Amaryllidaceae species at either the tribal (Haemantheae; Meerow and Clayton, 2004) or specific (*Allium wallichii*; Huang et al., 2000) levels. This chloroplastic DNA fragment was unable to resolve relationships within the tribe Haemantheae, producing a tree with low branch support for several clades, as well as only resolving two genera as monophyletic (Meerow and Clayton, 2004). However, using this region in combination with the *rps16* chloroplast region revealed more variation between populations of *Allium wallichii* Kunth than within them (Huang et al., 2000), thus indicating that although not sufficiently informative when used in isolation, trnL-F can be phylogenetically informative, if used in conjunction with another genetic region in members of the family.

Other studies have made use of *matK*, a chloroplast gene, which was informative enough to differentiate between geographically-separate genera within the same family, but not enough to differentiate between African Amaryllidaceae genera, grouping them into a polytomy (Ito et al., 1999) or between different members of the same species (Lledó et al., 2004). These results contradict results of a recent study examining the most informative genetic regions at the lower taxonomic levels among angiosperms, which found *matK* to be one of the more variable gene regions within the plastome, ranked twelfth out of 107 selected regions (Shaw et al., 2014).

Although plastid genes are useful for many interspecific relationship determinations, several researchers have noted the fallibility of plastid gene markers for differentiation of closely related species and identification of intra-species relationships due to the slow evolution of these genes (Ito et al., 1999; Huang et al., 2000; Lledó et al., 2004; Meerow and Clayton, 2004). Nuclear gene regions are therefore used together with plastid regions for phylogenetic reconstruction. One of the most common nuclear markers used is the internal transcribed spacer (ITS) that has been used in conjunction with several plastid markers (Lledó et al., 2004; Meerow et al., 2006; Conrad, 2008). For several Amaryllidaceae species, this non-coding nuclear DNA region was found to be more reliable, with better bootstrap support in constructed phylogenetic trees, compared to plastid regions (Conrad, 2008), as well as having more variation when comparing organisms of the same species within the same population (Huang et al., 2000).

Combined plastid and ITS sequence data have been successful at resolving species-level relationships (Lledó et al., 2004; Meerow et al., 2006; Conrad, 2008), but are seldom variable enough to reveal intra-specific variation. As a result, a range of different population genetic methods have been developed, including DNA fingerprinting techniques such as amplified fragment length polymorphisms (AFLP), inter-simple sequence repeats (ISSRs), start codon targeted (SCoT) polymorphism and random amplified polymorphic DNA (RAPD; Blears et al., 1998; Savelkoul et al., 1999; Reddy et al., 2002; Andersen and Lübbersted, 2003; Collard and Mackill, 2009; Ng and Tan, 2015; Costa et al., 2016). AFLP makes use of restriction digestion as well as PCR amplification,

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while the other three methods are PCR-based and rely on differential banding patterns on agarose gels to identify differences between organisms (Blears et al., 1998; Ng and Tan, 2015; Costa et al., 2016).

The AFLP technique begins with a restriction digest on purified genomic DNA using two restriction enzymes, and ligation of double-stranded adapters to the 3' and 5' ends of the fragments that remove the original restriction sites (Savelkoul et al., 1999). A PCR is then run using primers complementary to the adapters that extend into the flanking nucleotide sequence. The use of these extra nucleotides reduces the number of fragments amplified as only those with 3' ends complementary to the added nucleotide will be amplified. Following the PCR, a polyacrylamide gel is run, and the DNA fingerprints are compared between genotypes (Blears et al., 1998; Savelkoul et al., 1999).

Inter-simple sequence repeats are composed of several multi-allele loci flanked on both the 3' and 5' ends by microsatellites, regions with repeated DNA motifs of about two to five nucleotides long with varying numbers of repeats that are present in all eukaryotic organisms (Reddy et al., 2002; Ng and Tan, 2015). This technique works by using randomly designed nucleotide repeats, complimentary to microsatellites, as primers of about 16 to 25 bp long, to amplify random segments of DNA within the genome (Reddy et al., 2002). These primers are anchored at either the 3' or 5' ends with degenerate bases that continue into the adjacent sequence. A PCR is then run using a single ISSR primer, and once the reaction is completed, the product is run on an agarose or polyacrylamide gel (Ng and Tan, 2015).

Start codon targeted polymorphisms are another single primer DNA fingerprinting method. This method makes use of 18mer primers, with no degenerate bases, designed from consensus sequences of plant DNA regions surrounding the start codon (Collard and Mackill, 2009). Unlike the other DNA fingerprinting methods discussed here, SCoT markers are targeted to gene polymorphisms and not genomic polymorphisms (Andersen and Lübbersted, 2003).

The RAPD technique makes use of primers with random nucleotide sequences and is a simple, inexpensive and informative method of DNA fingerprinting. Arbitrarily designed 10-mer primers are used to randomly amplify extracted DNA at low annealing temperatures in PCRs using only one primer and the resulting PCR products are run on an agarose gel (Kumari and Thakur, 2014).

In all three fingerprinting methods, following the PCR, the resulting differential DNA banding patterns are noted and scored using binary notation, where a band is marked as '0' if absent and '1' if present (Ng and Tan, 2015; Costa et al., 2016). The most reproducible of these fingerprinting methods is the AFLP (Blears et al., 1998), but it comes at a higher cost (Reddy et al., 2002). Similarly, SCoT markers, which are gene-targeted and therefore more informative for applications in plant molecular genetics, are more expensive to develop than ISSRs and RAPD (Andersen and Lübbersted, 2003). On the other hand, the cheapest method, RAPD, suffers from low reproducibility. ISSRs, which are

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more highly reproducible than RAPD due to the longer primer sequences that allow the use of high annealing temperatures, are also more cost-effective than AFLPs (Reddy et al., 2002). Nevertheless, these techniques all analyse dominant markers and are, therefore, unable to show a clear difference between homozygous dominant and heterozygous individuals (Reddy et al., 2002; Ng and Tan, 2015).

2.4 *In vitro* propagation

Once the genetic variation of a population required for tissue culture has been noted, organisms within the population that have the most distant phylogenetic relationships can be cross-pollinated and the seeds used for *in vitro* tissue culture. As an alternative source of plant material to seeds or embryos, researchers can also choose to make use of various portions of vegetative plant material as explant sources for tissue culture. Tissue culture is a method of plant cultivation that makes use of controlled environments with plants grown on liquid, semi-solid or solid nutrient media with a carbon source. The plant material used for tissue culture must be decontaminated and placed in a sterile container with sterile nutrient media that contains essential elements, and certain vitamins for the supply of required micro- and macromolecules (George et al., 2008). This method affords the researcher more control of the biotic and abiotic interactions of cultured plants.

2.4.1 Micropropagation

Micropropagation is an application of tissue culture that uses explant material from mature plants and has been separated into four stages (George et al., 2008):

Stage I: Selection of donor plants and decontamination. During this stage, organs to be used are selected as explant material, and a surface decontamination protocol is performed to remove any microorganisms present. The decontaminated explants are then placed on aseptic media in sterile containers;

Stage II: Plantlet generation and multiplication. This stage usually involves the incorporation of growth-promoting phytohormones in the media to aid in the formation of recognisable plant organs from the explant. Generally, different hormone compositions are required for root and shoot development;

Stage III: Plantlet development. As the plantlets produced in stage II are too small to grow without the aid of nutrient media, they are separated from the mother explant and encouraged to grow. This also generally involves the addition of phytohormones to the media;

Stage IV: *Ex vitro* culture. This is the final stage of micropropagation and involves the transfer of tissue culture generated plantlets to soil and their acclimation to the *ex vitro* environment ("hardening off"). It is a vital stage in plant multiplication and must be done with great care to ensure the survival of the propagated plants.

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2.4.2 Explant selection

Several methods of micropropagation using explant material from mature plants have been successful on numerous Amaryllidaceae species. Most techniques reported employ the use of direct organogenesis of plantlets or somatic embryogenesis, the induction of embryogenic callus through hormone treatment of specialised explant material, from the explant material of mother plants (George et al., 2008). It is vital to select the best source of explant tissue for tissue culture to increase the possibility of successful micropropagation.

Embryo propagation *in vitro* is a method of plant propagation that begins by excising the embryo from within the seed or fruit (George et al., 2008). This method of plant tissue culture has been used to produce intraspecific hybrids of many Amaryllidaceae species, as embryos of these hybrids often suffer from high rates of abortion due to chromosomal imbalances (Coertze and Louw, 1990; Ma et al., 2001). Embryo propagation involves the excision of the embryo from the seed during developmental stages after the globular stage, that is, between the heart and early cotyledonary phases of embryo development, as later stage embryos are more difficult to culture (Smith, 2012). In one Amaryllidaceae species, *Sternbergia fischeriana* M.Roem., the use of immature embryos in tissue culture produced more bulblets than bulb scale explants (Mirici et al., 2005).

Many studies reported the use of twin scales, which involves the incision of bulbs transversely, then dividing each section longitudinally into twin-scales attached by a piece of the bulb's basal plate (Rabe and van Staden, 1999; Smith et al., 1999; Angulo et al., 2003; Moran et al., 2003; Rice et al., 2011; Daniels et al., 2013; Naidoo et al., 2017). This method of direct organogenesis tissue regeneration in Amaryllidaceae has been found to be more effective, yielding higher numbers of shoots (Fennell et al., 2001) and producing shoots earlier (Rabe and van Staden, 1999) than when leaves are used as explants. However, using bulbs also increases the risk of contamination of the explant (Rabe and van Staden, 1999; Smith et al., 1999; Fennell et al., 2001), due to the increased number of microorganisms in the soil compared to above ground, as well as the open arrangements allowing for the movement of microorganisms between the scales (Fennell et al., 2001). The use of leaf material is, however, non-destructive and is therefore preferred for the propagation of rare or endangered species as it does not lead to the loss of an entire mother plant.

In addition to leaves and bulbs, other organs that have previously been used as explants for micropropagation of bulbous plants include stems, peduncles and ovaries (Hussey, 1975; Novák and Petrů, 1981; Jacobs et al., 1992; Ziv and Lilien-Kipnis, 2000). In a study by Hussey (1975), the stems of *Hyacinth* Tourn. Ex L. plants generated as many bulblets as the leaf explants (45 bulblets per explant), which was double the number of bulblets per bulb explant, two to four weeks earlier than explants from bulb material. Hussey (1975) also used *Hyacinth* ovaries as explants; however,

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these produced no bulblets on basal media and managed to produce a maximum of five bulblets per explant in hormone-treated media, the lowest for all explants tested.

These results were echoed in the study by Ziv and Lilien-Kipnis (2000) using bulb and peduncle explants from four Amaryllidaceae species; two Alliaceae (Allioideae) species, and two Hyacinthaceae species. All peduncle explants used produced more shoots per explant than the bulb material. One species, *Haemanthus coccineus* L., did not produce any shoots from bulb explants but produced four shoots per peduncle explant. Contrary to these studies, *Nerine bowdenii* W.Watson peduncle explants failed to differentiate into shoots (Jacobs et al., 1992), regardless of the addition of growth-regulating hormones. These data show that the successful regeneration of the explant tissue is largely dependent on the plant species used. Therefore, one should take care in selecting the explant source best suited for the species to be studied.

Regardless of the type of explant used in tissue culture, efficient tissue propagation requires the consideration of several other factors. These include an efficient method of explant decontamination, optimal types and concentrations of plant growth regulators, as well as a favourable photoperiod. Additionally, the orientation that the explant material is placed in should ensure the best differentiation and multiplication in culture, as well as the use of activated charcoal and other additives to the media.

2.4.4 Decontamination

Prior to culture, decontamination of plant material harvested from non-sterile environments is required to minimise the loss of viable explants capable of differentiating into plantlets. The environment contains an ecosystem of organisms in intricate relationships that support the life cycles of microorganisms, plants and animals. For this reason, the surfaces of plants obtained from the external environment are likely to be covered in microorganisms. The nutrient-rich media on which plant material is cultured also supports the growth of these fungi and bacteria, which may then outcompete the cultured plant material. Hence, there is a need for an efficient decontamination protocol.

Decontamination of plant material meant for tissue culture is typically completed in a laminar flow hood, which excludes microorganisms through filtration and airflow (George et al., 2008). The use of sodium hypochlorite (NaOCl) or other chlorine-based bleaches in explant decontamination has been documented in several studies. Most protocols in surveyed literature making use of NaOCl utilise an initial rinse in tap or distilled water, followed by a wash step in ethanol then a wash with 0.5 to 2% (v/v) NaOCl, often with the addition of Tween 20 as a surfactant (Takayama et al., 1991; Ulrich et al., 1999; Ziv and Lilien-Kipnis, 2000; Mirici et al., 2005; Daniels et al., 2013; Rahimi Khonakdari et al., 2020). Some protocols omit the ethanol step and either replace it with a wash in antimicrobial

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pesticide or simply use NaOCl decontamination without additional steps (Hussey, 1975; Jacobs et al., 1992; Fennell et al., 2001; Angulo et al., 2003; Moran et al., 2003).

Despite this common use, decontamination with commercial bleach (active ingredient NaOCl) or chlorine gas yielded no clean cultures in a study investigating the efficacies of various decontamination protocols on *Hippeastrum* and *Zephyranthes* Herb. bulbs (Smith et al., 1999). The researchers discovered that the use of Plant Preservative Mixture (Plant Cell Technology, Inc., Washington, DC) yielded cultures with the lowest percentage of contamination, however, a small percentage of these clean cultures showed no signs of growth. The use of NaOCl was also found to be ineffective at decontaminating cultures of *Brunsvigia undulata* F.M.Leight. bulbs (Rice et al., 2011). Other studies have utilised mercuric chloride ($HgCl_2$) in conjunction with ethanol and a fungicide, in place of NaOCl (Anbari et al., 2007; Rice et al., 2011; Mujib et al., 2013; Ncube et al., 2015; Naidoo et al., 2017). One study even noted the use of both $HgCl_2$ and NaOCl in the decontamination of *Hippeastrum johnsonii* (Bury) Herb. (Zakizadeh et al., 2013). However, due to its highly toxic and polluting nature, $HgCl_2$ is not preferred by researchers (Rahimi Khonakdari et al., 2020).

Following decontamination, the explants are placed on tissue culture media to increase the number of individuals. However, there are several considerations to make in order to optimise growth conditions. One such consideration is the addition of plant growth regulators to the media.

2.5 Tissue culture *in vitro*

2.5.1 Media composition

Plant tissues require several elements in varying quantities for optimal growth. Elements that have a direct impact on the plant, are vital for the completion of its life-cycle, or have a specialised action that cannot be replaced in full by another element are regarded as essential (Epstein, 1971). The essential elements required in large amounts, known as macronutrients, are nitrogen, potassium, calcium, phosphorus, magnesium and sulfur (George et al., 2008). On the other hand, iron, nickel, chlorine, manganese, copper, zinc, molybdenum and boron are required in much lower amounts and are termed microelements (George et al., 2008). When propagating plants *in vitro,* media must ideally contain these macro- and micronutrients, vitamins and a source of carbon (Altman and Loberant, 1998).

Several media formulations have been designed to provide the best nutrient quantities for plants in tissue culture. These include Murashige and Skoog (MS; Murashige and Skoog, 1962), Linsmaier and Skoog (LS; Linsmaier and Skoog, 1965), Nitsch and Nitsch (NN; Nitsch and Nitsch, 1969) and Gamborg's (Gamborg, 1968) media, with MS being the most common of these. Additionally, compounds such as phytohormones, activated charcoal, myo-inositol or antibiotics may be required as an additive to media, depending on the purpose of tissue culture (George et al., 2008). Tissue

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culture media may be liquid or made semi-solid by the addition of a solidifying agent such as agar or gellan gum (Altman and Loberant, 1998; George et al., 2008).

2.5.2 Effect of phytohormones

Plant growth regulators are synthetic chemicals that act like natural phytohormones and are capable of altering growth and differentiation of plant tissues (Gaspar et al., 1996). Phytohormones are endogenous plant-growth regulating substances that include cytokinins, auxins, gibberellins, abscisic acid and ethylene. Cytokinins and auxins are the classes of plant hormones most frequently used for *in vitro* tissue culture as they are key components in the regulation of plant growth and development (George et al., 2008). These two classes of phytohormones function antagonistically and control the growth of callus and organised tissue as well as control the division and elongation of cells at the cellular level (Trigiano and Gray, 2005; George et al., 2008). Auxins are produced in the shoot apices of plants and are responsible for root development of plants *in vitro.* Likewise, cytokinins, produced in plant roots, encourage the growth of shoots in cultured plants. The addition of these hormones to tissue culture media in different concentrations promotes root and shoot development. Higher cytokinin and lower auxin concentrations promote shoot development, whereas the opposite is true for root development (Trigiano and Gray, 2005; George et al., 2008).

Different studies on the micropropagation of several Amaryllidaceae species made use of several cytokinin and auxin combinations, with 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) being the most used cytokinin and auxin, respectively (Table 2.1). Some studies also made use of alternative auxins such as indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-2-methoxybenzoic acid (dicamba), picloram, indole-3-butyric acid (IBA) and cytokinins such as zeatin, metatopolin (mT), 2-isopentenyladenine (2iP), kinetin, N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) and thidiazuron (TDZ) (Table 2.1). One study additionally made use of coconut milk, which contains the cytokinin 1,3-diphenylurea (DPU).

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Table 2.1: Cytokinins and auxins that have been used on explants from various organs of Amaryllidaceae species.

Organism	Explant Source	Cytokinin	Auxin	References
***Amaryllis* L.**	Bulbs; Peduncle; Flower Buds	kinetin	IAA[e]; NAA[f];2,4-D[g]	Bapat and Narayanaswamy, 1976
***Hippeastrum* Herb**	Bulbs; Peduncle; Flower Buds	Kinetin	2,4-D; IAA; NAA	Seabrook and Cumming, 1977
***Allium cepa* L.**	Bulb	BAP[a]	IBA[h]; NAA	Hussey and Falavigna, 1980
***Haemanthus katherinae* Baker*;* *Crinum abyssinicum* Hochst. ex A.Rich*;* *Leucojum vernum* L.**	Bulb	Kinetin	2,4-D; IAA, NAA	Kromer, 1985
***Hippeastrum hybridum* Hort**	Bulb	BAP; Kinetin; Zeatin	IAA; NAA	Huang et al., 1990
***Nerine bowdenii* W.Watson**	Bulb, Peduncle	BAP	IBA[h]; IAA; NAA; 2,4-D	Jacobs et al. 1992
***Allium sativum* L.**	Bulb	BAP	NAA	Ayabe and Sumi, 1998
***Hippeastrum* Herb*;* *Zephryanthes* L.**	Bulb	BAP	2,4-D; NAA	Smith et al., 1999
Crinum "Ellen bosanquet"	Bulb	BAP	NAA	Ulrich et al., 1999
***Nerine sarniensis* Herb*; Narcissus tazetta* L.*;* *Dichelostemma multiflorum* (Benth.) A.Heller*;* *Allium ampeloprasum* L.*;* *Eucrosia radiata* Ker Gawl*;* *Haemanthus coccineus* L.**	Peduncle; Pedicel-Peduncle Junction	BAP; kinetin	NAA; IBA; 2,4-D	Ziv and Lilien-Kipnis, 2000
***Crinum variabile* Herb.**	Bulb, Leaf	BAP	NAA	Fennell et al., 2001
***Eucrosia stricklandii* Baker**	Bulb	BAP; TDZ[b]	2,4-D; NAA	Colque et al., 2002
***Cyrtanthus loddigesianus* Herb*; Cyrtanthus speciosus* R.A Dyer**	Bulb	BAP	NAA	Angulo et al., 2003
***Cyrtanthus clavatus (L'Hér.) R.A.Dyer;* *Cyrtanthus spiralis* Burch. ex Ker Gawl**	Bulb	BAP	NAA	Moran et al., 2003

Sternbergia clusiana **Ker Gawl. ex Schult.**	Bulb	BAP	IBA	Oran and Fattash, 2005
Narcissus pseudonarcissus **L.;** ***Galanthus elwesii*** **Hook;** ***Leucojum aestivum*** **L.**	Leaf	BAP	2,4-D; NAA; Picloram	El Tahchy et al., 2011
Brunsvigia undulata **F.M.Leight.**	Bulb	BAP	NAA	Rice et al., 2011
Hymenocallis littoralis **Salisb.**	Root; Bulb	none	2,4-D; Dicamba[j]; IAA; NAA; Picloram	Noormi et al., 2012
Hippeastrum johnsonii **(Bury) Herb.**	Bulb	none	none	Zakizadeh et al., 2013
Gethylilis multifolia **L. Bolus**	Bulb	BAP	NAA	Daniels et al., 2013
Cyrtanthus contractus **N.E.Br;** ***Cyrtanthus obliquus*** **L.f;** ***Cyrtanthus guthrieae*** **L.Bolus**	Bulb	BAP; kinetin; mT[c]; zeatin; TDZ	NAA	Ncube et al., 2015
Allium schoenoprasum **Dumort.**	Bulb, Leaf	BAP; kinetin; Mt; zeatin; TDZ; CPPU[d]	None	Savic et al., 2016
Scadoxus punicus **(L.) Friis & Nordal**	Leaf	BAP; mT	NAA	Naidoo et al., 2017
Leucojum vernum **L.;** ***Leucojum aestivum;*** ***Galanthus woronowii*** **Losinsk;** ***Sernbergia lutea*** **(L.) Ker Gawl.**	Bulb	BAP	IAA; IBA; NAA	Resetár et al., 2017
Lapiedra martinezii **Lag.**	Bulb	2iP[i]; BAP; Kinetin	IBA; NAA	Juan-Vicedo et al., 2019
Leucojum aestivum	Bulb; Leaf	Zeatin	none	Ptak et al., 2019
Rhodophiala bifida **(Herb.) Cabrera**	Bulb	none	none	Reis et al., 2019
Narcissus tazetta	Bulb	BAP	NAA	Rahimi Khonakdari et al., 2020
Narcissus pseudonarcissus	Bulb	BAP	NAA	Ferdausi et al., 2020

a: 6-Benzylaminopurine b: thidiazuron; c: metatopolin; d: N-(2-chloro-4-pyridyl)-N′-phenylurea e: indole-3-acetic acid; f: 1-Naphthaleneacetic acid; g: 2,4-dichlorophenoxyacetic acid; h: indole-3-butyric acid; i: 2-isopentenyladenine; j: 3,6-dichloro-2-methoxybenzoic acid

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Lower auxin to cytokinin ratios are required for the induction of shoots on explants from many Amaryllidaceae plants. This has been demonstrated by studies using *Cyrtanthus* species and found that *C. loddigesianus* (Herb.) R.A.Dyer and *C. speciosus* R.A.Dyer produced the most shoots when grown in media containing an NAA:BAP ratio of 5.4:8.9, approximately 1.5 times as much BAP as NAA (Angulo et al., 2003). Furthermore, *C. clavatus* (L'Hér.) R.A.Dyer *and C. spiralis* Burch. ex Ker Gawl. required double the concentration of BAP to NAA for maximal shoot production from explants (Moran et al., 2003), and other *Cyrtanthus* cultivars produced the highest number of shoots per explant in media containing three to four times as much BAP as NAA (Ncube et al., 2015).

Similarly, in *Crinum variabile* Herb. and the *Crinum* cultivar "Ellen Bosanquet", an increase in the amount of NAA used led to a decrease in the number of shoots produced per explant, while the opposite was true for BAP concentrations (Ulrich et al., 1999; Fennell et al., 2001). However, this was not echoed in tissue culture of all Amaryllidaceae plants. For example, in *Allium sativum* L. cultures, the amount of hormones present in the media did not affect the shoot production by the explants (Ayabe and Sumi, 1998). No combination of NAA and BAP was significantly better than hormone-free media for shoot initiation in cultures of *Gethyllis multiflora* L.Bolus, whilst 0.9 μM BAP with 5.4 μM NAA produced the highest quality shoots (Daniels et al., 2013).

Somatic embryogenesis in *Narcissus papyraceus* Ker Gawl. bulbs required the addition of BAP or 2,4-D to the media (Anbari et al., 2007), and in *Narcissus confusus* Pugsley somatic embryogenesis was promoted in media containing picloram (Sellés et al., 1999). *Leucojum aestivum* L. embryogenesis was promoted by the addition of picloram, dicamba or 2,4-D to the medium (Ptak et al., 2013). However, in all these species, except *N. papyraceus*, somatic embryos were only generated from callus cells de-differentiated from specialised plant tissues. Mujib et al. (2013) observed that the addition of BAP was a requirement for somatic embryogenesis. In media supplemented with 2,4-D, NAA or IAA without a cytokinin present, the formation of somatic embryos was hindered in *Eucharis grandiflora* and *Hippeastrum hybridum*. Neither species formed embryos in the presence of NAA or IAA, but *H. hybridum* developed callus in the presence of 2,4-D. Meanwhile, the formation of *Crinum asiaticum* Blanco embryos was promoted by the addition of 2,4-D without a cytokinin present, but hindered by lone addition of NAA or IAA. In all three of these species, the addition of BAP yielded the highest number of embryos per explant (Mujib et al., 2013).

The best phytohormone concentrations for shoot multiplication were comparable to the hormones that had proved best for shoot induction (Ayabe and Sumi, 1998). However, for *Cyrtanthus clavatus* and *C. spiralis*, transfer to media with 2,4-D as the auxin instead of NAA led to an increase the number of shoots, albeit that these were much smaller than those developed on NAA (Moran et al., 2003). When the shoot induction of *Hymenocallis littoralis* (Jacq.) Salisb. by three cytokinins (2iP, TDZ and zeatin) were compared, 2iP was found to be the best, though no difference was found for

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shoot multiplication (Yew et al., 2010). For *Cyrtanthus contractus, C. guthrieae* and *C. obliquus,* substituting TDZ for BAP led to the highest multiplication of shoots produced, while having mT or kinetin as the cytokinins in media resulted in better quality shoots (Ncube et al., 2015). The use of TDZ as the cytokinin also increased the total number of shoots on *Allium schoenoprasm* explants (Tubic et al., 2016).

Micropropagation of geophytic plants may require a stage for the growth of bulbs and roots. In several studies using explants originating from bulb material, bulblets formed without the initial formation of shoots (Hussey, 1975; Novák and Petrů, 1981; Takayama et al., 1991; Rice et al., 2011). Media enhanced with IAA and NAA led to explant elongation after a few days of tissue culture of *Hyacinth* plants and eventually led to explants producing the highest number of bulblets, provided that there was a greater concentration of the cytokinin in the media (Hussey, 1975). However, increasing the NAA concentration in culture led to increased callus formation, thus reducing the differentiation of the cells. A high auxin concentration was also shown to lead to callus formation in *Amaryllis* (Bapat and Narayanaswamy, 1976) and, in the absence of cytokinin, *Hyacinthus orientalis* (Takayama et al., 1991) also formed callus on the explant, hindering the production of bulblets.

There is no single best phytohormone or phytohormone concentration for the induction and multiplication of Amaryllidaceae plantlets *in vitro.* Therefore the concentration and type of plant hormones in tissue culture can be concluded to be dependent on the plant species and the organ used as the explant source. In addition, it is important to consider other factors *in vitro* propagation of plants including, but not limited to, the photoperiod, explant orientation and the addition of activated charcoal to the medium.

2.5.3 Effect of the physical environment

The photoenvironment in micropropagation includes the flux intensity of light that cultures are exposed to, light wavelength and the photoperiod, that is the length of time tissue cultures are exposed to light in a 24 h period (George et al., 2008). This impacts the photosynthesis of plants, as photosynthesis requires light of wavelengths between 400 and 700 nm, as well as photomorphogenesis, that is the processes of plant development that are regulated by light (George et al., 2008). Photomorphogenesis processes include the internal circadian rhythm (Bastita et al., 2018) and secondary metabolite production (Higuchi and Hisamatsu, 2016) of plants.

Since tissue culture media usually contains a carbon source, photosynthesis is not required, and thus the main effect of the photoenvironment would be on photomorphogenesis (George et al., 2008). Several plant species show differential production of certain metabolites, including phenols and alkaloids, when maintained under different light wavelengths (Bastita et al., 2018). In addition, high light intensities have also been shown to reduce levels of cell division in tubers of *Helianthus tuberosus* L. (Fraser et al., 1967), thereby reducing the growth of these plant organs. The effect light

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has on plant morphogenesis may be due to its effect on several plant hormones, including auxins, brassinosteroids, cytokinins and gibberellins (Halliday et al., 2009; Sassi et al., 2013).

The induction and multiplication of shoots were found to be increased in a 16 h photoperiod compared to an 8 h photoperiod for *Allium cepa* L. (Hussey and Falavigna, 1980). A 16 h photoperiod throughout the culture period is the most commonly reported protocol for the Amaryllidaceae (Jacobs et al., 1992; Rabe and van Staden, 1999; Smith et al., 1999; Fennell et al., 2001; Daniels et al., 2013; Ncube et al., 2015; Tubic et al., 2016). Other studies have maintained explants in darkness for the shoot initiation period, then transferred to a 16 h photoperiod for shoot induction and bulb formation (Angulo et al., 2003; Moran et al., 2003).

The effect of the photo-environment on bulb development of *Amaryllis* plants *in vitro* appears to be species-dependent. *Nerine bowdenii* explants produced bulbs more readily when grown in a 16 h photoperiod as opposed to in complete darkness. Bulbs produced in light were moreover larger than those produced in the dark (Jacobs et al., 1992). This was also true for the size of *Hippeastrum johnsonii* bulbs, which exhibited a negative correlation with the photoperiod (Zakizadeh et al., 2013). Moreover, continuous light proved to be unfavourable to generate bulbs in *Brunsvigia undulata* (Rice et al., 2011). Bulb generation in other species, such as the *Crinum* variant “Ellen Bosanquet”, was unaffected by the photoperiod (Ulrich et al., 1999).

Darkness may be favourable in the production of bulblets as it mimics the light conditions within the soil where bulbs naturally occur in nature. This was found to be true for *Scadoxus puniceus,* which produced the greatest number of bulbs under continuous dark conditions (Naidoo et al., 2017). However, a study of the *Crinum* variant “Ellen Bosanquet” by Ulrich et al. (1999) found that the production of bulblets from tissue culture-generated shoots showed no correlation to light or dark growth conditions.

The orientation of explants on culture media has also been shown to influence the regeneration of plantlets. It has been observed that bulb explants of Amaryllidaceae plants placed with the adaxial side in contact with the media led to an increase in the number of bulblets produced per explant, with the requirement that the basal plate is also in contact with the media (Rice et al., 2011). On the other hand, shoot production in tissue cultures of leaf explants was found to be more successful when the abaxial side was in contact with the medium (Rabe and van Staden, 1999).

The effect of orientation on plantlet production from explants may be due to the polarity of the cells (Raven, 1979). There are two polarities of IAA in plant cells, IAA and IAA^-, and according to the chemiosmotic hypothesis proposed by Raven (1979), there is a greater concentration of IAA efflux carriers at the base of the cell than at the apical end. Since the cytoplasm is more alkaline than the cell wall, IAA^- is pushed out of the cell at a higher frequency at the base of the cell. This polar auxin transport is responsible for several growth and development processes in plants (Muday and

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DeLong, 2001). In shoots, auxin moves in a unidirectional manner from apical cells to basal cells, while in roots, it moves both towards the apex and in the opposite direction in the outer layers of the root cells (Muday and DeLong, 2001). This transport may explain why opposite orientations were found to be optimum for shoot tissue and subterranean tissue (Rabe and van Staden, 1999; Rice et al., 2011)

2.5.4 Activated charcoal

Certain plants, when injured, exude phenolic compounds into the media, which cause phenolic oxidation and darkening of the media. If re-absorbed by the plant, these phenols can cause an autotoxic reaction, discolouration and death of tissue. This issue becomes more common with the increased maturity of the explants (George et al., 2008). A way to mitigate this problem is to subculture onto fresh media every two to three days within the first two weeks from the day of initial culture (George et al., 2008). Another commonly used solution is the addition of activated charcoal, a carbon compound with an unorganised graphite structure, to the media. This structure of activated charcoal gives it a uniquely high adsorption capacity, owing to its large surface area to volume ratio. Although it adsorbs several growth-inhibiting organic molecules, such as phenols (Fridborg et al., 1978; Weatherhead et al., 1979), the carbon compound has also been observed to adsorb beneficial molecules including inorganic ions such as iron and magnesium, as well as plant-growth regulators. For this reason, activated charcoal has primarily been used in tissue culture without the addition of plant growth regulators.

The supplementation of 5 g/L activated charcoal to nutrient media led to an increase in the number of shoots produced by bulb explants of *Cyrtanthus clavatus* and *C. spiralis* (Moran et al., 2003), but a decrease in the number of shoots produced by *C. speciosus* bulb explants (Angulo et al., 2003). One study on *Crinum variabile* bulbs and leaves made use of activated charcoal-containing media supplemented with up to 10 mg/L BAP and 2 mg/L NAA and noted that this medium produced more, and larger bulbs in culture (Fennell et al., 2001). Another found that media containing both activated charcoal and growth hormones did not perform significantly better than media only supplemented with growth hormones (Rice et al., 2011). However, as Weatherhead et al. (1978) showed, activated charcoal can adsorb up to 300 mg/L NAA and up to 10 mg/L BAP, it is thus highly likely that the plant-growth regulators in media with activated charcoal have no effect. The differing effects that activated charcoal has on bulblet and shoot generation may be due to the darkening of media, which mimics soil conditions and thus may promote the growth of bulbs (Thomas, 2008).

2.5.5 Hardening off

The final stage of *in vitro* propagation is the hardening off, or *ex vitro* acclimatisation, stage in which tissue culture generated plantlets are transferred to soil. It is a critical stage in tissue culture that can result in considerable loss of propagules as in tissue culture, plantlets are grown in high humidity

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and are supplied with a carbon source. These plantlets are therefore not fully capable of photosynthesis and lack a thick enough waxy cuticle to prevent water loss through transpiration. Therefore, plants must spend several days growing *ex vitro* to develop these necessary characteristics for growth outside the laboratory environment (George et al., 2008). These plants are transferred to a rooting media and kept in high humidity and low light intensity conditions until they develop to a point where they can survive in the natural environment.

Hardening off *Hippeastrum reticulatum* Herb. was found to be equally supported by rooting in a substrate composed of vermiculite, carbonised rice husk, composted *Pinus* bark, coconut fibre and a combined substrate containing peat, carbonised rice husk and vermiculite (Heintze et al., 2018). In some studies, storage of generated bulblets in dark, cold environments for a few weeks to a few months was done before *ex vitro* acclimatisation in order to avoid the dormancy of the bulbs (Angulo et al., 2003). However, this was found to be unnecessary for *Cyrtanthus loddigesianus, C. speciosus* and *C. clavatus,* as neither the bulbs in cold storage nor those stored at 25°C entered dormancy (Angulo et al., 2003; Moran et al., 2003). Conversely, cold storage of *Galanthus ikariae* Baker bulblets increased the number of bulblets surviving the transfer to *ex vitro* conditions (Tipirdamaz, 2003). *Crinum variabile* plants transferred to soil without prior cold storage did not enter a dormancy period (Fennell et al., 2001). Plantlets that survive *ex vitro* acclimatisation can then be transferred from the highly regulated soil conditions to soil in the natural environment for conservation, cultivation for medicinal research or sale.

2.6 Conclusion

The family Amaryllidaceae is comprised of several endangered, economically, or scientifically important genera. It is therefore vital to multiply these populations using individuals that are not too closely related. Ensuring the highest levels of genetic diversity requires phylogenetic and population genetics analysis and should be done prior to *in vitro* propagation. *In vitro* propagation is a method of increasing the number of individuals using mature explant material or embryos, while considering the various environmental requirements of the organisms in culture. Finding the most effective methods of micropropagation tissue culture is, however, highly dependent on the species being cultured, the explant material in use, the composition of the culture medium as well as the conditions of the culturing environment.

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CHAPTER 3: EXAMINING THE GENETIC VARIABILITY BETWEEN AND WITHIN TWO *HAEMANTHUS PUMILIO* POPULATIONS

3.1 Introduction

The genus *Haemanthus*, in family Amaryllidaceae, subfamily Amaryllidoideae, contains 21 species that are all endemic to southern Africa. Fifteen *Haemanthus* species are found exclusively in the winter rainfall region of southern Africa, five in the summer rainfall region and only one, *H. albiflos,* occurs in both regions (Snijman, 1984). These plants are all geophytes possessing true bulbs and fleshy, distichous leaves (Snijman, 1984). The subterranean bulbs are tunicated, with new tunics being added each year as a new set of leaves forms above ground (Snijman, 1984).

Snijman (1984) separated *Haemanthus* into four groups based on phytogeographic distribution, cytology and morphology, namely, the *H. humilis*, *H. crispus*, *H. albiflos* and *H. coccineus* groups. *Haemanthus pumilio* is included in the *H. coccineus* group, characterised by unequal distichous tunication of the subterranean plant parts. In *H. pumilio*, the bulbs are narrow and ovoid with cream-coloured, fleshy tunics, which are formed annually as new leaf bases are added (Snijman, 1984; Summerfield, 1990). The leaves of this species are narrow and often twisted, with purple veins at the base and typically emerge after the inflorescence (Summerfield, 1990).

Haemanthus pumilio individuals tend to show clustered growth, with clumps of individual plants forming, most likely due to their method of seed dispersal. Once the flowers have been fertilised and seeds begin to develop, the peduncle lengthens, eventually senescing and collapsing with the weight of the fruits, leading to seeds germinating and new seedlings growing near the mother plant (Summerfield, 1990). The most likely method of distribution is through the fruit rolling a short distance or being washed away by rainwater to a distance slightly further away from the mother plant (Snijman, 1984).

Haemanthus pumilio is one of several threatened Amaryllidaceae species and is characterised as endangered on the SANBI Red List of Endangered Wildlife (South African National Biodiversity Institute, 2020b). Like many other species endemic to the Core Cape Subregion (CCR) of South Africa, *H. pumilio* requires specific habitat conditions that are dry during summer with wet, marshy winters (Summerfield, 1990). The CCR contains discrete phytogeographic centres with differing topographies and climatic conditions, which restrict several species within this region to specific microhabitats (Linder, 2003). It is, therefore, challenging to establish populations in other habitats, leading to very few known locations that still contain populations of *H. pumilio* (Summerfield, 1990). Since 1990, the number of known populations, as well as the number of individuals observed per population, has declined dramatically.

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In 1990, four sites with an estimated total of 1108 *H. pumilio* individuals were known, namely the Duthie Reserve at Stellenbosch University, a site in Klapmuts, and two sites on the Meerlust farm in the Stellenbosch district (Summerfield, 1990). Another population, not mentioned in the 1990 study, was later discovered at the Newton Commonage in Wellington. Eleven of the Wellington plants were transferred to the Stellenbosch University Botanical Garden (SUBG) when housing was being developed on the site (D. Kirkwood, *pers. comm.*). This is a relatively small distribution range, as the most distant locations, Wellington and the Meerlust farm, are within 70 km of each other. Three of these populations had gone completely extinct, by 2017, only the Duthie Reserve, remained, but with its number of individuals reduced to 37 flowering in that year (West, 2017) and 43 flowering individuals observed in 2019 (*pers. obs.*).

In 2019, plants from only two remaining populations of *H. pumilio* were known, those in the Duthie Reserve and the individuals relocated to the SUBG from Wellington. Interestingly, plants from these two populations, although generally regarded as the same species, exhibit consistent phenotypic differences. The Duthie Reserve plants are notably smaller than those from the Wellington population, while also producing sap that stains the skin a bright orange colour when handled. This staining does not happen with individuals from the Wellington population. These phenotypic differences and the declining *H. pumilio* habitat, in addition to its method of short-distance seed dispersal, highlight the need to determine both the inter- and intrapopulation genetic diversities of this endangered species for conservation purposes.

The observed differences in phenotype within *H. pumilio* could be a result of either genetic variability or environmentally-induced phenotypic factors. Genetic variability is the tendency of individuals within a population to possess varying alleles and is determined by the genotypes available within that gene pool (Laikre et al., 2010; Klug et al., 2016; Verma et al., 2017). Environmental conditions for natural populations can vary both geographically and over time, which may lead to increased expression of certain traits in different environments (Weinig and Schmitt, 2004). Several plants species present different morphologies in soils with lower nutrient density or as a result of water availability (Jones and Luchsinger, 1987). Molecular markers are regions within genomes or plastomes with high variability that are able to differentiate between taxa at the lower taxonomic ranges, down to intra-specific variation. Such markers can be used to determine whether the observed phenotypic differences are environmentally induced or due to genetic diversity. In the case of the latter, a re-evaluation of the specific classification of individuals in these populations will be required.

Chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) genomes are highly conserved and can be used for differentiation at higher taxonomic levels (Demesure et al., 1995). Their non-coding regions have a higher frequency of mutation and have, therefore, been used to differentiate at the

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lower levels from tribal and subfamilial relationships to inter- and intraspecific relationships (Taberlet et al., 1991; Drábková et al., 2004).

Non-coding regions within chloroplast DNA can be amplified by anchoring primers to the highly conserved coding regions. This makes it possible to use the same primer pair across several, distantly related species. The chloroplast genome codes for approximately 100 protein-coding genes as well as several rRNA and tRNA genes (Sugiura, 1992). The *trnL-trnF* intergenic spacer (trnL-F) is a commonly used non-coding region in cpDNA that separates two tRNA genes, *trnL* and *trnF* (Taberlet et al., 1991). Due to the low conservation of this region, it has been used for the reconstruction of interspecific phylogenies in many, diverse plant genera (Taberlet et al., 1991; Conrad, 2008).

The slowest evolving of the three plant genomes, the mtDNA genome, can be used for inference of phylogenetic relationships at the higher taxonomic levels of land plants (Dombrovska and Qiu, 2004). It is also easier to identify conserved regions to anchor primers for amplification than nuclear or cpDNA (Demesure et al., 1995; Knoop, 2004). Introns within mtDNA can be lost in some plant species, allowing for inference of phylogenetic relationships at the higher taxonomic levels of land plants (Dombrovska and Qiu, 2004). The plant mitochondrial NADH-ubiquinone oxidoreductase is a protein complex essential in the electron transport of NADH (Mower et al., 2012). Several genes code the proteins within this complex. One such gene is *nad1,* which contains four introns, of which one, *nad1i477,* has been amplified in *H. pumilio* (West, 2017). However, chloroplast and mitochondrial markers alone are not robust enough for phylogenetics studies, as both these genomes are maternally inherited. For this reason, nuclear gene regions are mostly used to complement these markers (Ito et al., 1999; Huang et al., 2000; Lledó et al., 2004; Meerow and Clayton, 2004; Conrad, 2008). One of the most commonly used nuclear gene regions for phylogenetics is the transcribed spacer region within ribosomal RNA, the internal transcribed spacer (ITS; White et al., 1990).

Ribosomal RNA genes are useful for the reconstruction of phylogenetic relationships of taxa from higher taxonomic levels through to the intraspecific level (White et al., 1990). The ITS regions are intergenic regions between nuclear rRNA genes, which have been used in techniques developed to target and sequence these regions to complement the extensive data provided by chloroplast DNA (White et al., 1990; Jobes and Thien, 1997). These regions are fast-evolving and may be informative for the separation of organisms at the population level (Wang et al., 1999). There are two ITS regions in nuclear DNA, ITS1 (187~298bp) and ITS2 (187~252bp), which flank the 5.8S nuclear rDNA region (White et al., 1990; Wang et al., 1999). Upstream of the ITS1 region and downstream of the ITS2 region are the coding 18S rDNA region and 28S rDNA regions, respectively (White et al., 1990),

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which are more conserved than the ITS regions and are, therefore, used to anchor the primers for ITS amplification.

These organellar and ITS markers are suitable for phylogenetic analysis within genera and may be able to show whether the two populations represent distinct species. They may, however, not be robust enough to determine intra-specific relationships. Microsatellites or simple sequence repeats (SSRs) can been used to reconstruct intraspecific phylogenetic relationships (Van der Zwan et al., 2000). They are nucleotide repeats of about 1 to 6 base pairs dispersed throughout the genomes of all eukaryotic organisms (Kalia et al., 2011). SSRs primers can be specifically designed for the target organism, however, this requires knowledge of the species genome sequence (Kalia et al., 2011). As there is no genome sequence information available for *H. pumilio* or any other *Haemanthus* species, an alternative molecular marker system was utilised for this study.

DNA fingerprinting techniques have been developed for population genetic analysis, which is used to reveal the levels of genetic diversity both between and within the populations. Two of these methods are the inter-simple sequence repeats (ISSRs) and the start codon targeted (SCoT) polymorphisms. Both of these are inexpensive, technically simple and comparatively more reproducible than other PCR-based fingerprinting methods that make use of a single primer as both the forward and reverse primer (Reddy et al., 2002; Collard and Mackill, 2009; Ng and Tan, 2015).The ISSR marker system amplifies regions between microsatellites (Reddy et al., 2002) using primers that are 16-25bp long and comprised of di-, tri-, tetra-or pentanucleotide repeats, anchored at the 3' or 5' end with degenerate bases (Godwin et al., 1997; Reddy et al., 2002). The SCoT primers are 18mer DNA molecules that bind to the conserved regions around start (ATG) codons (Collard and Mackill, 2009). These two fingerprinting techniques were selected to reveal the level of genetic diversity within and between populations of *H. pumilio*.

This study thus had three main aims:

1. To investigate the phylogenetic relationship between *H. pumilio* plants from the *ex-situ* Wellington population at the SUBG and Duthie population, using both chloroplast markers and ITS.
2. To examine the population genetic diversity of individuals from both the Duthie population and the SUBG population.
3. To determine whether or not the individuals from the SUBG and the Duthie reserve are conspecific by using both DNA sequence data and examining the ploidy levels of these populations.

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3.2 Materials and methods

3.2.1 Sample collection and DNA extraction

Tissue samples, consisting of approximately 2 cm of the terminal end of one leaf per plant, were collected for genetic analysis. Samples were taken from one individual from each separate clump at the Duthie Reserve (12 samples), and all 12 individuals of the largest single clump. One of the Wellington plants at the SUBG had replicated to produce three individuals between the time of relocation and sampling. Therefore, 13 individuals from the Wellington population held in the SUBG were sampled. Each sample was immediately snap-frozen in liquid nitrogen and stored at -80°C. DNA extraction was performed according to Hills and van Staden (2002). DNA concentration was determined using the ThermoScientific NanoDrop™ Lite Spectrophotometer (ThermoFisher Scientific), and DNA quality was assessed by gel electrophoresis using a 1% agarose gel. Extracted DNA was stored at -20 °C until required.

3.2.2 Phylogenetic analysis and tree construction

Polymerase chain reactions (PCRs) were carried out to amplify one nuclear and two organellar gene regions in the 25 sampled *H. pumilio* individuals. Primer names and sequences are displayed in Table 3.1.

Table 3.1: Primer sequences for the organellar (*nad1477* and trnL-F) and nuclear (internal transcribed spacer; ITS) gene regions used for phylogenetic analysis of *Haemanthus pumilio.*

Region	Forward primer (5'-3')	Reverse Primer (5'-3')	Reference
nad1i477	GCATTACGATCTGCAGCTCA	GGAGCTCGATTAGTTTCTGC	Demesure et al. (1995)
trnL-F	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	Taberlet et al. (1991)
ITS	GGAAGTAAAAGTCGTAACAAGG	TCCTCCGCTTATTGATATGC	White et al. (1990)

The two organellar regions used were the *nad1* intron *nad1i477* (Demesure et al., 1995) and *trnL-trnF* intergenic spacer (trnL-F; Table 3.1; Taberlet et al., 1991). Each reaction mixture was prepared to a total of 25 µL with 1X Ampliqon *Taq* DNA Polymerase Master Mix RED (Biomol, Germany), 0.3 µM forward and reverse primers, 50 ng DNA and sterile Milli-Q H_2O. Thirty PCR cycles were completed for both plastid gene regions from the denaturation to the extension phases (Table 3.2).

One nuclear region, ITS (internal transcribed spacer), was amplified (Table 3.1). The primers ITS4 and ITS5 (White et al., 1990) amplified a region of about 800 bp covering both ITS1 and ITS2 as well as the 5.8S rDNA region. Amplification reactions were conducted in a total volume of 25 µL containing 50 ng of gDNA, 1X Ampliqon *Taq* and 0.3 µM of each primer (Table 3.2).

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Table 3.2: PCR cycling temperatures and times for amplification of organellar (*nad1477* and trnL-F) and nuclear (internal transcribed spacer; ITS) gene regions used for phylogenetic analyses of *Haemanthus pumilio.*

	Initial denaturation		**Denaturation**		**Annealing**		**Extension**		**Final Extension**	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time
nad1i477	94 °C	4 min	92 °C	30 s	53.5 °C	45 s	72 °C	3 min	72 °C	10 min
trnL -F	94 °C	5 min	94 °C	1 min	45 °C	1 min	72°C	1 min	72 °C	10 min
ITS	94 °C	5 min	94 °C	45 s	55 °C	45 s	72 °C	1 min	72 °C	7 min

All PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced with the Sanger method at the Central Analytical Facility (CAF) at Stellenbosch University. The quality of each sequence was checked using SnapGene software (Insightful Science; available at snapgene.com), and low-quality bases at the beginning and end of the sequence were trimmed. For the plastid region (trnL-F) and one nuclear region (ITS), DNA sequences for 24 other *Haemanthus* species from a 2008 phylogenetic study (Conrad, 2008) were used in combination with the *H. pumilio* sequences for phylogeny reconstruction. Phylogenetic analysis for all five gene regions was carried out on NGPhylogeny.fr (Lemoine et al., 2019) using MAFFT version 7.4 for sequence alignment (Katoh and Standley, 2013), BMGE version 1.12 for alignment curation (Criscuolo and Gribaldo, 2010). Phylogenetic tree construction was performed using PhyML (Guindon et al., 2010) with smart model selection version 1.8.1 (Lefort et al., 2017). The reliability of internal branches was calculated using a Bayesian-like transformation of the approximate likelihood ratio test (aBayes; Anisimova et al., 2011). Phylogenetic trees were constructed for each DNA region as well as one tree constructed using the concatenated alignments. *Scadoxus* was selected as the outgroup for the *Haemanthus* phylogenetic reconstruction. These are the only two genera within the Amaryllidaceae subtribe Haemanthinae (Snijman 1984, Conrad 2008). Additionally, other phylogenetic studies consistently resolve *Haemanthus* and *Scadoxus* as sister clades (Ito et al., 1999; Meerow and Clayton, 2004; Conrad, 2008).

3.2.4 DNA fingerprinting

Thirteen ISSR and nine SCoT primers were used to amplify 37 *H. pumilio* samples in reactions of 15µL total volume. Each PCR utilised only one ISSR or SCoT primer for both the forward and reverse amplifications. The reaction mixture consisted of 1X Ampliqon Taq, 0.3 µM primer and 50 ng DNA. Both ISSR and SCoT PCR amplifications consisted of 35 cycles from the denaturation phase to the extension phase (Table 3.3), and the annealing temperature was optimised for each primer individually (Table 3.4).

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Table 3.3: PCR cycling temperatures and times for amplification DNA regions used for population genetic analyses of *Haemanthus pumilio.*

	Initial denaturation		**Denaturation**		**Annealing**		**Extension**		**Final Extension**	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time
ISSR	95°C	5min	94 °C	30 s	Ta [a]	1 min	72 °C	2 min	72 °C	5 min
SCoT	94 °C	3 min	94 °C	1 min	Ta [a]	1 min	72 °C	2 min	72 °C	5 min

a: Each primer required a different annealing temperature. Shown in Table 3.4

Three primers (Oligo2, SCoT12 and SCoT19) failed to amplify using Ampliqon *Taq,* and Hot Start PCR Master Mix, lyophilized, 2× (Rabbit Biotech, Germany) was used instead. The resulting PCR products were separated on a 2% agarose gel at a constant 80 V with λ*Pst*I ladder (New England BioLabs) for 1 h 40 min. Gel visualisation and image capture were completed using G:Box (Syngene).

Table 3.4: Start Codon Targeted (SCoT) primers and Inter-Simple Sequence Repeat (ISSR) primers used for DNA fingerprinting in *Haemanthus pumilio*

Marker type	**Primer name**	**Sequence (5'-3')**	**Ta[a] °C**	**Total bands**	**Polymorphic bands**	**% Polymorphism**	**PIC[b]**	**EMR[c]**	**MI[d]**
	SCoT13	ACGACATGGCGACCATCG	53	16	16	100	0.487	16	7.792
	SCoT30	CCATGGCTACCACCGGCG	50	21	21	100	0.433	21	9.033
SCoT	SCoT34	ACCATGGCTACCACCGCA	50	17	17	100	0.450	17	7.650
	SCoT12	ACGACATGGCGACCAACG	53	4	4	100	0.263	4	1.052
	SCoT19	ACCATGGCTACCACCGGC	55	19	18	94.7	0.468	17.1	8.008
	OW4	GAGAGAGAGAGAGAGAYA	50	16	16	100	0.269	16	4.304
ISSR	CA4	GAGAGAGAGAGAGAGYT	50	14	14	100	0.424	14	5.936
	Oligo2	CTCTCTCTCTCTCTCTTG	50	16	16	100	0.358	16	5.728

a: Annealing temperature; b: Polymorphism information content; c: Effective multiplex ratio d: Marker index

To ensure reproducibility, PCRs were repeated three times on three *H. pumilio* test samples. Only primers producing reproducible bands in all of the test samples were applied to the two populations. Each band was considered to represent a single locus and loci were scored visually, noting presence and absence in binary notation (0 = absent; 1 = present) for each individual. The capacity of these reproducible primers to accurately estimate genetic variability was evaluated through measurement of the polymorphism information content (PIC), the marker index (MI), and the effective multiplex ratio (EMR; Table 3.4). The formula $1-\sum Pi2$, *pi* being the frequency of the *i*th allele, was used to calculate PIC (Smith et al., 1997). The EMR was calculated as the product of the total number of polymorphic loci and the fraction of polymorphic loci and the primer's ability to detect polymorphic loci, MI, was calculated as PIC × EMR (Kumar and Agrawal, 2019).

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Jaccard's similarity coefficient was calculated from 30 000 bootstraps in DARwin 6 (Perrier and Jacquemoud-Collet, 2006) and used to reconstruct Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrograms to estimate the relationships between individuals. The means of the effective number of alleles (Ne), Shannon's information index (I), Nei's gene diversity (H), total genetic diversity (Ht), genetic diversity within populations (Hs), genetic differentiation (Gst) and gene flow (Nm) were calculated in Popgene v1.32 (Yeh et al., 1997). The significance of the calculated genetic dissimilarity was calculated using an Analysis of Molecular Variance (AMOVA) with the Excel add-on GenAlEx 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012)

3.2.5 Ploidy level analysis

Leaf material was collected from three plants in the Duthie Reserve and three from the cultivated Wellington population in the SUBG. The nuclei were isolated from cells within the tissue, and relative fluorescence measured using the 4',6-diamidino-2-phenylindole flow cytometry method (Doležel et al., 2007). Pentaploid plants collected from the Iberian Peninsula and fresh *Glycine max* were used as references. The recorded values were analysed using the GLM procedure in Statistica version 10.

3.3 Results

3.3.1 Phylogenetic analysis

Haemanthus pumilio material sampled from 24 Duthie Reserve and 13 SUBG plants was used for phylogenetic reconstruction of three DNA regions, ITS, trnL-F and *nadi1477*. Twelve of the Duthie Reserve samples (labelled Dut_A to Dut_L) represent all the plants in the largest clump at the reserve, the remaining Duthie Reserve samples represent one plant from each of the 12 smaller clumps. The clumps had been previously labelled for a previous study (West, 2017) and these labels were maintained for the present study. Additional sequence data from 24 *Haemanthus* and three *Scadoxus* samples from Conrad (2008) were included for the trnL-F and ITS regions (Figure 3.1A and C). Furthermore, five *Haemanthus* and one *Scadoxus* accession, available from the NCBI database, were used for phylogenetic reconstruction of the *nad1i477* intron (Figure 3.1B). For each of these individual regions, *H. pumilio* could not be confidently separated from other *Haemanthus* species. Several clades, across all gene regions, had less than 50% support, both within *H. pumilio* and the genus *Haemanthus* overall.

The trnL-F tree (Figure 3.1A) was able to separate the winter rainfall species, including *H. pumilio*, from the summer rainfall species (84% support). The summer rainfall species *H. montanus* was, however, resolved within a well-supported (99%) clade of winter rainfall species. Twenty-two of the *H. pumilio* samples, comprised of both SUBG and Duthie individuals, along with *H. sanguineus* and *H. pubescens* subsp. *pubescens,* were sister to this well-supported group. The remaining *H. pumilio* samples occurred outside of these two clades, though within the winter rainfall clade.

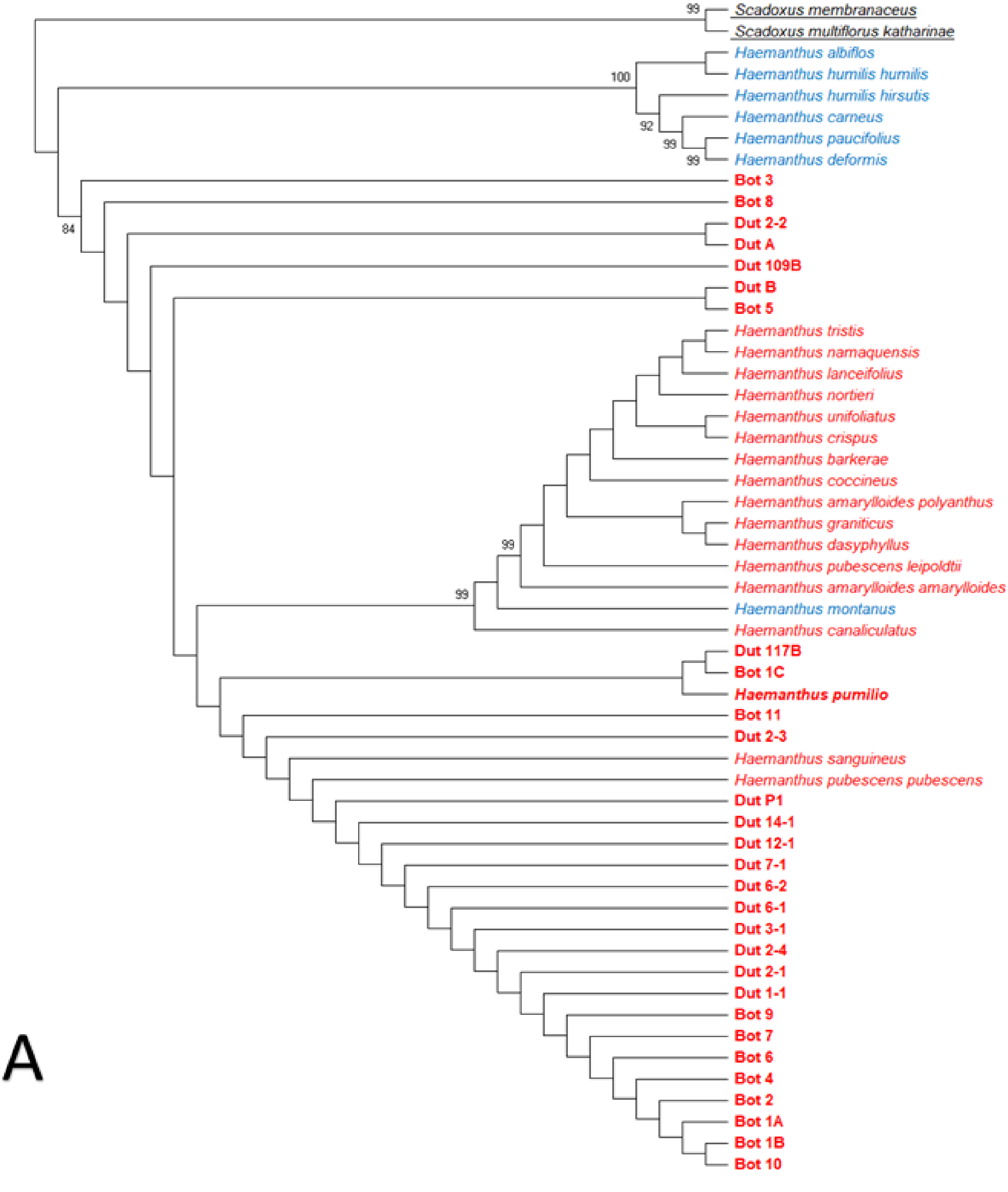

Figure 3.1: Maximum likelihood phylogenies of *Haemanthus pumilio* samples and other *Haemanthus* taxa with *Scadoxus* (underlined) taxa as outgroups. Trees were constructed with the (A) trnL-F intergenic spacer, (B) *nad1i477* intron and (C) ITS region. *Haemanthus* summer rainfall species, including *H. albiflos,* which occurs in both rainfall regions, are shown in blue and winter rainfall plants in red. The *H. pumilio* taxa collected in this study from the Stellenbosch University Botanical Gardens (Bot), Duthie reserve (Dut), as well as one from Conrad (for [A] and [C]; 2008), are shown in bold. Branch support values are based on a Bayesian-like transformation of the approximate likelihood ratio test (aBayes; Anisimova et al., 2011). Only support values ≥70% are shown.

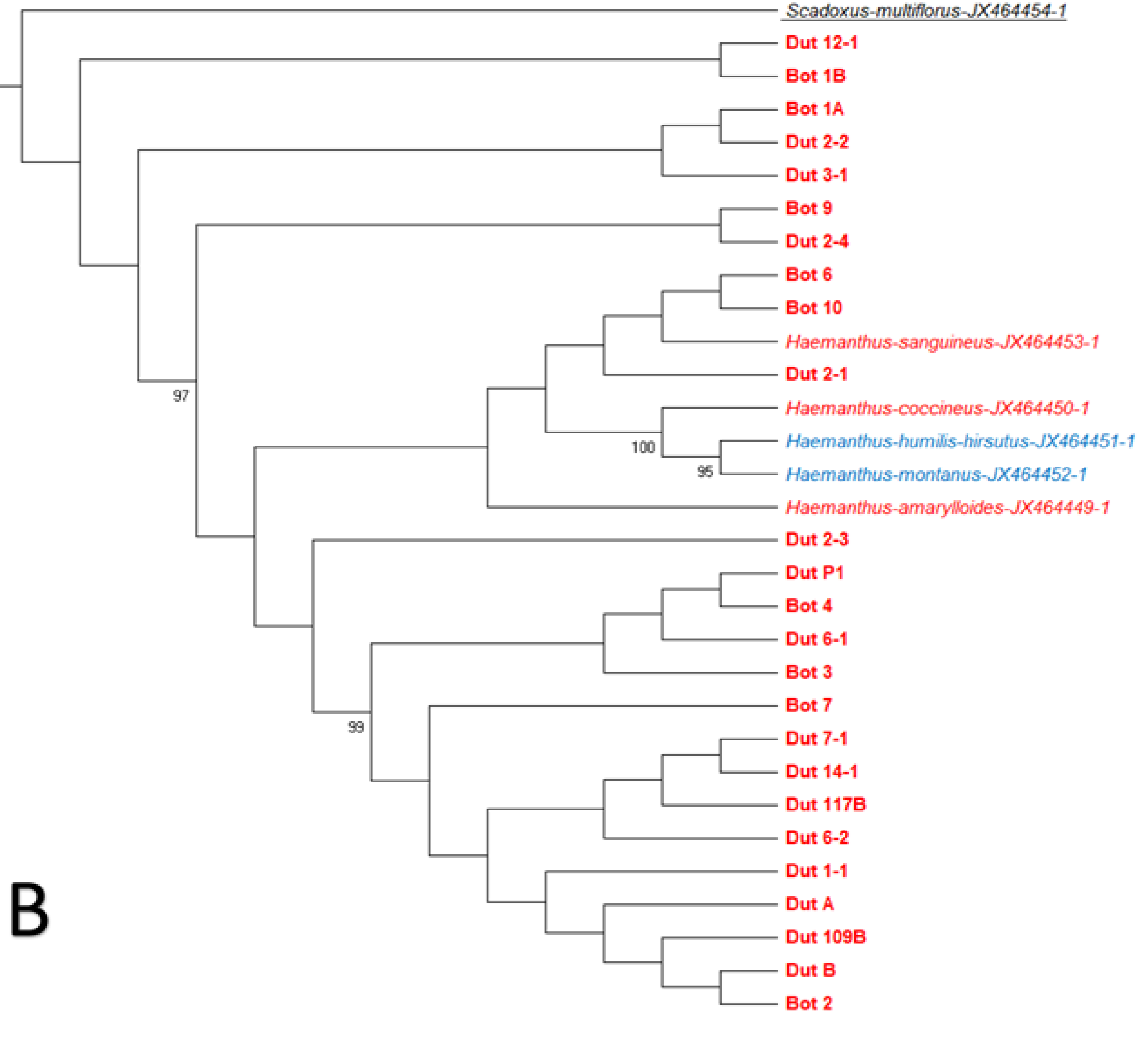

Figure 3.1 (cont): Maximum likelihood phylogenies of *Haemanthus pumilio* samples and other *Haemanthus* taxa with *Scadoxus* (underlined) taxa as outgroups. Trees were constructed with the (A) trnL-F intergenic spacer, (B) *nad1i477* intron and (C) ITS region. *Haemanthus* summer rainfall species, including *H. albiflos,* which occurs in both rainfall regions, are shown in blue and winter rainfall plants in red. The *H. pumilio* taxa collected in this study from the Stellenbosch University Botanical Gardens (Bot), Duthie reserve (Dut), as well as one from Conrad (for [A] and [C]; 2008), are shown in bold. Branch support values are based on a Bayesian-like transformation of the approximate likelihood ratio test (aBayes; Anisimova et al., 2011). Only support values ≥70% are shown.

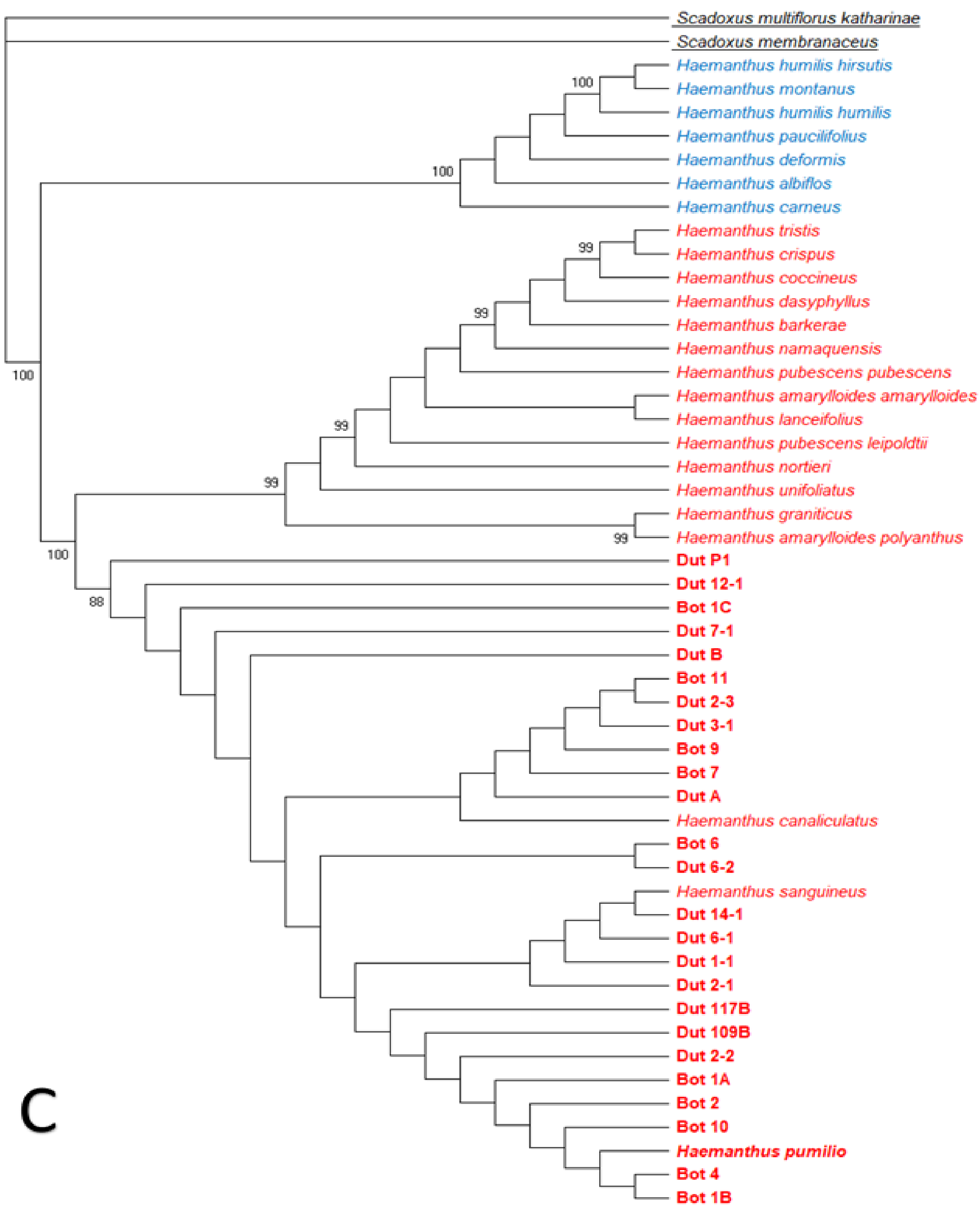

Figure 3.1 (cont): Maximum likelihood phylogenies of *Haemanthus pumilio* samples and other *Haemanthus* taxa with *Scadoxus* (underlined) taxa as outgroups. Trees were constructed with the (A) trnL-F intergenic spacer, (B) *nad1i477* intron and (C) ITS region. *Haemanthus* summer rainfall species, including *H. albiflos,* which occurs in both rainfall regions, are shown in blue and winter rainfall plants in red. The *H. pumilio* taxa collected in this study from the Stellenbosch University Botanical Gardens (Bot), Duthie reserve (Dut), as well as one from Conrad (for [A] and [C]; 2008), are shown in bold. Branch support values are based on a Bayesian-like transformation of the approximate likelihood ratio test (aBayes; Anisimova et al., 2011). Only support values ≥70% are shown.

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Only a few *Haemanthus* and *Scadoxus nad1i477* sequences are available on the NCBI database, and only one *Scadoxus* and five additional *Haemanthus* sequences could, therefore, be included in the phylogeny (Figure 3.1B). This marker did not separate *Haemanthus* into summer and winter rainfall groups, and the *H. pumilio* samples were not distinguished from the other *Haemanthus* species. Two *H. pumilio* samples (Bot 9 and Dut2.4) resolved together in a sister clade to the NCBI sequences as well as 15 other *H. pumilio* samples (97% support). However, *H. montanus* and *H. humilis* were confidently separated from *H. coccineus* (100 % support) and each other (95% support). Four *nad1i477* sequences (Bot 1C, Bot 5, Bot 8 and Bot 11) were excluded from phylogeny due to low-quality sequences.

The ITS phylogeny (Figure 3.1C) resolved a strongly supported dichotomy (100%) within *Haemanthus*, separating the summer and winter rainfall species. The smaller clade contained all summer rainfall species, including *H. albiflos,* which occurs in both rainfall regions, while the larger clade contained all winter rainfall species. The winter rainfall clade was also separated into two smaller clades (100% support), one consisting of *H. pumilio, H. canaliculatus* and *H. sanguineus* and another comprised of the remainder of the *Haemanthus* winter rainfall species. Within the *H. pumilio* clade, Dut P1 appeared to have diverged earliest from the samples taken, with 88% support. Two SUBG ITS sequences (Bot 3 and Bot 5) were excluded from phylogenetic analysis due to poor sequence quality.

For the construction of the combined phylogenetic tree (Figure 3.2), only species with sequences available for both ITS and trnL-F were used. The *nadi1477* intron was not included in the concatenated phylogeny, as the *Haemanthus* and *Scadoxus* individuals with available sequences on the NCBI were not the same as the ones used by Conrad (2008). The phylogenetic tree obtained from the combined ITS and trnL-F DNA sequence alignments clustered all the winter rainfall species into a single clade containing two well-supported subgroups. Despite the variation in topology between the trnL-F and the ITS phylogenies, the combined phylogenetic tree very closely resembles the ITS only phylogeny. The *H. pumilio* samples grouped together in a well-supported clade (99%) that also contained *H. sanguineus* and *H. canaliculatus*. None of the phylogenetic trees generated within this study separated *H. pumilio* plants originating from Wellington from those originating from Stellenbosch.

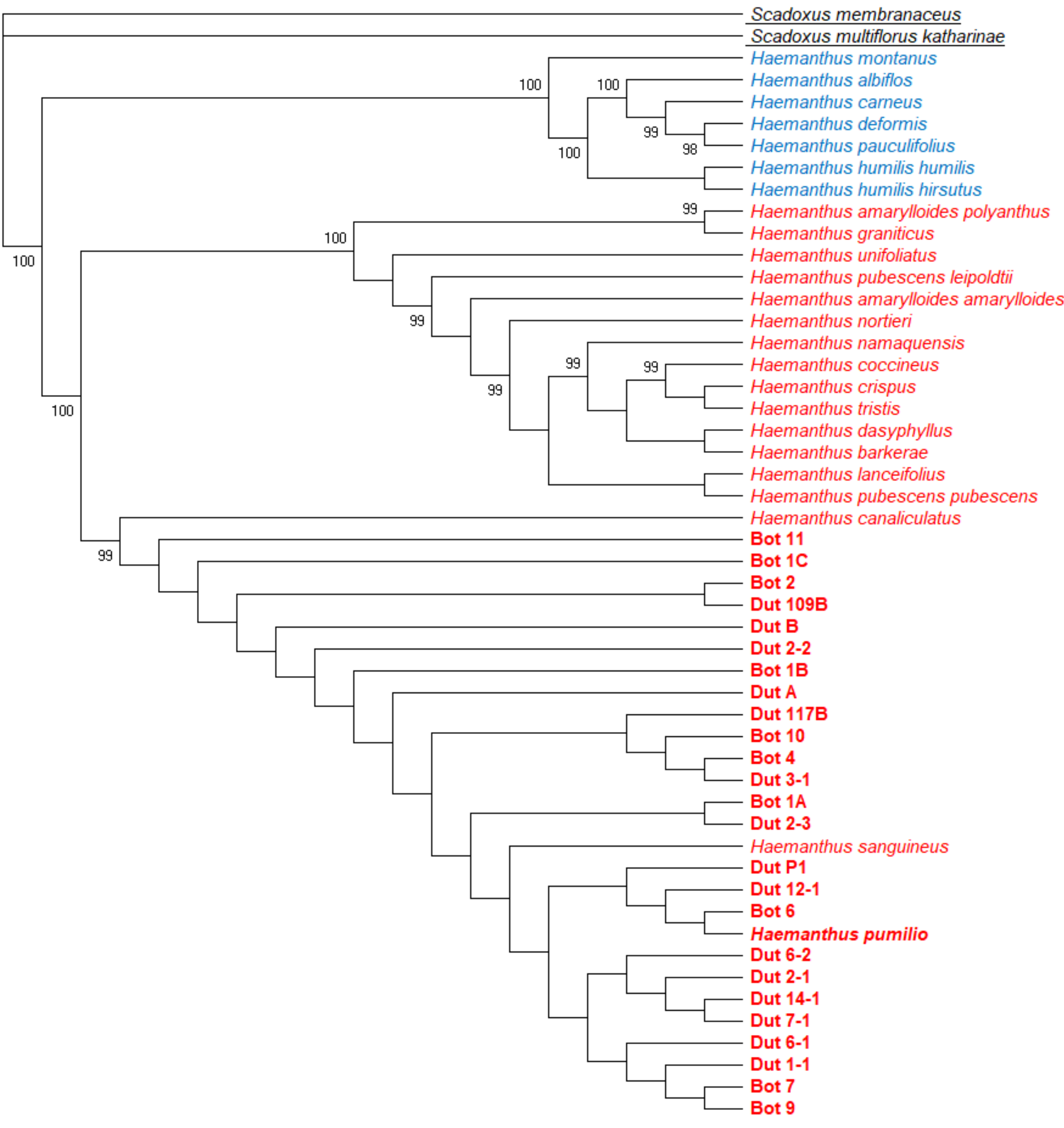

Figure 3.2: Combined maximum likelihood tree constructed using the ITS and trnL-F sequence data of 26 *Haemanthus pumilio* samples with 23 other *Haemanthus* taxa and two *Scadoxus* (underlined) taxa as outgroups. The support values shown on the branches were found using aBayes. *Haemanthus* summer rainfall species, including *H. albiflos* that occurs in both rainfall regions, are shown in blue and winter rainfall plants in red. The *H. pumilio* taxa collected from the Stellenbosch University Botanical Gardens (Bot), Duthie reserve (Dut), and one from Conrad (2008) are shown in bold. Only support values ≥70% are shown.

3.3.2 Analysis of DNA fingerprinting

Of the 13 ISSR and nine SCoT primers used in this study, seven ISSR and nine SCoT primers amplified and only three ISSR and five SCoT were reproducible (Table 3.4). These eight primers produced 123 bands with 99% polymorphism. This translates to an average of 15.38 bands per primer. Primer SCoT13 had the maximum PIC value of 0.487, whereas SCoT12 had the lowest value at 0.263. The maximum EMR and MI values, 22.00 and 9.033, respectively, were produced by

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SCoT30. This indicates that the SCoT primers were more robust and informative than the ISSR primers for *H. pumilio* analysis.

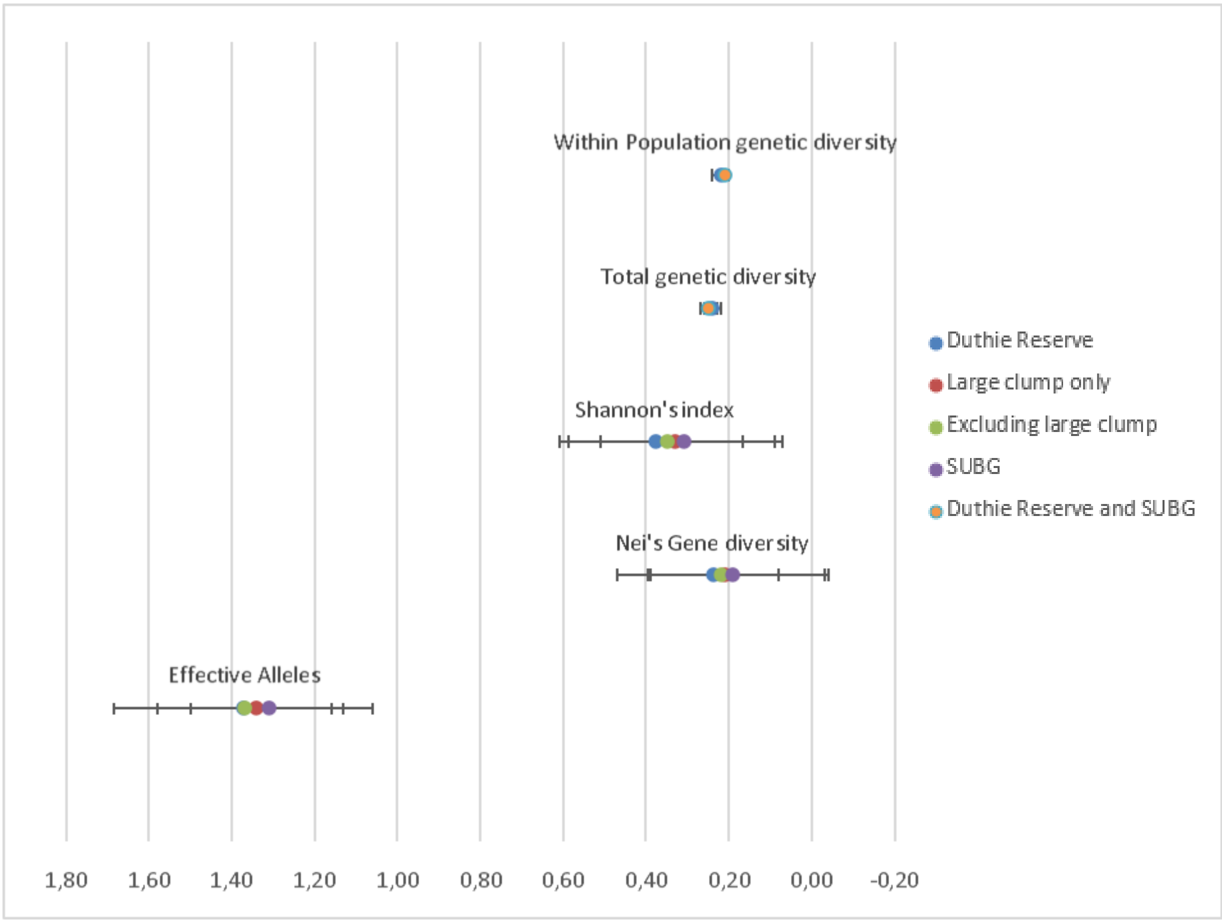

Figure 3.3: Scatter plot indicating the genetic variability estimates for *Haemanthus pumilio*. A total of 37 samples were analysed, 12 from the SUBG and 25 from the Duthie reserve. 12 Samples were from the large clump in the Duthie Reserve and 13 from the rest of the reserve.

The lowest effective number of alleles (Ne), 1.30, was produced from the analysis of *H. pumilio* individuals at the SUBG (Figure 3.3). The Ne in the Duthie Reserve and samples from the Reserve, excluding the large clump, were both 1.37, while the Ne for large clump at the Duthie Reserve was lower than both these values at 1.34. Nei's gene diversity (H), as well as Shannon's Information Index (I), were both somewhat higher in Duthie Reserve (0.24 and 0.38) compared to the SUBG (0.19 and 0.30); however, all indices had large standard deviations. Although the Duthie Reserve population showed slightly higher levels of genetic diversity, the standard deviations indicated that these differences are not significant. Overall, these diversity indices indicated only moderate levels of diversity in the *H. pumilio* populations.

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Table 3.5: Genetic differentiation and gene flow between the Duthie Reserve and Stellenbosch University Botanical gardens *Haemanthus pumilio* populations.

Population comparison	Gst[a]	Nm[b]
Within the Duthie Reserve	0.0895	5.0858
Between SUBG and Duthie Reserve	0.0911	4.9881

a: genetic differentiation, b: gene flow

Genetic variability was estimated between the Duthie Reserve and SUBG as well as within the Duthie reserve by comparing the large clump to the rest of the Duthie population (Figure 3.3). The mean total genetic diversity (Ht) and genetic diversity within populations (Hs) values were almost the same, between the SUBG and the Duthie Reserve, 0.25 and 0.21, and between the large clump and the rest of the Duthie Reserve which were 0.24 and 0.22, respectively. A slight difference was observed in the gene flow (Nm) values calculated between the large Duthie reserve clump and the rest of the Reserve (Table 3.5), 5.09, and between the SUBG and the Duthie Reserve, 5.00. The level of genetic differentiation (Gst) calculated was 0.09 both between the large Duthie reserve clump and the rest of the Reserve and between the SUBG and the Duthie Reserve (Table 3.5). These values indicate low levels of genetic variation within *H. pumilio.*

Table 3.6: Nei's unbiased genetic identity (above) and distance (below) between *Haemanthus pumilio* populations from Stellenbosch University Botanical Gardens (SUBG), the large clump at the Duthie Reserve and all smaller clumps at the Duthie Reserve**.**

	Duthie Reserve (large clump)	**Duthie Reserve (excluding large clump)**	**SUBG**
Duthie Reserve (large clump)		0.044	0.085
Duthie Reserve (Excluding large clump)	0.957		0.053
SUBG	0.919	0.948	

Nei's unbiased genetic identity showed a higher level of genetic identity between the SUBG plants and those from outside the large clump of the Duthie Reserve (0.95) than between the SUBG and the large clump (0.92; Table 3.6). The highest level of genetic identity was observed between the plants of the large clump and the rest of the Duthie Reserve (0.96). The AMOVA indicated a significantly higher estimated variance, based on 999 iterations, within (11.82) populations than among (3.45) them ($p < 0.001$) with most of the variation (77%) occurring within populations (Table 3.7).

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Table 3.7: Analysis of molecular variance (AMOVA) of 37 *Haemanthus pumilio* samples from within the large clump at Duthie Reserve, the smaller clumps at the Duthie Reserve and the SUBG plants.

Source	df	SS[a]	MS[b]	Est. Var.[c]	%	PhiPT[d]	P value
Among Populations	2	108.726	54.363	3.452	23%	0.206	0.001
Within Populations	34	401.814	11.818	11.818	77%		
Total	36	510.541		15.270	100%		

a: Sum-of-squares; b: Mean Squares; c: Estimated Variance; d: F-statistics analogue produced by GenAlEx

Dendrograms constructed from Jaccard's coefficient calculations revealed that a combination of SCoT and ISSR data was better able to separate the two populations into distinct clusters than data generated from ISSR or SCoT individually. The UPGMA dendrogram constructed from only ISSR data separated the species into two clusters, with one cluster containing all SUBG individuals, except for Bot 5 (Figure 3.4A). This cluster also contained five Duthie Reserve individuals. All individuals from the large clump at the Duthie Reserve resolved within the larger cluster. On the other hand, the dendrogram constructed from the five SCoT polymorphism analyses was unable to separate the two populations (Figure 3.4B).

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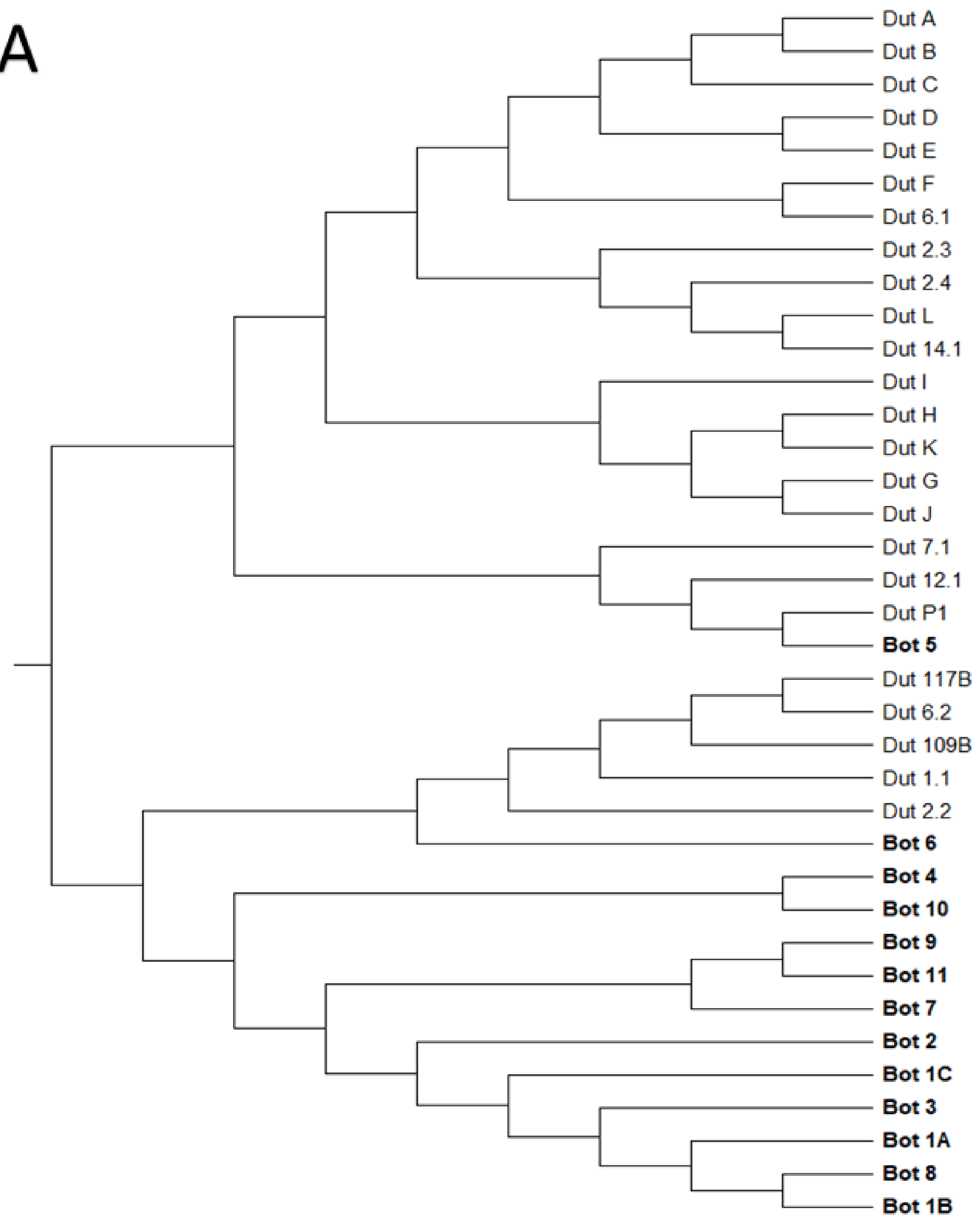

Figure 3.4: UPGMA dendrogram from Jaccard coefficient calculations performed on the presence/absence data of, **A:** three Inter-simple sequence repeat (ISSR) primers; **B:** five Start Codon Targeted polymorphism (SCoT) primers. *Haemanthus pumilio* samples collected from the Stellenbosch University Botanical Gardens (SUBG) are shown in bold. In total, 37 *H. pumilio* samples were used, 24 from the Duthie Reserve (Dut) and 13 from the SUBG (Bot).

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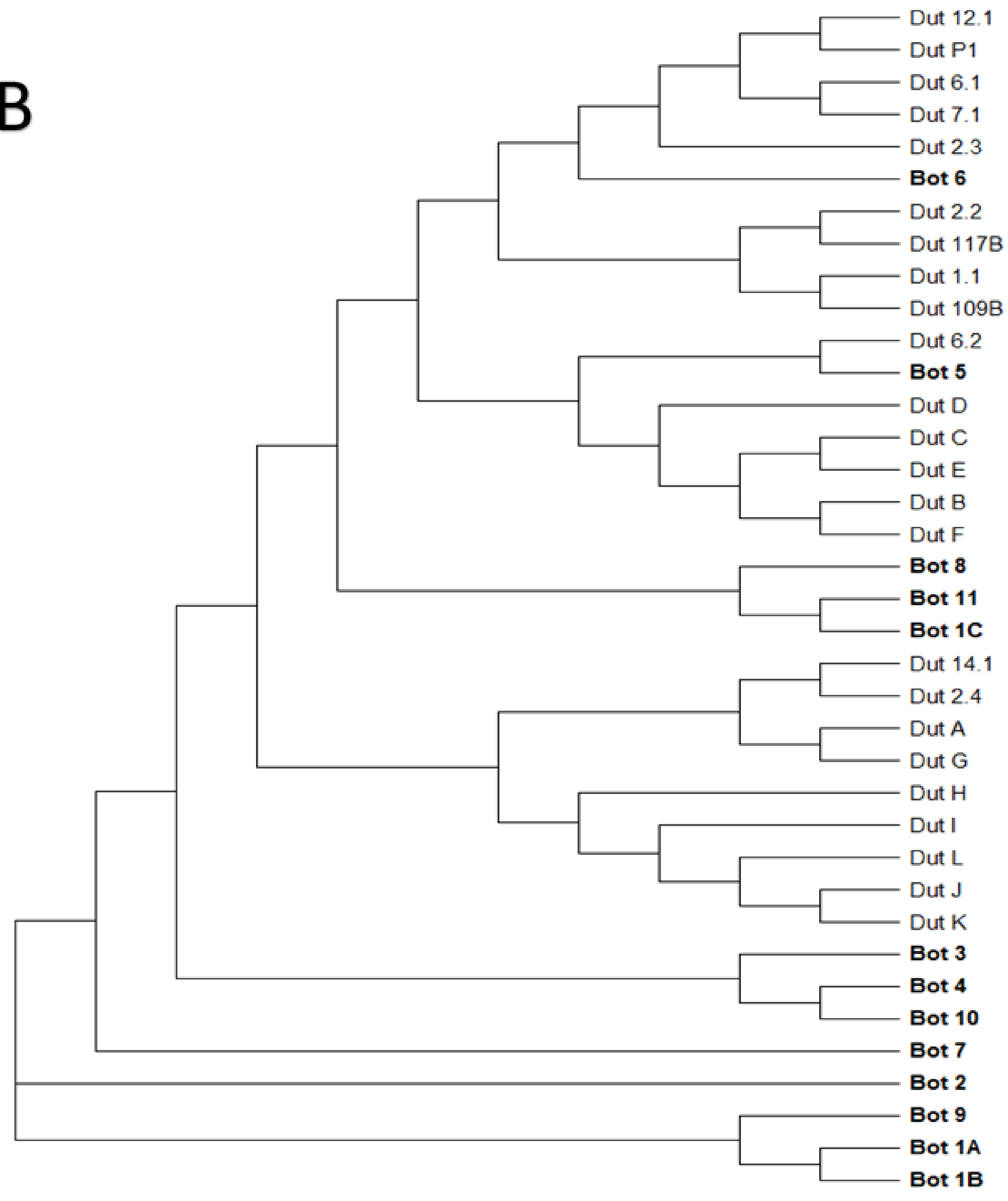

Figure 3.4 (cont): UPGMA dendrogram from Jaccard coefficient calculations performed on the presence/absence data of, **A:** three Inter-simple sequence repeat (ISSR) primers; **B:** five Start Codon Targeted polymorphism (SCoT) primers. *Haemanthus pumilio* samples collected from the Stellenbosch University Botanical Gardens (SUBG) are shown in bold. In total, 37 *H. pumilio* samples were used, 24 from the Duthie Reserve (Dut) and 13 from the SUBG (Bot).

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The combined ISSR and SCoT polymorphism dendrogram resolved a monophyletic group, including all Duthie Reserve samples and two SUBG samples (Bot 5 and Bot 6). This group was further divided into two sister clusters, with the larger cluster containing all individuals from the large clump (Dut A to Dut L) and the smaller cluster containing nine Duthie Reserve individuals and two SUBG samples. The remaining SUBG samples resolved outside of the Duthie Reserve clade. The SUBG samples Bot 1A, Bot 1B and Bot 1C samples were taken from the bulbs of a single plant that had separated into three individuals, however, only Bot 1A and Bot 1B resolved in the same cluster, while Bot 1C appeared more closely related to the Duthie reserve individuals (Figure 3.5).

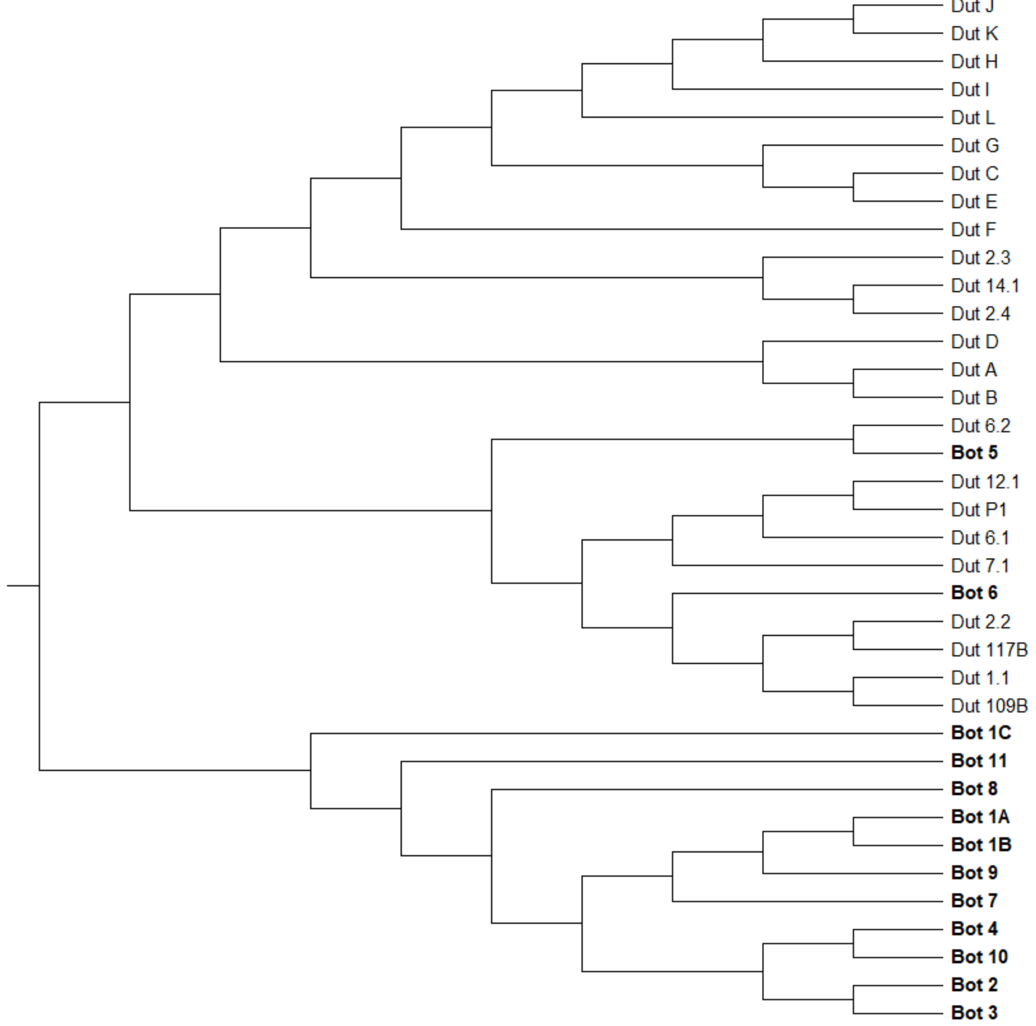

Figure 3.5: Combined UPGMA dendrogram from the Jaccard coefficient calculations performed on the binary information (1=present, 0=absent) from five Start Codon Targeted polymorphism (SCoT) and three Inter-simple sequence repeat (ISSR) primers. *Haemanthus pumilio* samples collected from the Stellenbosch University Botanical Gardens (SUBG) are shown in bold. In total, 37 *H. pumilio* samples were used, 24 from the Duthie Reserve (Dut) and 13 from the SUBG (Bot).

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3.3.4 Ploidy level analysis

Comparison of total DNA content revealed no difference in ploidy level between *H. pumilio* samples from the SUBG and the Duthie Reserve. The average difference, 1.1% was lower than the expected flow cytometry error rate of about 3% (Table 3.9). Thus, this difference was likely due to experimental error.

Table 3.8: Results of flow cytometry from six analysed samples of Haemanthus pumilio

***Haemanthus pumilio* sample**	**Relative fluorescence intensity**	**Population average**	**Difference**
SUBG K1	2.945		
SUBG K2	2.910	2.928	
SUBG K3	2.933		1.1
Duthie K1	2.894		
Duthie K2	2.903	2.895	
Duthie K3	2.888		

3.4 Discussion

The two populations of *H. pumilio* differed in size, with the SUBG plants three times larger than the Duthie Reserve plants. Additionally, the Duthie Reserve plants produce sap that stains surfaces a bright orange, while the SUBG plants do not. Both genetic and environmental factors can contribute to the morphological appearance of plants and several environmental variables including light, soil, nutrients, temperature and water can cause changes in appearance without an underlying genetic basis. This is known as phenoplasiticity and can alter plant dimensions, numbers of leaves, branches or flowers (Jones and Luchsinger, 1987; Weinig and Schmitt, 2004; Costa et al., 2016). Nevertheless, due to their close proximity, the climate and vegetation of both Wellington, the origin of the SUBG plants, and Stellenbosch are very similar (Climate-data.org, 2019); thus, light and temperature are unlikely to be the sources of this differentiation.

As *H. pumilio* is known to grow preferentially in soil that is waterlogged in winter, but dry in the summer, it is unlikely that soil moisture variation is the cause of the observed morphological differentiation. However, the soil nutrient content of the Duthie Reserve is notably low compared to the rest of the Stellenbosch flats. Moreover, Summerfield (1990) found that the soil in the Duthie Reserve is acidic and, as a result, several nutrients, including nitrogen, phosphorus, potassium magnesium, calcium and sulfur, are not easily obtainable from the soil. In contrast, aluminium, iron and manganese are easily obtained. Currently, no information is available on the soil nutrients and acidity of the Newton Commonage in Wellington.

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Variation in soil nutrient availability may also explain the presence of the orange sap in Duthie Reserve plants. Variations in phosphorus, potassium, nitrogen and sulfur levels affect the production of secondary metabolites such as cyanogenic glycosides, alkaloids and tannins (Bernays, 1989). Phenotypic differences caused by environmental factors do not persist when plants are cultivated, and the Wellington plants have maintained their increased size after being transplanted to the SUBG. However, the Duthie Reserve plants have not been cultivated, which may indicate that the low nutrient density and pH of the Duthie Reserve are the potential cause of the observed morphological variations. It is, however, difficult to attribute the observed morphological differences between the two populations solely to phenoplasticity because Summerfield (1990) also noted two *H. pumilio* groups of different size within the Duthie Reserve. One group of plants had broader leaves, thick fleshy peduncles (up to 10 mm) with more than 13 flowers, similar to the SUBG plants. The second group was comparable to plants presently found within the Duthie Reserve, with thinner peduncles (5 mm) that bore less than nine flowers and narrow leaves. Summerfield (1990) also noted that there were no transitional forms, with all plants sampled belonging to either group. This suggests a genetic basis for the observed phenotypic differences had a genetic basis and may indicate the same for the two populations in this study.

While polyploidy in plants can be a source of size variation (Klug et al., 2016), ploidy level analysis revealed no difference in DNA content between individuals from the two populations (Table 3.8). Several other genetic factors may affect plant morphologies and, in this study, both genetic markers and DNA fingerprinting were used to analyse genetic variation. The individual DNA markers applied in this study had varying abilities to differentiate closely related individuals. ITS was the only marker able to separate all the summer and winter rainfall *Haemanthus* species. Conversely, in a separate study, *H. montanus* resolved within the winter rainfall clade in an ITS tree (Bay-Smidt et al., 2011), similar to the result of the trnL-F region in this study. However, in a study of phylogenetic relationships among members of the tribe *Haemantheae* using several mitochondrial and plastid markers as well as ITS (Conrad, 2008), ITS was also the only region capable of resolving *H. montanus* within the winter rainfall clade. Despite the fast evolution of organellar introns, chloroplast and mitochondrial genomes are highly conserved by nature in comparison to nuclear genomes (Demesure et al., 1995). Therefore, these markers were unable to resolve *Haemanthus* taxa with smaller genetic differences.

In this study, the mitochondrial marker *nad1i477* was the least capable of differentiating between *Haemanthus* species. Mitochondrial DNA has long been preferred for use in distinguishing between lower taxonomic levels due to the low rate of recombination, uniparental mode of inheritance and low genetic complexity (Avise et al., 1983; Moritz et al., 1987; Piganeau et al., 2004). Several studies have found that phylogenies inferred from mtDNA are inconsistent with those of nuclear regions (Shaw, 2002; Ballard and Whitlock, 2004), implying that mtDNA may be inaccurate. Rubinoff and Holland (2005), however, argue that it would be incorrect to use these variations to assert that

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mtDNA should not be used for inference of phylogenetic relationships, as it represents a valid part of a species' evolution, divergence from nuclear DNA data does not signify an inaccurate phylogenetic analysis.

Regardless of the differences between nuclear, plastid and mitochondrial genomes, the markers used in this study could only differentiate the *Haemanthus* taxa at higher taxonomic, and not species, levels. *H. pumilio* consistently resolved in monophyletic clades with *H. canaliculatus* and *H. sanguineus* (Figure 3.4), which are both morphologically similar to *H. pumilio* (Snijman, 1984; Conrad, 2008). Similarly, Conrad (2008), using a combination of various plastid markers as well as ITS to reconstruct the Haemantheae phylogeny, found that *H. pumilio*, *H. cannaliculatus* and *H. sanguineus* resolved together.

DNA fingerprinting techniques have been used to differentiate organisms at lower taxonomic levels and have been found to detect more polymorphisms than traditional nuclear DNA and plastid DNA markers (Kothera et al., 2007; Akhavan et al., 2015; Rezaei et al., 2018; Sevindik et al., 2020). DNA fingerprinting using ISSR and SCoT markers was able to separate the Duthie Reserve population from the SUBG population, except for a few individuals (Figure 3.10). Using only unambiguous, bright bands, as done in this study, may lead to loss of information (Costa et al., 2016), but it increases the reproducibility and reliability of the obtained information. Though unable to explain the morphological differences between the populations and despite the loss of some information, the DNA fingerprinting in the present study was still sufficiently robust and informative enough to differentiate between individuals from the two populations.

Rare plants and plants with limited geographic coverage are expected to have low genetic variability compared to more widespread relatives (Wright, 1931; Hamrick and Godt, 1989). This may be due to chance events causing changes in allelic frequency, strong directional selection or an increased chance of inbreeding (Karron, 1991). As *H. pumilio* is a rare species, endemic to a narrow geographical region, it is expected to have low levels of within-population genetic variation, with high levels of differentiation among geographically separated populations (Hamrick and Godt, 1989; Karron, 1991; Ellstrand and Elam, 1993). The present study revealed a moderate level of genetic variability, which was higher than the low levels expected for a species this rare and geographically restricted. DNA fingerprinting analysis has been performed on a few widespread species of the subfamily Amaryllidoideae and *H. pumilio* genetic variation was higher than or equal to that of *Boophane distichia* (Cheesman, 2013) and *Lycoris longituba* (Deng et al., 2007). Therefore, it is possible that the genetic variability in the two remaining populations of *H. pumilio* is similar to that of widespread *Haemanthus* species. This is, however, speculative as no studies have considered the population genetics of other *Haemanthus* species. Additionally, the AMOVA indicated that most of

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this genetic diversity was within the two *H. pumilio* populations than between them, consistent with the low genetic differentiation observed.

Though contradictory to theoretical expectations, it is possible for endemic species with narrow geographical ranges to have higher levels of genetic differentiation than widespread counterparts (Smith and Pham, 1996; Gitzendanner and Soltis, 2000; Xue et al., 2004). Several factors can influence a species' level of genetic differentiation, including recent speciation from a wide-spread species, breeding system, multiple founder events, change in distribution and somatic mutation (Smith and Pham, 1996; Xue et al., 2004). *H. pumilio* may have recently separated from a more widespread *Haemanthus, H. sanguineus,* which is listed as least concern on the SANBI Red List (Snijman and Victor, 2004a). Phylogenetic analysis indicated that *H. pumilio, H. sangineus* and *H. canaliculatus* are very closely related and could not be separated by any of the markers used in this study. Additionally, *H. pumilio* and *H. sanguineus* resolved as sister clades in another phylogenetic study (Conrad, 2008). Moreover, most Amaryllidaceae plants, including *H. pumilio* are obligate out-crossers, and outcrossing species tend to have high levels of genetic diversity, which may also explain the higher than expected levels of genetic diversity observed.

H. pumilio individuals from the large clump were more closely related to each other than to plants from the rest of the Reserve. It was expected that plants within a single clump would be very closely related as the method of seed dispersal employed by *H. pumilio* that prevents seeds from spreading more than a few metres away from the mother plant. However, the diversity of these individuals was not significantly lower than that of the other populations. This high degree of within-population genetic variability also suggested low levels of inbreeding, thus, indicating that the clump is not comprised of clonal or near clonal individuals. Most genetic variability was found within the *H. pumilio* populations in contrast to the expectation that more variation would be present among populations of rare species (Xia et al., 2007). Despite the plants within the large clump being less diverse than the rest of the Duthie Reserve, the method of seed dispersal does not severely impact the genetic variation of the plants as previously thought (West, 2017).

The small numbers of individuals remaining in the two extant populations will, in future increase the effects of genetic drift and inbreeding depression. This is especially worrying for the SUBG plants as they have the smallest population size and, therefore, are more sensitive to stochastic events (Xia et al., 2007). Nevertheless, the higher within-population genetic variability may be encouraging for this endangered plant as it indicates a lack of genetic bottlenecking and increases the effective population size (Williamson and Werth, 1999).

Geographic separation of species establishes a barrier that limits gene flow (Jones and Luchsinger, 1987). Populations may evolve unique genotypic or phenotypic traits, with the populations becoming increasingly genetically different over time until they can no longer be classified as one species. This

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process, termed the geographical theory for speciation, additionally requires that different selective pressures be present in the separated populations (Jones and Luchsinger, 1987). Even though the UPGMA dendrogram separated the SUBG and Duthie Reserve individuals and genetic distance was greatest between the Duthie Reserve and the SUBG population, differentiation between these populations was low. Additionally, there was a moderate level of gene flow between populations, likely due to *H. pumilio* being insect-pollinated (Summerfield, 1990). This suggests that, although there were observable phenotypic differences between the populations, the genetic differences identified in this study and those observed by Summerfield (1990) were too small to consider them separate species. In fact, a much lower genetic diversity was detected than would be expected from two morphologically distinct populations.

Due to the obvious morphological differences between the two populations of *H. pumilio*, it was feared that gene flow might lead to a loss of genetic diversity. However, the low genetic differentiation observed in this study provides strong evidence that the two populations are not in the process of speciation. Based solely on genetic diversity it is, thus, not vital to prevent gene flow between the Duthie Reserve and SUBG. Additionally, crossbreeding these populations would increase the genetic diversity of future plants and would increase the population size, further shielding the species from the effects of genetic drift and stochastic events. However, it may still be important not to interbreed these populations because of the clear morphological differences, especially since the source of this variation is currently unknown.

3.5 Conclusions

Though genetic markers were unable to differentiate between the *ex-situ* Wellington population in the SUBG and the Duthie Reserve and no difference in ploidy level was observed, ISSR and SCoT polymorphism analysis revealed that there is a significant, albeit small, genetic difference between the populations. Based on the genetic diversity detected in each of the two populations, intrapopulation crossing is unlikely to lead to inbreeding depression. Moreover, such crossings are vital to protect the existing populations in order to preserve their genetic variation and to maintain this diversity by crossing distantly related plants. It is also important to increase the numbers of these plants through artificial propagation. Future research needs to determine whether the translocation of the Wellington population to the SUBG is leading to the reduction of interpopulation diversity. In addition, metabolite analysis of *H. pumilio* should be performed as differences in plant metabolites between the populations would further the understanding of their taxonomic relationship.

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CHAPTER 4: MICROPROPAGATION OF *HAEMANTHUS ALBIFLOS* AND *HAEMANTHUS PUMILIO* (AMARYLLIDACEAE)

4.1 Introduction

The genus *Haemanthus* is a member of the Amaryllidaceae, subfamily Amaryllidoideae native to the southern region of Africa, including Botswana, Lesotho, Mozambique, Namibia, South Africa and Swaziland (Snijman, 1984; Duncan, 2002; Fayaz, 2011). This genus is comprised of 21 species, 15 of which occur exclusively in the winter rainfall region of southern Africa, five in the summer rainfall region, with only one, *H. albiflos,* occurring in both (Snijman, 1984). *Haemanthus* lilies require regions with specific and predictable rainfall patterns for growth, restricting them to the temperate and arid environments of southern Africa (Snijman, 1984)

Fourteen of the 26 taxa (including subspecies) within this genus are classified as endangered, rare or vulnerable on the SANBI Red List (South African National Biodiversity Institute, 2020b). This is due to habitat loss and land cover alterations, over-harvesting for medicinal or horticultural purposes, and changes in populations of symbiotic species (Snijman and Raimondo, 2004; Snijman and Victor, 2004b; Snijman and Victor, 2004e; Snijman and Victor, 2004c; Snijman and Victor, 2004d; Snijman et al., 2007b; Snijman and Raimondo, 2007b; Snijman and Raimondo, 2007c; Snijman and Victor, 2011; Williams and Crouch, 2011).

It is therefore important to increase the numbers of these plants to re-introduce into nature or to grow in botanical gardens for conservation of these species. One way to do this is through micropropagation; that is the multiplication of individual organisms from vegetative explant material using tissue culture methods (George et al., 2008). Several studies have been performed on members of the family Amaryllidaceae (Table 4.1) using different plant growth regulators and different physical environments.

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Table 4.1: Tissue culture conditions published for *in vitro* propagation of several members of the Amaryllidaceae plants.

Organism	Explant source	Cytokinin	Auxin	Media	Gelling agent	Photoperiod	Media additions	References
***Amaryllis* L.**	Bulbs; Peduncle; Flower Buds	Kinetin	2,4-D[e]; IAA[f]; NAA[g]	MS; LS	Gellan gum	24 h	Iron; Coconut milk	Bapat and Narayanaswamy, 1976
***Hippeastrum* Herb.**	Bulb	BAP[a]	2,4-D	MS	Agar	15h	None	Seabrook and Cumming, 1977
***Allium cepa* L.**	Bulb	BAP	IBA[h]; NAA	MS	Agar	16 h; 8 h	Myo-Inositol, Nicotinic acid, Pyroxidine, Thiamine	Hussey and Falavigna, 1980
***Haemanthus katherinae* Baker*;* *Crinum abyssinicum* Hochst. ex A.Rich*;* *Leucojum vernum* L.**	Bulb	Kinetin	2,4-D; IAA, NAA	MS	Agar	24 h	None	Kromer, 1985
***Hippeastrum hybridum* Hort**	Bulb	BAP; Kinetin; Zeatin	IAA; NAA	MS	Agar	24 h	None	Huang et al., 1990
***Nerine bowdenii* W.Watson**	Bulb, Peduncle	BAP	2,4-D; IAA; IBA; NAA	MS	Agar	16 h	None	Jacobs et al., 1992
***Allium sativum* L.**	Bulb	BAP	NAA	LS	not mentioned	16 h	None	Ayabe and Sumi, 1998
***Haemanthus albiflos* Jacq.*;* *Haemanthus deformis* Hook.f *Haemanthus humilis* Jacq.**	Bulb; Leaf	None	None	MS	Agar	16 h	Myo-inositol	Rabe and van Staden, 1999

***Hippeastrum* Herb*;* *Zephryanthes* L.**	Bulb	BAP	2,4-D; NAA	MS	Gellan gum	16 h	Myo-Inositol, Nicotinic acid, Pyroxidine, Thiamine	Smith et al., 1999
Crinum "Ellen bosanquet"	Bulb	BAP	NAA	MS	Bacteriological-Agar	16 h	None	Ulrich et al., 1999
***Nerine sarniensis* Herb*;* *Narcissus tazetta* L.*;* *Dichelostemma multiflorum* (Benth.) A.Heller*; Allium ampeloprasum* L.*;* *Eucrosia radiata* Ker Gawl*;* *Haemanthus coccineus* L.**	Peduncle; Pedicel-Peduncle Junction	BAP; Kinetin	2,4-D; IBA; NAA	MS	Agar	16 h	AC; NaH2PO4; Adenine sulphate	Ziv and Lilien-Kipnis, 2000
***Crinum variabile* Herb.**	Bulb, Leaf	BAP	NAA	MS	Gellan gum	16 h	AC	Fennell et al., 2001
***Eucrosia stricklandii* Baker**	Bulb	BAP; TDZ[b]	2,4-D; NAA	MS	Agar; Phytagel	0 h	AC	Colque et al., 2002
***Cyrtanthus loddigesianus* Herb*; Cyrtanthus speciosus* R.A Dyer**	Bulb	BAP	NAA	MS	Agar	16 h	AC	Angulo et al., 2003
***Cyrtanthus clavatus (L'Hér.) R.A.Dyer;* *Cyrtanthus spiralis* Burch. ex Ker Gawl**	Bulb	BAP	NAA	MS	Agar	0 h; 16 h	AC	Moran et al., 2003
***Sternbergia clusiana* Ker Gawl. ex Schult.**	Bulb	BAP	IBA	MS	Agar	16 h	Inositol	Oran and Fattash, 2005
Narcissus pseudonarcissus* L.*;* *Galanthus elwesii* Hook*;	Leaf	BAP	2,4-D; NAA; Picloram	MS	Not mentioned	16 h	AC	El Tahchy et al., 2011

***Leucojum aestivum* L.**								
***Brunsvigia undulata* F.M.Leight.**	Bulb	BAP	NAA	MS	Bacteriological-Agar	16 h	AC	Rice et al., 2011
***Hymenocallis littoralis* Salisb.**	Root; Bulb	none	2,4-D; Dicamba[j]; IAA; NAA; Picloram	MS	Gellan gum	0 h	none	Noormi et al., 2012
***Hippeastrum johnsonii* (Bury) Herb.**	Bulb	BAP	NAA	MS	Agar	16 h	Myo-inositol	Daniels et al., 2013
***Gethylilis multifolia* L. Bolus**	Bulb	none	none	MS	not mentioned	12 h; 14 h; 16 h	None	Zakizadeh et al., 2013
***Cyrtanthus contractus* N.E.Br*;* *Cyrtanthus obliquus* L.f*;* *Cyrtanthus guthrieae* L.Bolus**	Bulb	BAP; Kinetin; mT[c]; TDZ; Zeatin	NAA	MS	Bacteriological-Agar	16h h	Myo-inositol	Ncube et al., 2015
***Allium schoenoprasum* Dumort.**	Bulb; Leaf	BAP; CPPU[d] Kinetin; mT; TDZ; Zeatin	none	MS	Agar	16 h	Myo-Inositol, Nicotinic acid, Pyroxidine, Thiamine, Adenine	Tubic et al., 2016
***Scadoxus punicus* (L.) Friis & Nordal**	Leaf	BAP; mT	NAA	MS	not mentioned	varied; 16 h	Phloroglucinol; Galic acid	Naidoo et al., 2017
***Leucojum vernum* L.*;* *Leucojum aestivum;* *Galanthus woronowii* Losinsk*;* *Sernbergia lutea* (L.) Ker Gawl.**	Bulb	BAP	IAA; IBA; NAA	MS	Bacteriological-Agar	14 h	Gamborg's vitamins;	Resetár et al., 2017
***Lapiedra martinezii* Lag.**	Bulb	2iP[i]; BAP; Kinetin	IBA; NAA	Gamborg's B5; MS	Agar	0 h; 16 h	Casein; Adenine; Glutathione	Juan-Vicedo et al., 2019

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Leucojum aestivum	Bulb; Leaf	Zeatin	none	MS	not mentioned	16 h	Melatonin; NaCl	Ptak et al., 2019
Rhodophiala bifida **(Herb.) Cabrera**	Bulb	none	none	MS	Agar	0 h	Plant Preservative Mixture	Reis et al., 2019
Narcissus tazetta	Bulb	BAP	NAA	MS	Agar	0 h; 16 h	None	Rahimi Khonakdari et al., 2020
Narcissus pseudonarcissus	Bulb	BAP	NAA	MS	Agar	12 h	None	Ferdausi et al., 2020

a: 6-Benzylaminopurine b: thidiazuron; c: metatopolin; d: N-(2-chloro-4-pyridyl)-N′-phenylurea e: 2,4-dichlorophenoxyacetic acid; f: indole-3-acetic acid; g: 1-Naphthaleneacetic acid; h: indole-3-butyric acid; i: 2-isopentenyladenine; j: 3,6-dichloro-2-methoxybenzoic acid

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Rabe and van Staden (1999) cultured three *Haemanthus* species *in vitro* using leaf and bulb material of *H. albiflos, H. humilis* and *H. deformis*. This study made use of 70% ethanol with Tween-20 and sodium hypochlorite solution (NaOCl) for decontamination. The decontaminated explants were placed on Murashige and Skoog media (MS; Murashige and Skoog, 1962) supplemented with 0.1 g/L myo-inositol with no added phytohormones. Bulb material, although more difficult to disinfect than leaf material, produced shoots more readily than leaf explants in all three species. In another study, the pedicel and peduncle tissues of *H. coccineus* were propagated on MS media supplemented with 5 µM NAA and 10 µM BAP, activated charcoal (2.5 g/l), adenine sulfate (100 mg/l) and NaH_2PO_4 (500 mg/l), resulting in an average of 4 buds per explant (Ziv and Lilien-Kipnis, 2000).

Haemanthus pumilio, a winter rainfall species (Snijman, 1984) is listed as endangered on the SANBI Red List for South African plants (South African National Biodiversity Institute, 2020b). It is one of only five taxa in its genus to have this indication, with the remaining 21 taxa listed as vulnerable, rare or least concern (South African National Biodiversity Institute, 2020b). It requires habitats that are dry during the summer, but marshy and waterlogged during the winter. Due to these specific habitat requirements, it is challenging to establish *H. pumilio* in alternative locations with different ecological attributes (Summerfield, 1990). To date, this species has also not yet been successfully micropropagated (West, 2017). With the dwindling numbers of these plants in the wild, the current conservation category may need to be revised to critically endangered.

Summerfield (1990) reported that the largest population in the Duthie Reserve at Stellenbosch University comprised of at least 1046 flowering individuals. These numbers had dropped significantly to about 45 flowering individuals in 2014 (West, 2017). In 2017, the number of observed flowering individuals had reduced even further to 37. However, 143 plants were later counted when the leaves emerged (West, 2017). In 2019, only 43 flowering plants were observed (*pers. obs.)*. Other populations listed by Summerfield in 1990 included about 50 individuals observed flowering 3km ENE of Klapmuts along the Old Paarl Road, but, only three flowering plants were seen at this site in 2017 (West, 2017). This agricultural land was subsequently ploughed in 2018, and there are likely no more *H. pumilio* plants in the area. Eleven specimens from a population discovered in Wellington that was not mentioned in the 1990 study were placed into the care of Stellenbosch University Botanical Gardens (SUBG) prior to the development of housing on the site.

The aims of this study were, therefore:

1. To adapt the tissue culture protocol of Rabe and van Staden (1999) on both leaf and bulb explants for micropropagation of *H. pumilio*. For this, *H. albiflos* was used for protocol design as it occurs in both the summer and winter rainfall regions, thus, leaf material for experimentation was available throughout the year. Additionally, this plant is listed as least

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concern on the SANBI Red List (South African National Biodiversity Institute, 2020b) ensuring the availability of ample material.

2. To use the most effective micropropagation protocols designed using *H. albiflos* on explants of *H. pumilio*, providing an effective protocol for the micropropagation and tissue culture of this endangered plant.

4.2 Materials and methods

4.2.1 Artificial pollination and *in vitro* germination

Flowers of the most distantly located plants at the Duthie Reserve were pollinated by hand using cotton buds. After pollination, lace bags were placed over the flowers to protect seeds from herbivory. At maturity, seeds were collected and either planted in soil or washed in 50% bleach (1.75% [m/v] NaOCl), rinsed in sterile distilled water and placed on hormone-free quarter MS nutrient media. All seeds collected in 2019 were planted in the SUBG, while seeds collected in 2020 were germinated *in vitro*. Pots for planting seeds contained three layers: the bottom layer made of cork and shade cloth to provide rooting space, a mixture of 1.5 parts compost, two parts sand, one part vermiculite and a handful of inorganic fertiliser, filling up three-quarters of the pot. Finally, the top layer comprised of a 1:1:1 ratio of perlite: vermiculite: sand. The seeds were watered daily with inorganic fertiliser containing bactericide and fungicide. The 2020 seeds were placed on quarter strength standard Murashige and Skoog (MS) media (Sigma), solidified with 5 g/L phytoagar at pH 5.8. Media were sterilised by autoclaving at 121°C for 20 min at a pressure of 1.05 kg/cm2. Seeds were maintained in a growth room at 25°C under cool white fluorescent lights (Osram L 58V/740, 50 μmol photons /m2/s) with a photoperiod of 16 h day: 8 h night until they germinated.

4.2.2 Plant material

Haemanthus albiflos plants were sourced from the SUBG and Kirstenbosch Botanical Gardens and stored in a semi-shaded area. Plants were watered three to four times a week during the winter growing season and treated with Plant Protector (Efekto) once a week until plant material was required for tissue culture. Samples were collected throughout the year at the beginning of the various experiments. *Haemanthus pumilio* plants were sourced from the Duthie Reserve and the SUBG, after the floweing season as the leaves emerged Some *H. pumilio* plants in the SUBG were watered with Plant Protector (Efekto) before harvest. All plant material was harvested using sterile scalpel blades, and each leaf was cut with a new blade to prevent the transfer of contaminants between samples.

4.2.3 Decontamination and media preparation

Several decontamination protocols were tested. *Haemanthus* leaves were placed in 1 g/L Benlate® SP (Du Pont) or 10 ml/L Funginex (Efekto) overnight and then transferred to 1 g/L Previcur® Fungicide (Bayer) solution for 5 min with intermittent shaking. The leaves were then transferred to

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70% ethanol containing two drops of Tween-20 added using a teat pipette for 2 min. They were finally placed in a 50% bleach solution (1.75% [m/v] NaOCl) for 15 min, before thoroughly rinsing three times in sterilised distilled water. The leaves were blotted dry on sterile filter paper and cut into approximately 1 cm^2 explants for culture.

Alternatively, leaves were washed under running tap water and transferred to a 1 g/L suspension of Benlate® SP (Du Pont) fungicide overnight. Following the overnight fungicide soak, the leaves were transferred to a laminar flow hood and soaked for 30 min in 1 g/L Benlate®, 1 g/L boric acid, 4.5 mg/L gentamicin and three drops of Tween-20. The leaves were then removed from the soaking solution and placed in a 50% bleach solution (1.75% [m/v] NaOCl) with two drops of Tween-20 for 5 min, then rinsed in sterile distilled water three times for 5 min each to ensure that no bleach remained on the plant. The leaves were blotted dry on sterilised filter paper and cut into explants of approximately 1 cm^2.

Haemanthus albiflos bulbs were cut longitudinally into quarters, rinsed thoroughly under running tap water and then placed in 2 g/L Benlate® suspension for two days. The bulbs were immersed in a solution containing 1 g/L Benlate®, 1 g/L boric acid, 4.5 mg/L gentamicin and three drops of Tween-20 added using a teat pipette for 30 min. They were then transferred into 50% bleach with two drops of Tween-20, whereafter they were rinsed three times in dH_2O for 5 min each. Following decontamination, the bulbs were dried on sterile filter paper and the lower half of each bulb cut into twin scales and explants of approximately 1 cm^2 cut from the top half of bulb material to ensure that no plant material went unused. *Haemanthus pumilio* bulbs were not used in this study, since bulb culture would destroy one of the few remaining individuals of this species.

Following decontamination, explants were placed on nutrient media in glass tubes or magenta jars. All media used comprised of full-strength or quarter-strength MS (Sigma) containing 0.1 g/L myo-inositol, 2% (m/v) sucrose, solidified with 5 g/L phytoagar at pH 5.8. Media were sterilised by autoclaving at 121°C for 20 min at a pressure of 1.05 kg/cm2. Phytohormones were sterilised by filtration through a 0.22 µm filter (ThermoScientific) and were added after autoclaved media had cooled to under 60°C. The type and concentration of phytohormones (Table 4.2, Table 4.3) and the use of activated charcoal (Table 4.2) varied between treatments.

4.2.4 Contaminant identification

Fungal contaminants were observed growing from the cut sites of several Haemanthus explants in tissue culture. Only contamination from *H. pumilio* explants was analysed. Samples were taken from pure cultures of morphologically distinct fungi and transferred to 50 g/L malt extract agar media (Merck) in petri dishes and grown at 30°C for 48 h. DNA was extracted from filamentous fungi as per the protocol by Zhang et al. (2010) and stored at -80°C. A PCR was then conducted to amplify the fungal Internal Transcribed Spacer (ITS) region using ITS4 and ITS5 primers designed by White et

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al. (1990). PCR conditions consisted of an initial denaturation of 5 min at 94°C followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 60 s at 72°C, with a final extension of 7 min at 72°C. The resulting PCR product was sequenced using Sanger sequencing conducted at Stellenbosch University's Central Analytical Facility, and a BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) alignment was conducted to identify the fungal species.

4.2.5 *Haemanthus albiflos* and *H. pumilio* tissue culture

To assess the efficacy of *H. albiflos* bulb decontamination, twin-scale and bulb explants were placed in individual containers with hormone-free full-strength MS media. The explants were maintained in a growth room at 25°C under cool white fluorescent lights (Osram L 58V/740, 50 µmol photons /m^2/s) with a photoperiod of 16 h day: 8 h night.

Several experiments were competed with *H. albiflos* and *H. pumilio* explants to identify the optimal growth conditions (Table 4.2). All explants were maintained at 25°C. *H. albiflos* explants were placed on media containing combinations of 0, 0.1, 0.5, 1.0 or 2.0 mg/L BAP with 0, 0.1 or 1 mg/L NAA with or without the addition of 5 g/L activated charcoal (AC). Five technical repeats were conducted for each of the hormone concentrations. Additionally, the effects of 0.5 mg/L of the cytokinins BAP, zeatin, metatopolin (mT) or 2-isopentenyladenine (2iP) on the differentiation of *H. albiflos* explants placed on quarter-strength standard media (in the absence of an auxin) was tested through comparison to a hormone-free control.

Table 4.2: Tissue culture experiments conducted on *Haemanthus albiflos* and *H. pumilio*

	2iP[a]	*BAP*[b]	*mT*[c]	*Zea*[d]	*NAA*[e]	*Photo-period*	*AC*[f] *(g/L)*	*MS strength*	*Explants per treatment*	*Length (weeks)*
Haemanthus albiflos		X			X	0 h	5	Quarter	5	8
		X			X	16 h	5	Quarter	5	8
	X	X	X	X		16 h		Quarter	10	16
		X			X	16 h		Quarter	7	-
		X			X	16 h		Full	7	-
		X			X	16 h		Full	5	12
Haemanthus pumilio	X	X	X	X		16 h	5	Quarter	5	16
		X			X	16 h		Full	10	12

a: 2-isopentenyladenine b: 6-Benzylaminopurine c: metatopolin; d: Zeatin e: 1-Naphthaleneacetic acid; f: activated charcoal

Quarter- and full-strength media was poured into sterilised individual tubes and supplemented with various combinations of NAA and BAP (Table 4.3). This experiment was initiated in February 2020, with the explants maintained at 25°C and transferred to fresh media once every four weeks. Levels of contamination were noted, and contaminated explants removed from the experiment. This

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experiment could not be completed due to the Covid-19 lockdown instituted from March to August 2020, however, some hormone combinations (0 mg/L NAA: 5 mg/L BAP, 0.1 mg/L NAA: 1 mg/L BAP, 0.1 mg/L NAA: 5mg/L BAP, 1 mg/L NAA: 0.1 mg/L BAP, 1 mg/L NAA: 0.5 mg/L BAP, 2 mg/L NAA: 0.1 mg/L BAP and 5 mg/L NAA: 0.1 mg/L BAP) had started to show signs of differentiation and were used to for the *H. pumilio* experiments post-lockdown. This experiment was repeated with *H. albiflos* explants on full-strength media only, post-lockdown (Table 4.3).

Table 4.3: Combinations of NAA and BAP used for tissue culture of *H. albiflos.*

		BAP (mg/L)					
		0	**0.1**	**0.5**	**1**	**2**	**5**
NAA (mg/L)	**0**	A1	B1	C1	D1	E1	F1
	0.1	A2	B2	C2	D2	E2	F2
	0.5	A3	B3	C3	D3	E3	F3
	1	A4	B4	C4	D4	E4	F4
	2	A5	B5	C5	D5	E5	F5
	5	A6	B6	C6	D6	E6	F6

Explants of *H. pumilio* were placed on quarter strength standard media containing 5 g/L AC or 0.5 mg/L cytokinin (Table 4.2) poured into sterilised containers. Additionally, *H. pumilio* explants were also maintained in individual tubes containing full strength standard media supplemented with NAA and BAP in combinations of 0 mg/L NAA: 5 mg/L BAP, 0.1 mg/L NAA: 1mg/L BAP, 0.1 mg/L NAA: 5mg/L BAP, 1 mg/L NAA: 0.1 mg/L BAP, 1 mg/L NAA: 0.5 mg/L BAP, 2 mg/L NAA: 0.1 mg/L BAP and 5 mg/L NAA: 0.1 mg/L BAP as well as hormone-free control. The explants were maintained in a growth cabinet at 25°C with subculture onto fresh media every four weeks. Contamination was noted and contaminated explants were removed from the experiment.

4.2.6 Hardening off

Plantlets, developing from the *H. albiflos* explants that were sufficiently large (about 2 cm from the base of the bulb to the leaf tip) were removed from tissue culture, any media washed off under running tap water and placed in pots containing perlite, vermiculite and sand in a 1:1:1 ratio. The pots were placed in a clear container, in a glasshouse, with eight holes on the lid. Initially, all the holes were plugged and each day for the first three days two plugs were removed. On the fourth day the lid was removed.

4.2.7 Statistical analysis

An Analysis of Variance (ANOVA) was conducted using the analysis Toolpak excel add on. If the ANOVA revealed a significant difference, Tukey’s HSD tests were used for pairwise comparisons.

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4.3 Results

4.3.1 Decontamination

The initial decontamination protocol using Funginex, Previcur® and ethanol resulted in 100% contamination of explants by the end of the second week. Decontamination with Benlate® resulted in varying levels of contamination in each treatment (Figure 4.1) but was more effective than Funginex. Therefore, all subsequent cultures used the Benlate® decontamination protocol.

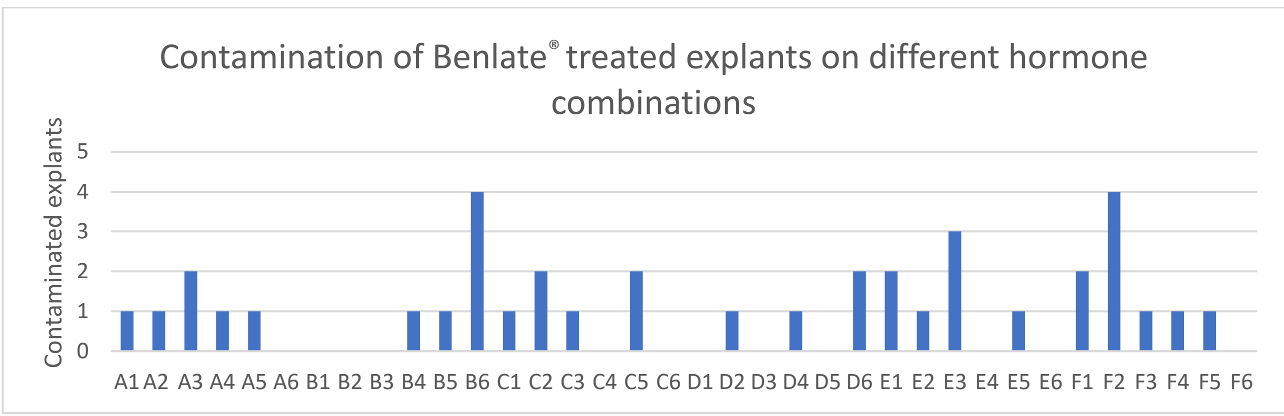
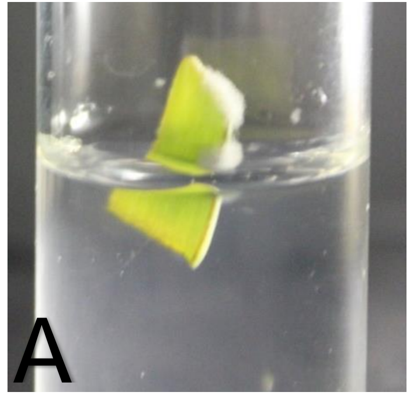


Figure 4.1: Contamination of *H. albiflos* plants by treatment. Definitions for the hormone combinations shown on the x-axis are given in Table 4.3.

4.3.2 Contaminant identification

The ITS regions of 12 fungal contaminant samples, observed emerging from the cut sites on *H. pumilio* explants in culture (Figure 4.2), were amplified and sequenced. Only seven of these samples were amplifiable, and of these seven, five returned sequences that produced significant levels of similarity after a BLAST search. Four samples were found to be *Meyerozyma guilliermondii* with more than 90% identity and an E value of 0.0. One sample was found to be *Sarocladium kiliense* with a 90.82% identity and an E value of 0.0.



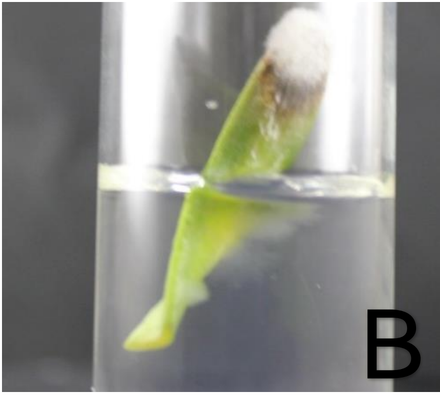


Figure 4.2: Contamination seen emerging from explant cut sites. A: shows contamination emerging from an *H. albiflos* explant and **B:** contamination from an *H. pumilio* explant.

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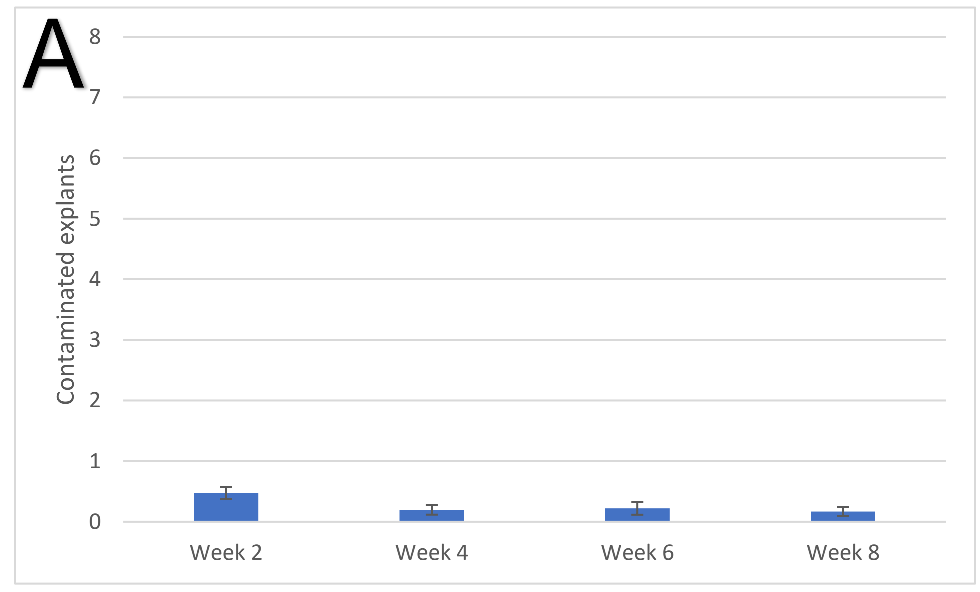
4.3.3 Explant contamination and *Haemanthus albiflos* micropropagation

Bulb material became contaminated within the first week of culture and subsequent washes with 50% bleach and 1 g/L Benlate® were insufficient to remove the contamination. All explants from bulb material were removed from the experiment by week four and attempts to decontaminate again were unsuccessful. No differentiation of cells was visible at this point. Explants of *H. albiflos* cultured on standard media with various combinations of NAA and BAP showed higher levels of contamination from explants on AC containing media than on media without AC within the first four weeks. However, by the end of the eight-week period, all but three explants had been contaminated or browned. Two of the differentiating explants were on 0 mg/L NAA:0 mg/L BAP and one on 1 mg/L NAA:2 mg/L BAP AC containing media.



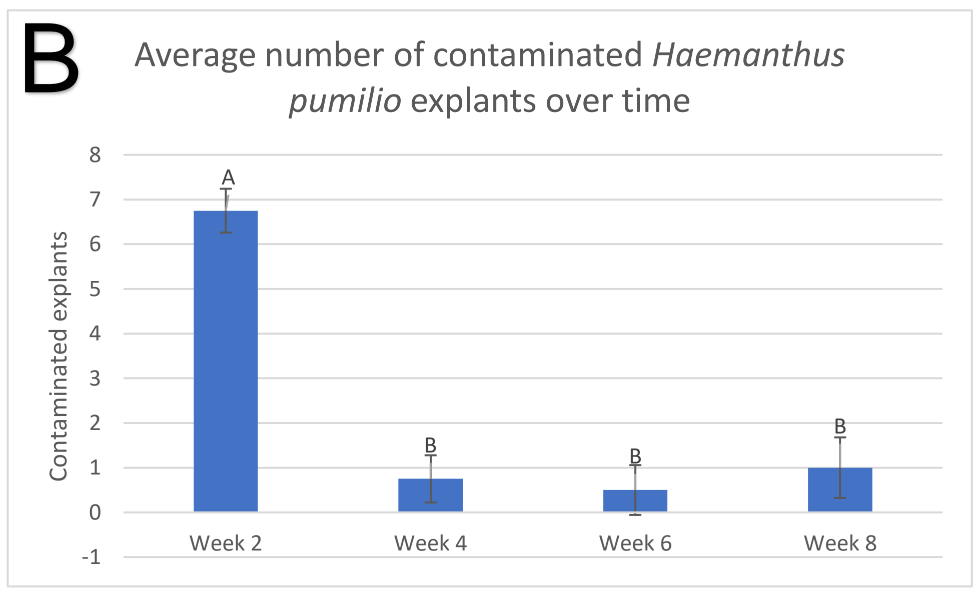


Figure 4.3: Average levels of explant contamination in **A:** *Haemanthus albiflos* and **B:** *Haemanthus pumilio* over time.

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When comparing the efficacy of different cytokinins (BAP, 2iP, zeatin and mT) to hormone-free media in initiating differentiation, explants maintained in the dark had higher levels of contamination than those in the light. All explants in the dark on BAP, 2iP, mT or hormone-free media were contaminated by the end of the eighth week. Nonetheless, the uncontaminated explants on zeatin media failed to differentiate by the end of 16 weeks. Of explants exposed to 16 h of light daily, hormone-free media and 0.5 mg/L BAP yielded the lowest levels of contamination. Levels of browning amongst explants were also lower in light conditions than in darkness. However, none of the explants from any of the cytokinin treatments had differentiated by the end of week 16.

Of the tested hormone combinations, only a few combinations of NAA and BAP initiated cell differentiation (Table 4.5). The levels of contamination within the first eight weeks of culture were noted (Figure 4.3A), though the difference in contamination per week was insignificant ($p>0.05$). Explants that differentiated started with an initial formation of callus-like structures that grew until a root, bulb or shoot was visible (Figure 4.4). The ANOVA comparing the numbers of explants per treatment, revealed that there were no significant differences between treatments ($p>0.05$)

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Table 4.5: Differentiation of *Haemanthus albiflos* explants on various combinations of NAA and BAP. The abbreviated combinations are explained in Table 4.3.

Treatment	No. plantlets	
B1	14	
B2	12	
C5	36	
C6	-	
D5	>100	
E3	14	
E5	>100	
F4	35	

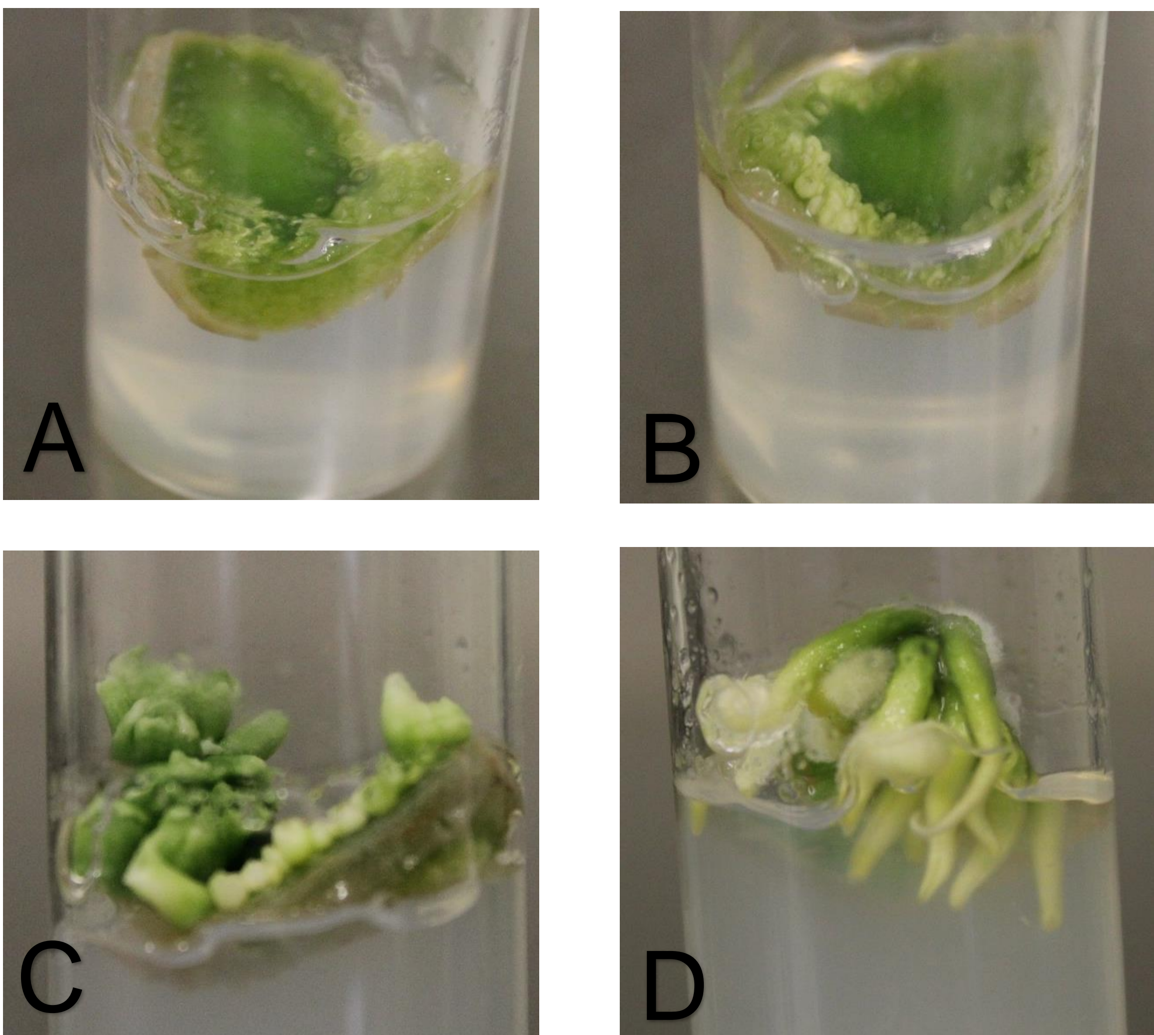

Figure 4.4 Stages of plantlet formation in *Haemanthus albiflos* explants. Explants begin by forming callus-like structures (**A**) that gradually become more distinguishable (**B**) and eventually form either leaf and bulblet structures (**C**) or root structures (**D**).

4.3.4 *Haemanthus pumilio* micropropagation

H. pumilio plants were decontaminated using Funginex and placed on quarter strength standard hormone-free media or supplemented with g 0.5mg/L cytokinin (BAP, 2iP, zeatin, mT). These explants developed only low levels of contamination. However, all explants on AC-containing media had become discoloured by the end of week 8, while only explants on zeatin and mT media became discoloured in the same period. Nonetheless, no differentiation was produced by any explant at the end of 12 weeks.

Secondly, some plants were treated with Plant Protector and placed on media containing various combinations of NAA and BAP (0 mg/L NAA: 5 mg/L BAP, 0.1 mg/L NAA: 1 mg/L BAP, 0.1 mg/L NAA: 5mg/L BAP, 1 mg/L NAA: 0.1 mg/L BAP, 1 mg/L NAA: 0.5 mg/L BAP, 2 mg/L NAA: 0.1 mg/L BAP and 5 mg/L NAA: 0.1 mg/L BAP). Levels of contamination over the first eight weeks were noted

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(Figure 4.3B), with a significant amount of the total contamination being produced within the first two weeks. Explants from plants treated with Plant Protector showed significantly less contamination (p<0.05) compared to untreated plants (Figure 4.5).

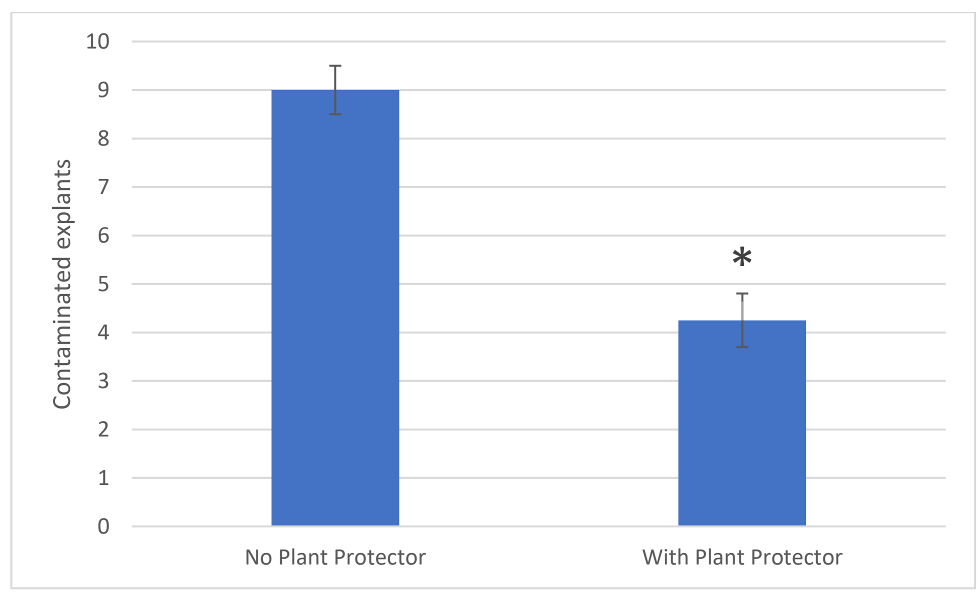

Figure 4.5: Effect of Plant Protector on explant contamination. Explants treated with Plant Protector produced significantly less contamination (p<0.05) than untreated plants.

Despite the reduced contamination levels, many remaining explants had browned by the end of 12 weeks. All surviving explants failed to show signs of differentiation after 12 weeks of tissue culture. However, at week 13, one explant, growing on media containing 0.1 mg/L BAP: 2 mg/L NAA began to show signs of differentiation (Figure 4.6).

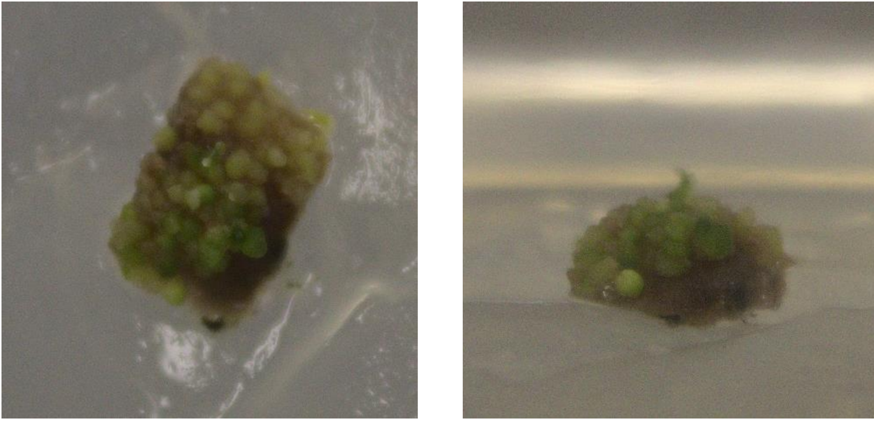

Figure 4.6 Differentiation on a *Haemanthus pumilio* explant Pictured from the top (left) and from the side (right) is the formation of *H. pumilio* plantlets on a single explant in the early stages of differentiation.

4.3.5 *In vitro* seed germination

In 2019, artificial pollination yielded 116 seeds that were planted in the SUBG. Less than 20 of these seeds germinated. In 2020 15 seeds (nine from the Wellington population and six from the Duthie Reserve) were obtained from artificial pollination experiments and 11 of these germinated *in vitro,*

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though 12 (including 10 of the seedlings) became contaminated, and thus were removed from the experiment and immediately planted out to save the plant.

4.4 Discussion

Contamination is a significant hindrance to successful plant tissue culture (Mng'omba et al., 2012). Root and bulb materials are usually more difficult to decontaminate than leaf and stem material (Hussey, 1975; Rabe and van Staden, 1999) due to the abundance of microorganisms in the soil as well as the structure of bulbs which allows for movement of microorganisms between the scales (Rabe and van Staden, 1999; Fennell et al., 2001). The presence of mites and thrips found in the soil compounds these problems (Cheesman, 2013). In the present study, none of the explants sourced from bulb material could be successfully decontaminated. This agrees with results of Rabe and van Staden (1999) who lost 65% of their *Haemanthus albiflos* and *H. deformis* bulb explants to contamination. Due to the conservation status of *H. pumilio*, an effective decontamination protocol would be required prior to attempting tissue culture using these bulbs, given that bulb tissue culture techniques lead to the destruction of an entire plant.

In the present study, fungal contamination was observed emanating from leaf and bulb explants. Plant-fungi symbioses are commonplace in nature. Endophytes, fungi or bacteria that grow within plant tissue, can do so without causing any harm to the plant itself and can, in some cases, benefit the plant (Barton and Northup, 2011). In mutually beneficial relationships, the plant provides nutrients, while the endophyte produces growth-promoting substances or protects the host plant from pathogenic microorganisms and herbivory (Barton and Northup, 2011).

Several genera of fungi have been found in association with Amaryllidaceae plants (Yang et al., 2009; Muvea et al., 2014; Zhou et al., 2020). *Allium cepa* L. was protected from *Thrips tabaci* Lind. herbivory by inoculation of fungal species isolated from *A. cepa* plants that did not display signs of infection (Muvea et al., 2014). Additionally, the production of several important Amaryllidaceae alkaloids was increased in cultures of *Lycoris radiata* Herb. inoculated with various fungal species (Zhou et al., 2020). However, in tissue culture, the usually harmless fungi can exit the plant material and outgrow the explant, leading to necrosis of plant tissue. Pathogenic fungi can also associate with plants. Although harmless to the plant while on the surface, the fungi can enter tissue through lesions and cause infection. *Clivia, Crinum*, *Hippeastrum* and *Hymenocallis* Salisb. species were all found to harbour pathogenic fungi from the genus *Colletotrichum* (Yang et al., 2009).

In the present study, several *H. pumilio* cultures produced fungal contaminants identified as either *Meyerozyma guilliermondii or Sarocladium kiliense*. As contaminants isolated and sequenced in the present study emerged from the cut sites of the explants (Figure 4.2), it is possible that these fungi may be endophytes of *Haemanthus* as both species possess characteristics that would make them

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advantageous to plants in symbiosis. As far as could be ascertained, there is no information currently available in literature on the endophytic fungi associated with *Haemanthus.*

Sarocladium kiliense, previously *Acremonium kiliense,* is a saprobic fungus that is an opportunistic human pathogen (Júnior et al., 2013). It is an endophyte (Choi et al., 2008; Piwoni, 2012; Teasdale et al., 2018) in some plants, but also exhibits pathogenicity towards other plant species (Choi et al., 2008). The fungus has been isolated from bulbs of the *Narcissus* cultivar fortissimo (Piwoni, 2012), the rhizosphere of *Zea mays* L. plants (Khonglah et al., 2015) as well as from the tillers of *Brachiaria jubata* Stapf (Teasdale et al., 2018). An association with this fungus may be advantageous to plants due to its mycotoxicity against plant pathogenic fungi such as *Diaporthe longicolla,* which is a cause of seed rot in soybean (Divilov and Waker, 2016) as well as *Fusarium oxysporum* and *Clavibacter michiganense,* which can cause wilting of tomato plants (Bargmann and Schönbeck, 1992).

Meyerozyma guilliermondii, previously *Pichia guilliermondii*, is the heterothallic type species of the genus *Meyerozyma* (Kurtzman, 2011). It has been isolated from the medicinal plant, *Paris polyphylla* Sm. (Zhao et al., 2010), the roots of *Drynaria quercifolia* (L.) J.Sm. (Aban et al., 2017) and the flowers of *Gentiana imbricate* Froel. (Kurtzman, 2011)*,* while its anamorph, *Candida guilliermondii,* has been isolated from seawater (Zaky et al., 2014), freshwater, soil, sand and various animals (Cooper, 2011). Associations with *M. guilliermondii* would be beneficial to plant hosts as it produces the auxin IAA. Aban et al. (2017) reported the amount of IAA produced per gram dry weight by *M. guilliermondii* to be significantly higher than that produced by other fungal isolates. Mycotoxic properties of *M. guilliermondii* against pathogenic and post-harvest spoilage fungi have also been documented (Droby et al., 1989; Petersson and Schnürer, 1995). Nevertheless, *C. guilliermondii* has been reported as a major contaminant in tissue culture of several plant species, representing the majority of yeast species contaminating cultures of *Rosa* E.Willm*, Astilbe* Buch.-Ham.*, Bergenia* Neck.*, Clematis* L.*, Delphinium* L. *and Hemerocallis* L. plants (Leifert et al., 1990). Leifert et al, (1990) found that it fermented up to 75% of the sucrose in media, thereby producing by-products such as ethanol and acetic acid which reduced the pH of the media to less than 3.0 (Leifert et al., 1990). These alterations to the *in vitro* environment could also be the reason that *H. pumilio* is difficult to culture as the endophytes may be increasing acidity of media and utilise the carbon source.

In order to improve the decontamination process and eliminate internal fungal contaminants, Rabe, and van Staden (1999) suggested the treatment of plants with a systemic fungicide before harvesting plant material. In the present study, explants from *H. pumilio* plants that were treated with Plant Protector showed significantly lowered levels of contamination in comparison to untreated explants (Figure 4.5). Mng'omba et al. (2007) found that treatment of *Uapaca kirkiana* Mull.Arg plants with a systemic fungicide also led to successful decontamination. However, this treatment is not viable for wild populations, as the addition of fungicides could alter and damage ecosystems. Due to the high

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levels of contamination, the efficacies of several treatments could not be examined. It is, therefore, important to continue to test and optimise decontamination protocols. To reduce the levels of contamination in explants, seeds obtained from artificial pollination could be germinated *in vitro*, resulting in axenic seedlings which can be used as sources of explant material.

In the present study, more seeds were obtained in 2019 via artificial pollination than in 2020. This large difference in seeds acquired from artificial pollination between 2019 and 2020 could be explained by the protandrous nature of *H. pumilio,* in which male reproductive organs mature before female reproductive organs, (Summerfield, 1990). It is possible that the 2019 artificial pollination experiment occurred at a time when the majority of plant stigma were receptive to pollen, but that the second (2020) experiment occurred too early. Nonetheless, only 10 of the 2019 seeds geminated in soil, whereas 11 of the 15 seeds from the 2020 group germinated. Summerfield (1990) was able to germinate 82% of seeds obtained by artificial pollination *in vitro*, using only water and filter paper. She also noted that only one seed became contaminated before germination and was removed from the experiment. It is possible that using growth media in the present study contributed to the high level of contamination. As mentioned above, explants obtained from *in vitro* germinated seedlings could then be placed on media with hormone combinations to induce production of new plantlets without the need for decontamination.

Two of the few *H. albiflos* treatments that did not get contaminated and were able to produce plantlets were hormone-free media, and media with 1 mg/L NAA and 2 mg/L BAP and AC. These results are similar to those of Rabe and van Staden (1999), where plantlets were produced from *H. humilis, H. deformis* and *H. albiflos* explants on hormone-free media. Additionally, supplementation of media with AC led to an increase in the number of *Cyrthanthus clavatus* (L'Hér.) R.A.Dyer*, Cyrtanthus spiralis* Berch ex. Ker Gawl. and *Crinum variabile* Herb. plantlets produced in culture (Fennell et al., 2001; Moran et al., 2003). Interestingly, it did not affect plantlet production in *Brunsvigia undulata* F.M.Leight. (Rice et al., 2011) and reduced production of *Cyrtanthus speciosus* R.A.Dyer plantlets (Angulo et al., 2003). Activated charcoal is capable of adsorbing several chemicals, such as phenolics, which can cause deleterious effects on plantlet growth (Fridborg et al., 1978). However, it also adsorbs phytohormones from the media, making them inaccessible for plant tissues (Weatherhead et al., 1978). Therefore, the varying effects of AC on plantlet formation and growth is dependent on the requirement levels of the explants.

The most prolific *H. albiflos* treatments in the present study contained BAP and NAA at ratios of 1:2 (1 mg/L and 2 mg/L) and 1:1 (2 mg/L). These results are contrary to previous studies on Amaryllidaceae that found that a higher BAP:NAA ratio led to the highest number of explants produced (Ulrich et al., 1999; Angulo et al., 2003; Moran et al., 2003; Ncube et al., 2015) and that having a higher NAA concentration hindered the production of plantlets (Fennell et al., 2001). These

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variations may be due to differences at the time of harvest, as the explants that differentiated most at these lower BAP:NAA ratios were harvested in the winter, while the higher BAP: NAA ratios had begun to initiate differentiation of explants harvested in summer, before lockdown. The initial harvest was prior to the flowering period of *H. albiflos*, while the second harvest was done towards the end of the peak flowering time between April to August (Snijman, 1984). It has been suggested that the levels of cytokinin increase as plants begin to flower (Bernier et al., 1993), so the elevated levels of internal cytokinin may have reduced the need for BAP in culture, thus lowering the BAP:NAA optimum ratio. However, while this may be true for evergreen species, like *H. albiflos*, the same cannot be expected of *H. pumilio* as it flowers before the leaves emerge.

While most combinations of NAA and BAP in *H. albiflos* either produced bulbs or shoots, treatment with 0.5 mg/L BAP and 2 mg/L NAA led to the production of roots. This is due to the antagonistic nature of cytokinins and auxins. A high auxin concentration with a low cytokinin concentration generally leads to the formation of root tissue (Skoog and Miller, 1957). At the root apical meristem, cytokinins promote cell differentiation by suppressing auxin, while auxins promote cell division (Moubayidin et al., 2009) and as such, a high auxin concentration increases root size.

Though it was the last of the three species tested by Rabe and van Staden (1999) to produce plantlets, *H. albiflos*, is one of the easiest species in the genus to grow (Duncan, 2002). The ease of micropropagation of this species is likely due to its ability to reproduce clonally by offsets (Rabe and van Staden, 1999) in nature. It is even known to readily form bulblets in leaf axils (Snijman, 1984). While these characteristics make it difficult to use the same micropropagation protocol to multiply other species of *Haemanthus*, a single *H. pumilio* explant began to differentiate using the protocol designed with *H. albiflos* as the model. This shows that *H. pumilio* can be propagated *in vitro* from vegetative material, though the process may take longer than in *H. albiflos.* The treatment that produced differentiation of *H. pumilio* contained high NAA (2 mg/L) and low BAP (0.1 mg/L) and was the only one to induce cell differentiation in this species. At the time of writing, the tissue differentiation had not progressed sufficiently to determine whether it would result in the production of roots, shoots or bulblets. It appears likely that roots will develop on the explant, as the concentrations of phytohormone in the treatment are optimal for root formation. Though, due to the possibly elevated cytokinin levels in *H. albiflos* at the time of harvest, this hormone combination induced shoot production.

4.5 Conclusion

This study built upon the work of Rabe and van Staden through testing various hormone combinations on plantlet production of *H. albiflos.* However, it is difficult to ascertain whether the use of phytohormones in the tissue culture of *H. albiflos* improved plantlet production as Rabe and van Staden (1999) did not provide numbers of plantlets produced per explant. Nonetheless, a successful

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micropropagation protocol was developed using *H. albiflos* explants, and a treatment from this protocol initiated differentiation in a single *H. pumilio* leaf explant. This provides a valuable first step in forming an effective micropropagation protocol for *H. pumilio*. Future studies should focus on using cytokinin and auxin concentrations in similar concentrations to the 0.1 mg/L BAP and 2 mg/L NAA that were most successful in the present study. The relationship between *H. pumilio* and the two endophytic fungi identified from them in this study should be further investigated. These fungi could be providing the plant with vital growth compounds that may improve tissue culture if added to the media in future experiments.

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CHAPTER 5: CONCLUSIONS AND FUTURE STUDIES

This study aimed to establish a micropropagation protocol for the endangered *Haemanthus pumilio* as well as to create an understanding of the population genetics of this species. The first experimental chapter (Chapter 3) used two organellar and one nuclear gene region to determine the phylogenetic position of *H. pumilio* within the genus. However, these regions could not differentiate this species from *H. sanguineus* and *H. canaliculatus*. Additionally, the resulting trees had vastly different topologies. This incongruence may be due to variations in phylogenetic histories of the plastid and nuclear genomes or sampling error. It is important to determine what the cause of this incongruence is as this would inform decisions on whether or not to partition the datasets. It also employed several DNA fingerprinting markers to describe the population genetics of the remaining two populations of this critically endangered species. Although the gene regions were unable to, DNA fingerprinting methods were mostly able to partition the two populations into separate subclades and found greater diversity within the populations than between them. High intraspecies diversity suggests that the mode of seed dispersal of *H. pumilio* does not greatly impact its genetic diversity, indicating that propagation of the plants from the same population will not have severe negative genetic consequences.

The second experimental chapter (Chapter 4) aimed to produce an effective *H. pumilio* micropropagation protocol by creating and optimising one for *H. albiflos*, a non-threatened plant that has leaf material available throughout the year. Effective methods of decontamination and plantlet initiation in *H. albiflos* were developed. This study was, however, unable to successfully decontaminate the majority of *H. pumilio* explants without irrigation using Plant Protector™. Therefore, a decontamination protocol must be designed and optimised for *H. pumilio* before further tissue culture experiments are conducted. Additionally, the micropropagation protocol used for *H. albiflos* protocol was not fully transferable to *H. pumilio,* as *H. albiflos* easily propagates by cloning, even in nature. Nevertheless, a single *H. pumilio* explant had begun to differentiate by the time of writing, which provided crucial preliminary information on efficient *H. pumilio* propagation, and, most importantly, confirms that micropropagation of *H. pumilio* is possible. Moreover, artificial pollination and *in vitro* seed propagation were successful in germinating seedlings of *H. pumilio*, which could be a useful source of explant material once an ideal medium is identified.

Although this study was able to delineate the two populations of *H. pumilio*, further analysis must be conducted to determine whether the populations are becoming more differentiated, or less so. In light of the skin-staining sap which is specific to the plants from the Duthie Reserve, metabolite analysis of plants from each of the populations should also be conducted as this will increase the understanding of the relationship between them. Additionally, future tissue culture research should focus on improving the tissue culture protocol that allowed for explant differentiation. Plant-symbiont

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relationships of *H. pumilio* should also be examined to determine whether the contaminants produced by explants play a crucial role in plant development.

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