Quantifying pollen transfer between cultivated and wild *Cyclopia* species in South Africa

by

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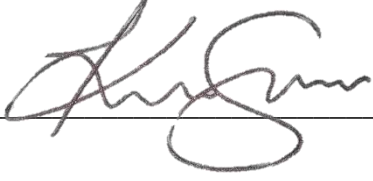
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Abstract

Gene flow between cultivated populations and their co-occurring wild relatives can result in genetic contamination, with significant consequences in terms of the loss of genetic resources, possible extinction of locally adapted variations, and hybridization. Honeybush, *Cyclopia* Vent., is an endemic genus in the fynbos biome of South Africa with commercial value in the tea industry. With the growth of the industry, the shift from wild-harvested material to cultivated biomass is promoted to ensure ecological sustainability and avoid over harvesting. However, cultivating indigenous species in their native range may cause pollen-mediated gene flow between cultivated and wild populations, causing depletion of the genetic resources essential for the tea industry of South Africa, threatening local gene pools and possibly resulting in hybridization in the wild. To establish the potential for gene flow through pollen-transfer, the pollinators of four commercially important *Cyclopia* species; namely *Cyclopia genistoides*, *C. subternata*, *C. maculata* and *C. intermedia* were identified. Pollinator movement was investigated using mark-release-recapture and radio-tagging. Pollen longevity was determined under field conditions to indicate the distance at which pollen can be transferred. Hand-crossing experiments were conducted to determine the capacity for between and within species crosses in *Cyclopia*. Commercially important *Cyclopia* species are pollinated only by carpenter bees, *Xylocopa*, including *Xylocopa capitata, X. flavorufa, X. caffra, X. rufitarsis, X. scioensis* and *X. sicheli*. Carpenter bees are generalist foragers with indiscriminate foraging behaviours on a variety of *Cyclopia* species. Mark-release-recapture of carpenter bees revealed numerous cases of movement between cultivated and wild *Cyclopia* populations. In addition, radio-tracking confirmed between-site movement, with a maximum distance of 1194 m travelled in a single foraging bout and daily home range sizes (of up to 23 893 m²) spanning across cultivated and wild *Cyclopia* populations. Additionally, *Cyclopia* pollen remains viable for at least five days. Crosses between *C. subternata* x *C. genistoides* and *C. subternata* x *C. maculata* produced hybrid seeds, revealing the likelihood for hybridization to occur with pollen-transfer between cultivated and wild populations. These results signify the necessity for a protocol guiding the planting of *Cyclopia,* in order to avoid genetic homogenization and erosion, which is presented in preliminary form for the use by the honeybush tea industry. This protocol considers various barriers to genetic contamination, including the natural range, ploidy, seed dispersal and pollen-flow distances, and seed source. The protocol provides a valuable tool for determining the risk of introgression at individual plantations that can be used not only for *Cyclopia,* but adapted to other indigenous agricultural flora. In order to ensure the sustainable use of indigenous crops, management in the form of a planting protocol, like the one presented here, is critical.

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Abbreviations and Acronyms

Abbreviation/Acronym	
˚C	Degrees Celsius
μL	Microlitre (millionth of a litre)
ACF	Western Cape Department of Agriculture - Alternative Crop Fund
AGM	Annual General Meeting
ARC	Agricultural Research Council
COVID-19	Coronavirus disease caused by the SARS-CoV-2 virus
d	Day/s
et al.	And others
FCR	Fluorochromatic Reaction test
g	Gram/s
GBIF	Global Biodiversity Information Facility
GPS	Global Positioning System
ha	Hectare/s
hr	Hour/s
IQR	Interquartile Range
km	Kilometre/s
LC	Least Concern
m	Metre/s
MCP	Minimum Convex Polygon
mm	Millimetre/s
No.	Number
n	Number/replicates
NT	Near Threatened
QGIS	An open source Geographic Information System
SAHTA	South African Honeybush Tea Association
spp.	Species plural

Glossary

Term	
Autonomous autogamy	Also termed "selfing". Involves the deposition of self-pollen for reproductive assurance in plants.
Camera trapping	The use of a camera for monitoring activity through automatic triggering.
Cross-pollination	The pollination of a flower with pollen of a different plant.
Cultivated-caught	Bees caught and marked in the cultivated site.
Daily MCP home range	An estimation of daily home range size using the Minimum Convex Polygon (MCP) method of connecting outer GPS point locations to form a convex polygon.
Floral observation	Involves direct observation of a patch of flowers noting any interactions from visitors to the flowers within a certain timeframe.

Fluorescence	A type of luminescence brought about through the absorption of light.
Fluorochromatic Reaction test	A test for determining the viability of pollen grains through their reaction to a chemical, fluorescein diacetate, causing bright fluorescence.
Foraging bout	The length of time whereby foraging takes place.
Gene flow	The movement or transfer of genetic material from one population to another.
Genetic contamination	The uncontrolled transfer of genetic material into wild populations.
Genetic drift	The change in frequency of different genotypes in a populations with the disappearance of gene variants by chance.
Genetic homogenisation	Reduction in variation in the gene pool typically through hybridization.
Hand-pollinate	Human induced pollination through manual transfer of pollen from the anther/s to the stigma.
Heterozygosity	A genetic condition where two alleles are present at a locus.
Hybrid vigour	The improved functioning of hybrid offspring, superior to the two parents.
Hybridization	The production of offspring through mating by parents of different species.
Inbreeding depression	Decreased fitness of offspring as a result of increased homozygosity (possessing to identical forms of the same gene) in parents.
Introgression	Transferring genetic material from one species to another as a product of hybridization with continuous backcrossing.
Maximum forage distance	The maximum flight distance reached from the release point.
Natural pollination	The transfer of pollen from anther to stigma without any intervention from humans.
Outbreeding depression	The reduction of fitness as a result of crossing between two genetically distant groups.
Outcrossing	The transfer of genetic material between species that are not closely related.
Planting protocol	A guide for biodiversity-friendly cultivation of indigenous flora aimed at minimizing genetic contamination of wild populations.
Ploidy variation	Variation in the number of sets of chromosomes an organism possess.
Pollen limitation	A method to test whether plants are resource or pollen-limited. Also referred to as pollen addition as it involves the supplementation of pollen to an open flower.
Pollen viability	The survivability of pollen, synonymous with pollen longevity or the duration which pollen remains alive.
Pollination network	A visual network displaying the interaction between species of plants and their pollinations.
Polyploid	Containing more than two homologous sets of chromosomes.
Radio-tagging	Attaching a radio-transmitter device on an organism intended to aid with tracking and monitoring of their natural movements.
Radio-transmitter	Equipment that is used to generate electromagnetic waves intended to deliver signals or messages.
Re-observation	The observation of a marked bee after it had been marked and released.
Site fidelity	The tendency to return multiple times to the same location previously visited.

Telemetry	The measurement of data collected from remote points. Telemetry range is the spatial distance at which telemetry is operational.
Tripping	The separation of the lateral and ventral petals of a flower through active handling by its pollinators.
Visitation rate	The rate at which visitors are interacting with a plant. Calculated by visits/flower/hour.
Wild-caught	Bees caught and marked in the wild site.

Chapter 1 : General introduction

1.1.1. Pollen-mediated gene flow

The reproduction of 80% of angiosperms or flowering plants relies on animal-mediated pollination (Rodger *et al.,* 2021). This confirms the value of plant-pollinator interactions in maintaining the functioning of terrestrial ecosystems (Ollerton *et al.,* 2011). Additionally, the movement of pollinators between habitat patches is necessary to avoid genetic erosion of isolated plant populations by increasing the movement of genetic material, allowing for species adaptation to a changing environment (Ellstrand & Elam, 1993). In some cases, isolated populations face the threat of genetic drift and inbreeding depression, resulting in the loss of heterozygosity or genetic variability (Ellstrand & Elam, 1993). Thus, gene flow through pollen-transfer may have considerable value in the maintenance of ecological and genetic integrity of plant populations. On the other hand, pollen-mediated gene flow between agricultural crops and nearby wild relatives may result in genetic contamination and hybridization (Ellstrand *et al.,* 1999). This not only impacts the wild relatives of transgenic crops (Hancock *et al.,* 1996, Ellstrand, 2001), but also the wild relatives of indigenous species that have been domesticated (Ellstrand *et al.,* 1999; Andersson & de Vicente, 2010).

1.1.2. Gene flow between cultivated and wild conspecifics/congenerics

Genetic introgression of wild plant populations with cultivated varieties may occur when cultivated plants are planted within the natural range of a closely related species. The loss of rare alleles, due to selective breeding, for cultivation has been recorded (e.g., *Scutellaria baicalensis* (Yuan *et al.,* 2010)) or is likely (e.g., *Cyclopia* (Potts, 2017)) resulting in a reduction in genetic diversity, which in turn, could reduce the phenotypic resilience of native relatives with gene flow from cultivated plants. Gene flow between species can result in hybridization, introgression and possibly hybrid vigour (Van der Bank *et al.,* 1996). Uwimana *et al.,* 2012), potentially threatening the survivability of native plants. Being the result of human activities, either on purpose or accidently, these are activities that can be monitored and managed. Additionally, the homogenising effects of plant breeding and agricultural farming practices has resulted in a loss of genetic resources which play a key role in agriculture as well as biodiversity (Millennium Ecosystem Assessment, 2005). In cultivation, genetic diversity is important for the strength of crops; for example, their resistance to diseases (Hammer & Teklu, 2008). In wild populations, when different locally adapted genetic varieties are planted in near proximity, introgression may result in the homogenisation of alleles and the loss of locally

adapted distinctiveness, and possibly the extinction of the locally adapted variation (Ellstrand *et al.,* 1999; Wolf *et al.,* 2001; Gepts & Papa, 2003). This movement of genetic material is mostly unidirectional from crops to wild populations (Papa & Gepts, 2003; Trucco *et al.*, 2009; Jencewski *et al.,* 1999). The potential of genetic contamination to occur between cultivated populations and their wild relatives through cross-pollination has been widely studied globally (see Table 1.1 for examples). Notably 12 of the world's 13 most important crops have resulted in hybrid formation with wild relatives within their agricultural distribution (Ellstrand *et al.*, 1999).

Table 1.1. Examples of some of the most well-known crops and the threat of gene flow between these and their wild conspecifics/congenerics

Source	Location of study	Species under study (common name)	Hybridization/ gene flow evident	Key result
Arias & Rieseberg (1994)	Morelos, Mexico	*Helianthus annuus* (sunflower)	Gene flow evident	Using a homozygous allele as a molecular marker, gene flow was revealed at increased rates with proximity to the cultivated site.
Jenczewski *et al.* (1999)	Spain	*Medicago sativa* (alfalfa)	Gene flow through cross-pollination by honey bees, bumble bees, megachilids	Wild populations were different to cultivated populations in terms of allozymes and quantitative traits.
Burke *et al.* (2002)	North/ South Dakota, Kansas/ Colorado/ Nebraska, and western Texas	*Helianthus annuus* (sunflower)	Hybridization and therefore gene flow through cross-pollination by honey bees, bumble bees, and solitary bees	Morphological evidence of hybridization in sunflowers of 10-33% of the populations surveyed.
Song *et al.* (2003)	Hunan Province, southern China	Cultivated rice variety Minghui-63 and *Oryza rufipogon*	Hybridization and therefore gene flow through wind-pollination	Hybridization between the two species was detected. Gene flow was observed at a considerable rate, however decreased with an increase in distance between the two species.
Ureta *et al.* (2008)	Six provinces of central Argentina	*Helianthus annuus* (sunflower)	Hybridization and therefore gene flow though cross-pollination by honeybees	Gene flow between cultivated and wild *Helianthus annnus* accelerated with a decreased distance from the cultivated population. Hybridization was evident and evaluated using a crop specific isozyme marker.
Delplancke *et al.* (2011)	Lebanon, Turkey, and Syria	*Prunus dulcis* (almond) and *Prunus orientalis*	Gene flow through insect-mediated pollination	Detected substantial and symmetric gene flow between a cultivated *Prunus dulcis* and wild *Prunus orientalis* population, as well as high genetic diversity levels.
De Schawe *et al.* (2013)	Northeast lowlands of Bolivia	*Theobroma cacao* (cacao)	Pollen movement – gene flow through insect-mediated pollination (mostly bees and flies)	Pollen movement was detected 16-20% of pollination events between wild and cultivated cacao – a high pollen exchange rate.
Cornille *et al.* (2013)	Europe, Caucasus, and Central Asia	Cultivated *Malus domestica* (apple) and closest wild relatives	Hybridization and therefore gene flow through insect-mediated pollination (mostly Ceratopogonid midges)	Introgression tested using micro-satellites was indicated in all wild species although at a varied extent.
Zhou *et al.* (2020)	QinLing Mountains, China	*Juglans regia* (walnut)	Pollen flow – gene flow through wind-pollination	An elevated level of genetic variation and pollen flow was detected using 12 micro-satellite markers between wild and cultivated populations.

1.1.3. Indigenous plants of commercial value in South Africa

The agricultural sector has the largest labour force in Africa and contributes significantly to the economy of African countries (Chauvin *et al.*, 2012). South Africa has a rich biodiversity, with numerous native plant species of commercial importance including indigenous vegetables, medicinal plants (Street & Prinsloo, 2013; Mahomoodally, 2013), and floricultural crops (Reinten & Coetzee, 2002). The global export of indigenous crops is valuable for the economy of South Africa (Reinten & Coetzee, 2002; Street & Prinsloo, 2013; Mahomoodally, 2013). South Africa has ~350 indigenous species being commonly used and traded (Van Wyk *et al.,* 2013). Popular native species with medicinal value include buchu or *Agathosma betulina* (Berg.) Pillans (Huisamen, 2019), devil's claw or *Harpagophytum procumbens* (Burch.) DC. (Mncwangi *et al.,* 2012; Gurib-Fakim & Mahomoodally, 2013), cancer bush or *Sutherlandia frutescens* (L.) R.Br. (Van Wyk, 2011), rooibos or *Aspalathus linearis* (Burm.f.) Dahlg. (Joubert & de Beer, 2011), and honeybush or *Cyclopia* Vent. (Ajuwon *et al.,* 2018; McKay & Blumberg, 2006) among many others.

1.1.4. The herbal tea industry of South Africa

Over the past few decades there has been a worldwide increase in tea demand, prompting an increase in the hectarages under tea plantations (Basu *et al.,* 2010; FAO, 2019). *Aspalathus linearis* (rooibos) and *Cyclopia* (honeybush spp.) have been identified as two commercial "success stories" in indigenous plant cultivation in South Africa (Joubert *et al.,* 2008). Rooibos and honeybush have been studied for various medicinal properties, including bioactive compounds that assist with type 2 diabetes mellitus (Ajuwon *et al.,* 2018), as well as potent antioxidants, chemo-preventatives, and immuno-modulators (McKay & Blumberg, 2006). Carl Thunberg, a Swedish botanist, was the first to observe *Aspalathus* (*A. cordata)*brewed into a beverage by the Khoi in 1772 (Forbes, 1986). *Aspalathus linearis* is still enjoyed as a healthy alternative to caffeinated traditional teas, and as such has been extensively cultivated in South Africa (Joubert *et al.,* 2008). The earliest record of *Cyclopia* dates back to 1830, when it was first recorded by Bowie (1830). Currently there are six *Cyclopia* species recognized for their commercial value, *Cyclopia genistoides* (L.) R. Br*, C. intermedia* E. Mey*, C. maculata* (Andrews) Kies*, C. subternata* Vogel*, C. sessiliflora* Eckl. & Zeyh.*,* and more recently *C. longifolia* Vogel, with the first four species most frequently cultivated (Joubert *et al.*, 2011). *Cyclopia* tea is marketed both locally and internationally on an increasing scale (Bester, 2013).

A survey conducted by the South African Honeybush Tea Associated in 2016 analysed the extent of cultivation of five commercially important *Cyclopia* species (*C. subternata, C. genistoides, C. intermedia, C. longifolia* and *C. maculata*) and found that a total of ~147 ha is cultivated across the Western and Eastern Cape (McGregor, 2017). Since then, it is expected that the extent of cultivation has increased, with the growth from ~250 tonnes in 2016 to ~580 tonnes in 2019 of bulk processed honeybush exports (PPECB, 2020). Cultivation is necessary to prevent over-exploitation of wild populations (Bester, 2013), which are thought to be at risk of depletion (McGregor, 2017). As the honeybush tea industry moves toward becoming more commercialized, it is necessary to consider how best to develop biodiversity-friendly farming practises (Potts, 2017). With *Cyclopia* being cultivated in the native range, the probability of gene flow occurring between cultivated and wild populations – of the same and different species – may pose a threat to the genetic integrity of all *Cyclopia* species (Galuszynski & Potts, 2020; Galuszynski, 2021). Specifically, since these commercially important species are moved across the range for cultivation (Joubert *et al.,* 2011), including into areas where they do not naturally occur. Additionally, each of the 21 current *Cyclopia* species have different range sizes, some with large distributions and different genetic lineages in different parts of the range (Schutte, 1997; Galuszynski, 2020). Therefore, *Cyclopia* is an ideal genus for a pollen-mediated gene flow study, particularly owing to the commercial value of the genus (Joubert *et al.,* 2011) and its expansive natural range in which it is widely planted (Schutte, 1997).

1.1.5. Honeybush (*Cyclopia* Vent.)

The *Cyclopia* genus belongs to the pea-family, Fabaceae (Schutte, 1997). The common name "honeybush" is derived from the honey-like scent when flowering (Joubert *et al.*, 2011). The genus name is derived from the Greek words for circle "cyclos" and foot "pous" referring to the depression at bottom of the calyx (Schutte, 1997). *Cyclopia* is a papilionoid legume with bright yellow distinctive flowers, trifoliolate leaves and unifloral inflorescences (Schutte, 1997). There are two strategies for fire survival in *Cyclopia* spp. in the fire-driven fynbos vegetation complex: reseeders and resprouters (Schutte, 1997). Resprouters have buds in underground organs that germinate in response to fire, whereas reseeders, or simply seeders, release seed in response to fire while the parent plant dies (Bond & Van Wilgen, 1996). The fire survival strategies may be beneficial to the honeybush tea industry as it may offer understanding of the response of different species to disturbances such as pests and harvesting (Slabbert *et al.,* 2019). All species in the genus *Cyclopia* are endemic to the the fynbos biome of South Africa (Schutte, 1997). Two of the 23 species, *Cyclopia filiformis* Kies and *C. laxiflora* Benth.,

are considered Extinct (Hilton-Taylor, 1996), with an additional four species Critically Endangered; *C. latifolia* DC., *C. longifolia* Vogel (commercialised), *C. pubescens* Eckl. & Zeyh. and *C. squamosa* A.L.Schutte. Three species are listed as Rare; *C. aurescens* Kies, *C. falcata* Kies and *C. glabra* (Hofmeyr & E.Phillips) A.L.Schutte, another two species are Vulnerable; *C. bolusii* Hofmeyr & E.Phillips and *C. burtonii* Hofmeyr & E.Phillips, and another two are Endangered; *C. alopecuroides* A.L.Schutte and *C. plicata* Kies (IUCN Red List, 2021; Raimondo *et al.,* 2009). In fact, only six species are listed as Least Concern, including the commercially important *C. intermedia* and *C. subternata*, while the remaining three species are Near Threatened, all of which have been commercialised; *C. maculata, C. genistoides* and *C. sessiliflora* (IUCN Red List, 2021; Raimondo *et al.,* 2009). The four *Cyclopia* study species are listed as Least Concern (LC) and Near Threatened (NT), i.e. *C. subternata* (LC)*, C. intermedia* (LC)*, C. maculata* (NT) and *C. genistoides* (NT)*.* The *Cyclopia* species have varied distribution ranges with some species occurring over extensive ranges and others limited to even just one locality (Schutte, 1997; Joubert *et al.*, 2011). The potential for hybridization between naturally occurring species and those that are moved across the range for cultivation is likely, a concern particularly for narrow endemics and the species that are already threatened. Additionally, gene flow between different genetic varieties can result in genetic homogenization, particularly since some *Cyclopia* species have different genetic lineages resulting in varied growth form across the landscape (e.g. *C. genistoides* has a denser growth in the Overberg, with more branching and leafiness of the lower parts than the plants growing on the West Coast; Bester pers. comm. 2021).

Fortunately, gene flow via seed dispersal in *Cyclopia* is probably low since ants, as the main seed dispersers, rarely move seeds further than 10 m and up to a maximum of 180 m (Schutte *et al.*, 1995; Schutte, 1997; Gómez & Espadaler, 2013). Therefore, to assess gene flow the focus should be on pollination, in particular pollinator movement and foraging distances. The type of primary pollinators for *Cyclopia* species, their movement (between wild and cultivated populations) and their foraging distance needs to be determined to quantify the potential for cross-pollination to occur. Carpenter bees are most likely the primary pollinators of all commercialised *Cyclopia* species based on their shared floral morphology (Schutte, 1997), for example *Cyclopia pubescens* Eckl. & Zeyh. is pollinated by *Xylocopa flavorufa* De Geer, *X. caffra* Linnaeus and *Apis mellifera* Linnaeus (Western honey bee) (Grobler & Campbell, 2020). Honey bee foraging ranges are well studied, revealing distances further than 9.5 km from the hive in some cases (Beekman & Ratnieks, 2000). However, whether honey bees transfer pollen remains to be determined, since honeybees might be too small to legitimately visit these larger leguminous flowers (Córdoba & Cocucci, 2011). The forage distance for carpenter bees is known to be to be up to 6 km for *Xylocopa flavorufa* (Pasquet *et al.*, 2008). Even though

Pasquet *et al.*'s study was undertaken in Kenya, *X. flavorufa* has a large distribution range that overlaps with that of *Cyclopia* species in the Cape Floral Region. Together with pollinator movement, information on *Cyclopia* pollen viability under field conditions needs to be determined. Limited pollen longevity under field conditions might limit outcrossing potential, as the pollen of many plant species only stay viable for a few hours and this will thus limit the gene flow to the distance a carpenter bee can travel in that time (see for examples Dafni & Firmage, 2000).

1.2. Research problem

There is a risk of introgression through pollen transfer between cultivated and neighbouring wild populations of *Cyclopia* (honeybush). With the expansion of the honeybush tea industry, the risk is intensifying. To minimize genetic contamination, it is necessary to determine the potential for cross-pollination and subsequent hybridization between cultivated and wild *Cyclopia* species. This ecological study is necessary to develop guidelines ensuring the conservation of the wild *Cyclopia* gene pool and the sustainable cultivation of *Cyclopia*.

1.3. Research aim and objectives

This study aims to determine the risk of cross-pollination and thus genetic contamination between wild and cultivated *Cyclopia* species and populations.

The following objectives were identified and used to address this aim:

- To determine the pollinators in the wild and in cultivation of the four cultivated *Cyclopia* species.

- To determine the movement of pollinators between planted and wild *Cyclopia*, and therefore cross-pollination potential.

- To determine whether cross-pollination between planted and wild *Cyclopia* of the same and different species can produce hybrid plants.

1.4. Thesis outline

Pollen transfer between cultivated and co-occurring wild populations of *Cyclopia* may result in introgression, a progressive threat with the growth of the honeybush tea industry. Therefore, it is necessary to determine the potential for cross-pollination to occur by identifying the

primary pollinators of the *Cyclopia* genus. In addition, the likelihood of pollen-transfer needs to be determined through exploring pollinator movement and flight distance, as well as through hand-pollination experiments. This information will aid in the development of guidelines in the form of a planting protocol for utilization by the honeybush tea industry.

In Chapter 2 I highlight the value of native non-*Apis* insects for indigenous crop-pollination through identifying the pollinators of four commercially important *Cyclopia* species. From this, I ask in Chapter 3 whether there is a risk of gene flow between wild and cultivated in *Cyclopia* by tracking pollinator movement. I use mark-release-recapture methods to determine the potential for pollinator movement between cultivated and wild *Cyclopia* populations. Additionally, I identify the daily foraging ranges of pollinators using radio-tagging and tracking. The viability and thus longevity of *Cyclopia* pollen is also quantified. Hand-pollination experiments reveal the potential for hybridization in *Cyclopia*.

I then use this information to develop a general protocol guiding the planting of indigenous species, with commercial value, within their native range. I use *Cyclopia* as a case study and refined the protocol through facilitated workshops, with input from industry and academics. In the final chapter of this thesis, I provide an overview of the results, including the important research contributions of the study, and link this to recommendations and potential future research opportunities in this field. Each of the content chapters will be submitted as standalone papers for publication, there might thus be some overlap in methods and context for the three content chapters.

1.5.   References

Ajuwon, O., Ayeleso, A. & Adefolaju, G. 2018. The potential of South African herbal tisanes, rooibos and honeybush in the management of type 2 diabetes mellitus. *Molecules, 23*, 3207.

Andersson, M. & de Vicente, M. 2010. *Gene flow between crops and their wild relatives.* Baltimore, MD: Johns Hopkins University Press.

Arias, D. & Rieseberg, L. 1994. Gene flow between cultivated and wild sunflowers. *Theoretical and Applied Genetics*, *89*, 655–660.

Basu, M., Bera B. & Rajan, A. 2010. Tea Statistics: Global Scenario. *Journal of Tea Science, 8*, 121–124.

Beekman, M. & Ratnieks, F. 2000. Long-range foraging by the honey bee, *Apis mellifera* L. *Functional Ecology, 14*, 490–496.

Bester, C. 2013. A model for commercialisation of honeybush tea, an indigenous crop. *Acta Horticulturae, 1007*, 889–894.

Bond, W. & Van Wilgen, B. 1996. *Fire and plants*. Population and community biology series 14. Chapman & Hall, London.

Bowie, J. 1830. Sketches on the botany of South Africa. *South African Quarterly Journal*, 1, 27–36.

Burke, J., Gardner, K. & Rieseberg, L. 2002. The potential for gene flow between cultivated and wild sunflower (*Helianthus annuus*) in the United States. *American Journal of Botany, 89*, 1550–1552.

Chauvin, N., Mulangu, F. & Porto, G. 2012. Food production and consumption trends in Sub-Saharan Africa: Prospects for the transformation of the agricultural sector. United Nations Development Programme (UNDP). Regional Bureau for Africa. WP 2012-011.

Córdoba, S. & Cocucci, A. 2011. Flower power: its association with bee power and floral functional morphology in papilionate legumes. *Annals of Botany, 108*, 919–931.

Cornille, A., Gladieux, P. & Giraud, T. 2013. Crop-to-wild gene flow and spatial genetic structure in the closest wild relatives of the cultivated apple. *Evolutionary Applications, 6*, 737–748.

Dafni, A., & Firmage, D. 2000. Pollen viability and longevity: Practical, ecological and evolutionary implications. *Pollen and Pollination,* 113–132.

Delplancke, M., Alvarez, N., Espíndola, A., Joly, H., Benoit, L., Brouck, E. & Arrigo, N. 2011. Gene flow among wild and domesticated almond species: insights from chloroplast and nuclear markers. *Evolutionary Applications, 5*, 317–329.

De Schawe, C., Durka, W., Tscharntke, T., Hensen, I. & Kessler, M. 2013. Gene flow and genetic diversity in cultivated and wild cacao (*Theobroma cacao*) in Bolivia. *American Journal of Botany, 100*, 2271–2279.

Duraiappah, A., Naeem, S., Agardy, T., Ash, N., Cooper, H., Diaz, S., Faith, D., Mace, G., McNeely, J., Mooney, H., Oteng-Yeboah, A., Pereira, H., Polasky, S., Prip, C., Reid, W., Samper, C., Schei, P., Scholes, R., Schutyser, F. & Van Jaarsveld, A. 2005. *Ecosystems and human well-being: biodiversity synthesis; a report of the Millennium Ecosystem Assessment*. World Resources Institute.

Ellstrand, N. 2001. When transgenes wander, should we worry? *Plant Physiology, 125*, 1543–1545.

Ellstrand, N. & Elam, D. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics, 24*, 217–242.

Ellstrand, N., Prentice, H. & Hancock, J. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology, Evolution, and Systematics*, *30*, 539–563.

FAO (Food and Agriculture Organization). 2019. Detailed trade matrix. ://www.fao.org/faostat/en/#data/TM [4 October 2022].

V. Forbes (Ed.). 1986. Carl Peter Thunberg travels at the Cape of Good Hope 1772–1775, Van Riebeeck Society, Cape Town.

Galuszynski, N. 2020. *Applied phylogeography: mapping the genetic resource of Honeybush across the Cape Floristic Region* (Doctoral thesis, George: Nelson Mandela University).

Galuszynski, N. 2021. The transition to agricultural cultivation of neo-crops may fail to account for wild genetic diversity patterns: insights from the Cape Floristic Region. *PeerJ,* 1–21.

Galuszynski, N. & Potts, A. 2020. Applied phylogeography of *Cyclopia intermedia* (Fabaceae) highlights the need for `duty of care' when cultivating honeybush. *PeerJ*, 1–24.

Gepts, P. & Papa, R. 2003. Possible effects of (trans)gene flow from crops on the genetic diversity from landraces and wild relatives. *Environmental Biosafety Research, 2*, 89–103.

Gómez, C. & Espadaler, X. 2013. An update of the world survey of myrmecochorous dispersal distances. *Ecography*, *36*, 1193–1201.

Grobler, B. & Campbell, E. 2020. Pollinator activity and the fecundity of a rare and highly threatened honeybush species along a highway in the Cape Floristic Region. *International Journal of Plant Sciences, 181*, 581–593.

Gurib-Fakim, A. & Mahomoodally, M. 2013. African flora as potential sources of medicinal plants: towards the chemotherapy of major parasitic and other infectious diseases: A Review. *Jordan Journal of Biological Sciences, 6*, 77–84.

Hammer, K. & Teklu, Y. 2008. Plant genetic resources: selected issues from genetic erosion to genetic engineering. *Journal of Agriculture and Rural Development in the Tropics and Subtropics*, *109*, 15–50.

Hancock, J., Grumet, R. & Hokanson, S. 1996. The opportunity for escape of engineered genes from transgenic crops. *Hortscience, 31*, 1080–1085.

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

Huisamen, B. 2019. *Medicinal effects of* Agathosma *(buchu) extracts*. AOSIS, Cape Town..

Jenczewski, E., Prosperi, J. & Ronfort, J. 1999. Evidence for gene flow between wild and cultivated *Medicago sativa* (Leguminosae) based on allozyme markers and quantitative traits. *American Journal of Botany, 86*, 677–687.

Joubert, E. & de Beer, D. 2011. Rooibos (*Aspalathus linearis*) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany, 77*, 869–886.

Joubert, E., Gelderblom, W., Louw, A. & de Beer, D. 2008. South African herbal teas: *Aspalathus linearis, Cyclopia* spp. and *Athrixia phylicoides* — A review. *Journal of Ethnopharmacology, 119*, 376–412.

Joubert, E., Joubert, M., Bester, C., de Beer, D. & De Lange, J. 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets — The catalytic and supporting role of research. *South African Journal of Botany, 77*, 887–907.

Mahomoodally, M. 2013. Traditional medicines in Africa: an appraisal of ten potent African medicinal plants. *Evidence-Based Complementary and Alternative Medicine, 2013,* 1–14.

McGregor, G. 2017. Industry review: an overview of the Honeybush industry. Department of Environmental Affairs and Development Planning, Cape Town.

McKay, D. & Blumberg, J. 2006. A review of the bioactivity of South African herbal teas: rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*). *Phytotherapy Research*, *21*, 1–16.

Mncwangi, N., Chen, W., Vermaak, I., Viljoen, A. & Gericke, N. 2012. Devil's Claw—A review of the ethnobotany, phytochemistry and biological activity of *Harpagophytum procumbens. Journal of Ethnopharmacology*, *143*, 755–771.

Ollerton, J., Winfree, R. & Tarrant, S. 2011. How many flowering plants are pollinated by animals? *Oikos, 120*, 321–326.

Papa, R. & Gepts, P. 2003. Asymmetry of gene flow and differential geographical structure of molecular diversity in wild and domesticated common bean (*Phaseolus vulgaris* L.) from Mesoamerica. *Theoretical and Applied Genetics, 106*, 239–250.

Pasquet, R., Peltier, A., Hufford, M., Oudin, E., Saulnier, J., Paul, L., Knudsen, J., Herren, H. & Gepts, P. 2008. Long–distance pollen flow assessment through evaluation of pollinator

foraging range suggests transgene escape distances. *Proceedings of the National Academy of Sciences, 105*, 13456–13461.

Potts, A. 2017. Genetic risk and the transition to cultivation in Cape endemic crops—The example of honeybush (*Cyclopia*)? *South African Journal of Botany, 110*, 52–56.

PPECB. 2020. Perishable Produce Export Certification Agency, Annual reports.

Raimondo, D., von Staden, L., Foden, W., Victor, J., Helme, N., Turner, R., Kamundi, D. & Manyama, P. 2009. *Red List of South African Plants.* Strelitzia 25. South African National Biodiversity Institute, Pretoria.

Reinten, E. & Coetzee, J. 2002. Commercialization of South African indigenous crops: aspects of research and cultivation of products. In: Janick, J., Whipkey, A. (Eds.), Trends in New Crops and New Uses. ASHS Press, Alexandria, VA.

Rodger, J., Bennett, J., Razanajatovo, M., Knight, T., Van Kleunen, M., Ashman, T., Steets, J., Hui, C., Burd, M., Burkle, L., Burns, J., Durka, W., Freitas, L., Kemp, J., Li, J., Pauw, A., Vamosi. J., Wolowski, M., Xia, J. & Ellis, A. 2021. Widespread vulnerability of flowering plant seed production to pollinator declines. *Science Advances, 7*, 1–10.

Schutte, A. 1997. Systematics of the genus *Cyclopia* Vent. (Fabaceae, Podalyrieae). *Edinburgh Journal of Botany, 54*, 125–170.

Schutte, A., Vlok, J., Van Wyk, B. & Van Wyk, B-E. 1995. Fire-survival strategy—a character of taxonomic, ecological and evolutionary importance in fynbos legumes. *Plant Systematics and Evolution,* 195, 243–259.

Slabbert, E., Malgas, R., Veldtman, R. & Addison, P. 2019. Honeybush (*Cyclopia* spp.) phenology and associated arthropod diversity in the Overberg region, South Africa. *Bothalia, 49*, 1–13.

Smith, C. 1966. *Common names of South African plants*. Botanical Survey Memoir No. 35.Department of Agricultural Technical Services, Pretoria, South Africa, 247.

Song, Z., Lu, B., Zhu, Y. & Chen, J. 2003. Gene flow from cultivated rice to the wild species *Oryza rufipogon* under experimental field conditions. *New Phytologist, 157*, 657–665.

Street, R. & Prinsloo, G. 2013. Commercially important medicinal plants of South Africa: A review. *Journal of Chemistry, 2013*, 1–16.

Trucco, F., Tatum, T., Rayburn, A. & Tranel, P. 2009. Out of the swamp: unidirectional hybridization with weedy species may explain the prevalence of *Amaranthus tuberculatus* as a weed. *New Phytologist, 184*, 819–827.

Ureta, M., Carrera, A., Cantamutto, M. & Poverene, M. 2008. Gene flow among wild and cultivated sunflower, *Helianthus annuus* in Argentina. *Agriculture, Ecosystems & Environment, 123*, 343–349.

Uwimana, B., Smulders, M., Hooftman, D., Hartman, Y., Van Tienderen, P., Jansen, J., McHale, L., Michelmore, R. Van de Wiel, C. & Visser, R. 2012. Hybridization between crops and wild relatives: The contribution of cultivated lettuce to the vigour of crop–wild hybrids under drought, salinity and nutrient deficiency conditions. *Theoretical and Applied Genetics, 125*, 1097–1111.

Van der Bank, M., Van Wyk, B.-E. & Van der Bank, H. 1996. Speciation in Virgilia: allopatric divergence followed by introgression? *Plant Systematics and Evolution*, 201, 51–73.

Van Wyk, B-E. 2011. The potential of South African plants in the development of new medicinal products. *South African Journal of Botany, 77*, 812–829.

Van Wyk, B.-E., Van oudtshoorn, B. & Gericke, N. 2013. *Medicinal plants of South Africa*. Briza Publications, Pretoria.

Wolf, D., Takebayashi, N. & Rieseberg, L. 2001. Predicting the risk of extinction through hybridization. *Conservation Biology, 15*, 1039–1053.

Yuan, Q., Zhang, Z., Hu, J., Guo, L., Shao, A. & Huang, L. 2010. Impacts of recent cultivation on genetic diversity pattern of a medicinal plant*, Scutellaria baicalensis* (Lamiaceae). *BMC Genetics, 11*, 1–13.

Zhou, H., Zhao, P. & Woeste, K. & Zhang, S. 2020. Gene flow among wild and cultivated common walnut (*Juglans regia*) trees in the QinLing Mountains revealed by microsatellite markers. *Journal of Forestry Research, 32*, 2189–2201.

Chapter 2 : The importance of wild pollinators for indigenous crop pollination: the case

of *Cyclopia* (honeybush)

Abstract

Pollination is an important ecosystem service. Animal-mediated pollination (mostly insects) increases the production of 35% of global crops. Honey bees (*Apis mellifera*) are considered important crop pollinators globally, but are under pressure and therefore the value of wild pollinators for crop production is receiving more attention. The importance of pollinators (including non-*Apis*) has been extensively studied in the agricultural sector of South Africa, however little research is available on the pollination of indigenous crops. With the considerable value of the indigenous honeybush (*Cyclopia* Vent.) in the tea industry, it is important to determine the pollinators since *Cyclopia* is widespread across the fynbos biome and the pollinators are largely unknown. Here I ask whether carpenter bees are the only pollinators of commercially important *Cyclopia* species, or if honey bees contribute to pollination. Floral observations and camera trapping confirmed that six species of xylocopid bees were the only pollinators of four commercially important *Cyclopia* species. Honey bees were observed to be ineffective pollinators of *Cyclopia* owing to their inability to trip *Cyclopia* flowers and gain access to the floral reproductive parts. Similarly, an additional seven species including Diptera, Apidae and *Lema* sp. were unable to gain access to *Cyclopia* flowers, although were observed visiting. Nectar measurements were computed for each study species revealing the highest volume of nectar belonging to *C. genistoides,* while *C. intermedia* had the highest nectar sugar concentration (above 35% for all species). The value of native non-*Apis* insects for crop pollination in a changing world is highlighted in *Cyclopia,* an indigenous legume gaining traction in the global tea market and therefore in cultivation.

2.1.   Introduction

Pollination is a valuable ecosystem service, essential for crop productivity (Garibaldi *et al.,* 2020) and ecosystem functioning (Genung *et al.,* 2017). Approximately 80% of the 250 000 global angiosperms rely on animal-mediated pollination for sexual reproduction (Rodger *et al.,* 2021). Animal-mediated pollination (mainly insects) increases the production of approximately 35% of global crops (Klein *et al.,* 2007), illustrating the exceedingly dependent relationship of humans on insect pollinators (Potts *et al.,* 2016). Honey bees (*Apis mellifera*) are considered economically important pollinators owing to their widespread distribution and generalist foraging behaviour (Hung *et al.,* 2018). The drastic population declines of honey bees in North

America (Stokstad, 2007; Watanabe, 1994; Pettis & Delaplane, 2010) and Europe (Potts *et al.,* 2010; Biesmeijer *et al.,* 2006) have urged the question of whether wild pollinators may fulfil the role of honey bees in terms of crop pollination (Winfree *et al.,* 2007, Garibaldi *et al.,* 2013).

There are a handful of studies confirming the importance of pollinator species richness for some commercially important crops in South Africa (reviewed by Melin *et al.,* 2014), but little is known about the pollination of indigenous crops (but see Melin *et al.,* 2014). In South Africa, there are numerous indigenous plant species used in cultivation, viz buchu (*Agathosma* spp.) (Moolla & Viljoen, 2008; Van Wyk, 2008), and *Aloe* L. (Cousins & Witkowski, 2012; Cowling *et al.,* 1996). Many of these indigenous species, e.g., the leguminous rooibos tea (*Aspalathus linearis*) have floral traits adapted to pollination by non-Apis pollinators, including Megachilinae, Masarinae and Xylocopinae (Gess & Gess, 2014). The pollinators of many potentially economically important plants in South Africa are however, unknown. For successful conservation, it is necessary to determine the identity and availability of pollinators of these commercially valuable indigenous plant species.

Honeybush (*Cyclopia* Vent.) is a model genus for such a pollination study as it is widespread across the fynbos biome (Schutte, 1997), a number of species are cultivated, and it has considerable value in the tea industry of South Africa (McKay & Blumberg, 2006; Koen *et al.*, 2019). Smith (1966) claimed that the earliest indication of *Cyclopia genistoides* being used as tea, is implied by the vernacular name "Honigtee" [honey tea] that was recorded by Carl Thunberg in the 1770's, during his botanical explorations in the Cape (Smith, 1966). However, the name Honigthee does not appear in his travel journal, but the possibility exists that the name was recorded on the reverse of some of the herbarium specimens of the species that he collected. The first explicit record of *Cyclopia* species being used for tea (actually as medicinal tea) was that of Bowie in 1830 (Van Wyk & Gorelik, 2017). There are six *Cyclopia* species recognised for their value in the tea industry; *Cyclopia genistoides* (L.) R. Br*, C. intermedia* E. Mey*, C. maculata* (Andrews) Kies*, C. subternata* Vogel*, C. sessiliflora* Eckl. & Zeyh.*,* and more recently *C. longifolia* Vogel (Joubert *et al.*, 2011), but the pollinators of *Cyclopia* species still remain largely unknown.

The calyx in the *Cyclopia* genus is characterized by having two fused upper lobes and three lower lobes with varied appearance of size and shape between species (Schutte, 1997). All species have a bright yellow, continually structured corolla (in exception of the duller *C. sessiliflora*) with sweetly-scented, rigid flowers (Schutte, 1997). The moveable lateral and ventral petals require active handling by pollinators in order to reveal the reproductive parts of the plant and perform successful pollination (Córdoba & Cocucci, 2011). This mechanism is referred to as "tripping" (Córdoba & Cocucci, 2011), and large bees, such as carpenter bees

(Xylocopinae) have the ability to trip leguminous papilionoids (Westerkamp, 1992; Etcheverry *et al*., 2008; Grobler & Campbell, 2020).

Based on research conducted on other leguminous papilionoids, it is likely that carpenter bees are the primary pollinators of *Cyclopia.* However, the lack of research conducted on pollination of *Cyclopia* provides little validation (but see Schutte (1997) and Grobler & Campbell (2020)). Therefore, this study aims to investigate what pollinators are responsible for pollination of wild and cultivated populations of four commercially important *Cyclopia* species. Specifically, I asked (1) whether carpenter bees are the only pollinators of commercially important *Cyclopia* species, and (2) can honey bees contribute to pollination of *Cyclopia*?

2.2.   Methods

2.2.1. *Study area, site selection and study species*

The study was conducted in the fynbos biome of the Western and Eastern Cape provinces in South Africa (Fig 2.1 A). Sites were chosen according to the availability of both cultivated and wild-growing *Cyclopia*. At these sites, harvesting of cultivated plants was postponed (since flowers were needed for the pollination aspect of the study), whilst no wild harvesting is conducted. Four of the six commercialized *Cyclopia* species were selected according to their importance in the honeybush tea industry of South Africa (Joubert *et al*., 2011).

Two commercial honeybush farms were identified as suitable study sites based on proximity of planted *Cyclopia* to wild populations (Fig. 2.1). The first was located at Pearly Beach in the Overberg Municipality (34˚41'43.1''S 19˚36'15.5''E), Western Cape (Fig. 2.1 B). This study site was dominated by Overberg sandstone fynbos vegetation. This vegetation type has a mean annual precipitation of 450 – 830 mm (with most rainfall in July and August), mean daily temperatures ranging between 6.3˚C and 25.6˚C, and a low frost incidence of 2-3 days is experienced in this region per year (Mucina & Rutherford, 2011). This farm consisted of 2.7 ha of cultivated *Cyclopia genistoides*, with a few *C. subternata* plants under cultivation, and a patch of wild growing *C. genistoides. Cyclopia genistoides* "kustee" or coastal tea, flowers between September and November (Motsa *et al*., 2017; Slabbert *et al*., 2019) and *C. subternata* "vleitee" or marshland tea, flowers between September and October (Motsa *et al*., 2017).

The second site was located at Twee Riviere (33˚52'08.6''S 23˚54'54.2''E) just outside of Joubertina, in the Eastern Cape (Fig. 2.1 C). This study site was dominated by Tsitsikamma sandstone vegetation, with a mean annual precipitation of 575 mm throughout the year (Cape Farm Mapper 2.6.15). The mean annual temperature is 15.7˚C (Cape Farm Mapper 2.6.15),

with 2-10 days of frost incidence per year (Mucina & Rutherford, 2011). The cultivated species on this farm included *Cyclopia subternata* and *C. maculata*, with wild-growing *C. subternata* along the riverbanks, as well as wild *C. intermedia* higher up in the surrounding mountains within 1 km from the cultivated site. *Cyclopia maculata* "needle-leaf honeybush" and *C. intermedia* "bergtee" or mountain tea flowers between September and November (Slabbert *et al.*, 2019; Barnado, 2013)*.*
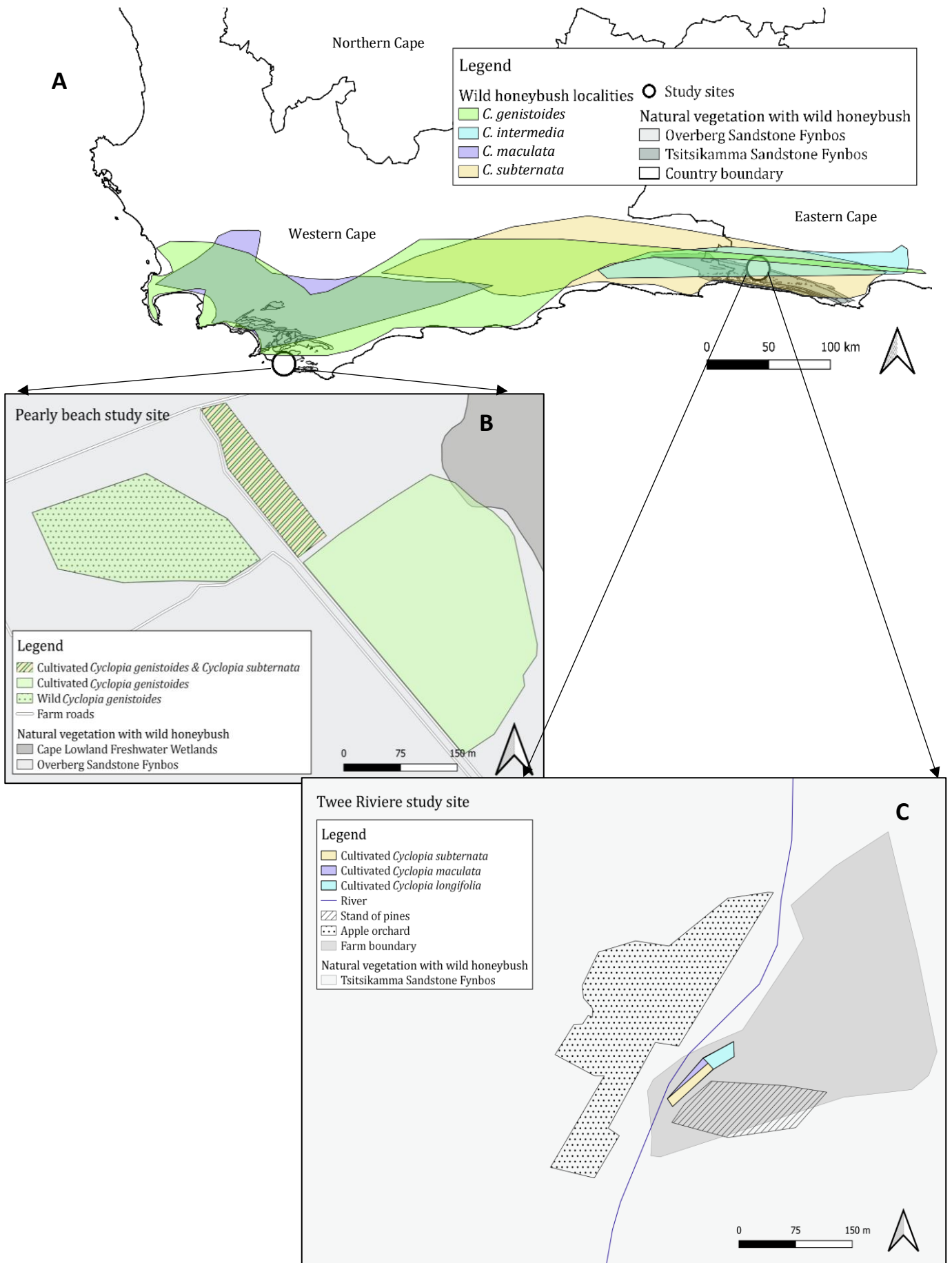
Fig. 2.1. (A) Location of the two study sites, (B) the wild and cultivated *Cyclopia* population localities at the Pearly Beach study site and (C) the natural vegetation areas abundant with native *Cyclopia,* and the cultivated *Cyclopia* at the Twee Riviere study site.

2.2.2. Pollinator observations

Floral observations were conducted to determine whether carpenter bees are the primary pollinators of commercially important *Cyclopia* species. Observations took place during warm and sunny conditions, with inclement weather avoided, from September to October 2021. A total of ~46 hours of observations were conducted at the Twee Riviere study site, and ~19 hours of observations were conducted at the Pearly Beach study site (Table 2.1). Insect identifications were conducted in field using a *Xylocopa* key where possible (Eardley, 1983). If identification was not possible, a description of the visitor was recorded in the place of the species name on observation logs, for later identification purposes. The gender of floral visitors was not recorded, as the identifying characteristics allowing for distinguishability between male and female were not easily observable. Representatives of the flower visitors observed accessing the *Cyclopia* flowers were collected for more accurate identification purposes where gender was also identified. These specimens were pinned and accessioned into the Iziko South African Museum (Appendix 2 A).

Cyclopia plants were observed over a recorded period of time, noting the number of flowers visible to the observer, the number of flower visits made by each flower visitor and the type of interaction observed (pollination, robbing or thieving). To avoid the use of ambiguous terminology regarding floral larceny, the definitions outlined by Inouye (1980) were used. Primary nectar robbing is defined as: "a hole is made and used to obtain nectar, bypassing the opening used by pollinators" and secondary nectar robbing occurs when visitors make use of holes created by primary nectar robbers (Inouye, 1980). Nectar thieving is defined as: "no hole is made in the flower, the thief uses the opening of the flower which is used by pollinators however due to a mismatch in morphologies pollination is precluded" (Inouye, 1980).

Floral visitors were scored as potential pollinators when observed tripping the flowers and gaining access to the anthers and stigma (Fig. 2.2). If insect visitors did not trip flowers and failed to gain access to the reproductive floral parts, the visit was deemed ineffective. These visitors were recorded as floral robbers or thieves.

Fig. 2.2. A *Xylocopa capitata* tripping (actively handling the lateral "wing" and ventral "keel" petals) a *Cyclopia* flower to gain access to floral rewards.

Observations were supported with the use of camera traps (Bushnell Trophy Cam HD Max trail camera and Bushnell Core Low Glow trail camera) taking photographs when triggered by floral visitors, placed at a distance approximately 30-50 cm away from flowers. Camera traps were tested at the start of the field season to assess the settings required for sound pollinator observations. Visitors the size of carpenter bees and larger were able to trigger the camera trap, however smaller visitors such as honeybees were not observed in camera trap footage. The camera traps were set to trigger during the day and at night to identify whether nocturnal pollination was occurring (Somanathan *et al.,* 2019).

A camera trap picture demonstrating noticeable tripping by a floral visitor was recorded as one visit to one flower. There were visitors that triggered the camera trap multiple times within a short (30 second) timeframe while visiting a single flower. In this case, all of the pictures that were captured within a 30 second range were recorded as one visit (unless movement to a new flower was clearly visible). This was to ensure that a single visit was not counted more than once.

Camera traps were placed in the wild and cultivated study sites between September and November 2021. Since camera traps were used in supplementation to direct floral observations, priority was given to *Cyclopia* species that were not as frequently observed. This was to ensure that all *Cyclopia* species received adequate pollinator observation. Therefore, *Cyclopia genistoides* had the highest number of hours of camera trap observation, and *C. subternata* had the least (Table 2.2).

2.2.3. Nectar properties
In order to determine nectar volume and concentration, *Cyclopia* flowers were collected from secured mesh bags which were placed over branches for 24 hr period prior. The volume of nectar for each species was measured using micro-capillary tubes, and nectar sugar concentration was measured using a 0-55% handheld refractometer (Bellingham and Stanley, UK). Flowers were collected from each *Cyclopia* study species for measurement (cultivated *C. subternata* n = 30, cultivated *C. maculata* n = 39, cultivated *C. genistoides* n = 48, wild *C. subternata* n = 7, wild *C. intermedia* n = 32).

2.2.4. Data analyses
The visitation rate of pollinators to each *Cyclopia* species in both the cultivated and wild study sites was calculated as visits/flower/hour. The visitation rate was calculated using the data

collected from the floral observation plots and camera traps. Camera trap visitation rate was calculated by determining the number of visitors recorded by each camera trap, approximating the number of flowers visible on the camera trap images and finding the sum of hours each camera trap was set up for. The visitation rate calculated from the camera trap data was adjusted to exclude night time hours when no pollination was observed.

The visitation rates for each pollinator to each *Cyclopia* species was calculated separately using the floral observation data and the camera trap data. The daily mean visitation rate was used in all data analyses to avoid bias toward *Cyclopia* species with a higher number of observation hours. These visitation rates were then used to construct pollination networks visualizing pollinator sharing among *Cyclopia* species using the "bipartite" package (v2.16; Dormann *et al.*, 2009) in R Studio (R Core Team, 2021). Thereafter, the mean visitation rate between the floral observations and camera traps was calculated for each pollinator to each *Cyclopia* species under study, and additional pollination networks were constructed using the "bipartite" package (v2.16; Dormann *et al.*, 2009) in R Studio (R Core Team, 2021). No difference was observed between these two methods, but combined they paint a more complete picture (Fig. 2.3, Appendix 2 B & C). Network metrics (links per species and connectance) were calculated in R Studio (R Core Team, 2021) to aid with data interpretation. Links are the number of unique interactions between plants and pollinators. Connectance is defined as the realized proportion of possible links (Dunne *et al.,* 2002), by taking into consideration the total number of links and network size (Jordano, 1987).

The difference in nectar volume and sugar concentration was analysed using a Kruskal-Wallis test in the native stats package in R. Statistically pairwise comparisons of nectar volume and concentration between *Cyclopia* species were calculated using a Dunn Test (Dunn, 1964) computed in R Studio (R Core Team, 2021). Data was not normally distributed and therefore a non-parametric test was used.

2.3. Results

2.3.1. Pollinator observations

A total of 65.23 hours of direct observation revealed six different carpenter bee species visiting wild and cultivated *Cyclopia* plants, these included; *Xylocopa capitata* Smith*, X. flavorufa* De Geer*, X. rufitarsis* Lepeletier*, X. caffra* Linnaeus*, X. scioensis* Gribodo and *X. sicheli* Vachal (Table 2.1; Appendix 2 A&B). *Xylocopa capitata* was the most frequent visitor in the wild and cultivated sites (Fig. 2.3 A, Table 2.1, Fig. 2.4 A, B&C)*. Xylocopa caffra* had the second highest visitation rate to wild *C. genistoides* and wild *C. intermedia,* while the remaining visitors maintained comparatively low visitation frequencies (Fig. 2.3 A & Table 2.1)*.* Wild *Cyclopia*

genistoides and wild *Cyclopia subternata* was visited by four pollinator species (Fig. 2.3 A & Table 2.1). Wild *Cyclopia intermedia* had only three recorded pollinator species with similar visitation rates, *X. caffra, X. rufitarsis* and *X. capitata* (Fig. 2.3 A). There were overall fewer visits in the cultivated site (Fig. 2.3 B), with *C. genistoides* rarely receiving visitation (Fig. 2.3 B).

No night time pollination was observed in camera trap pictures; pollination triggers occurred only between 08:30 and 17:45. Three pollinator species were caught by the camera traps; *Xylocopa capitata* visiting *Cyclopia subternata, C. intermedia* and *C. maculata, X. caffra* visiting *C. maculata,* and *X. rufitarsis* visiting *C. genistoides* and *C. intermedia* (Table 2.2). The camera trap caught little visitation to wild *C. genistoides,* with only one pollinator species, *X. rufitarsis,* observed interacting with wild *C. genistoides* (Table 2.2). Wild *Cyclopia genistoides* had the lowest visitation rate, with only four individual *X. rufitarsis* captured in over 1544 hours of observation time (Table 2.2). The camera trap data revealed only two pollinator species, *X. capitata* and *X. caffra,* visiting the cultivated *Cyclopia,* with *X. capitata* making up the vast majority of visitation (Appendix 2 B). Cultivated *Cyclopia subternata* had the highest camera trap visitation rate from only one species, *X. capitata,* in just over 20 hours of observation time (Table 2.2). Cultivated *C. maculata* was visited primarily by *X. capitata* with some *X. caffra* camera trap observations (Appendix 2 B).

2.3.2. Network metrics

The links per species in the wild site was lower than the cultivated site (1.2 and 1.5 links, respectively). Fewer links indicate a higher degree of specialization. The cultivated site was slightly more connected than the wild site (connectance 0.778 and 0.6111, respectively; Fig. 2.3).

2.3.3. Other floral visitors

Other species were observed visiting the *Cyclopia* flowers, attempting to gain access to floral rewards, including rodents (Fig. 2.4 D) and honey bees (*Apis mellifera* Linnaeus) (Fig. 2.4 E & Table 2.3). The camera trap captured five individual striped field mice (*Rhabdomys* sp.) visiting (likely consuming) *C. genistoides* flowers (Fig. 2.4 D). . During floral observations a total of 69 honey bees (*Apis mellifera*) were observed attempting to gain access to *Cyclopia* floral rewards (63 observed on cultivated *C. subternata,* three observed on wild *C. subternata* and three on wild *C. intermedia*) without contributing to pollination (Table 2.3). Other visitors

were also observed on *Cyclopia* flowers during floral observations (Table 2.3) however, none were able to successfully trip the flowers and perform pollination.

2.3.4. Nectar properties

The highest mean volume of nectar was extracted from cultivated *C. genistoides*, while the lowest mean volume was recorded in cultivated *C. subternata* (Fig. 2.5 A). There was a significant difference in nectar volume between the study species (Kruskal-Wallis χ2= 33.47, df = 4, P < 0.001 (Fig. 2.5 A). Cultivated *C. genistoides* flowers has significantly more nectar than the other study species besides from wild *C. subternata* (Fig. 2.5 A).

The mean nectar sugar concentration percentage remained above 35% for all species, with *C. intermedia* the highest at over 55% (Fig. 2.5 B). Nectar sugar concentration was significantly different between the study species (Kruskal-Wallis χ2 = 40.15, df = 4, P < 0.001; Fig. 2.5 B), with wild *C. intermedia* significantly higher (Fig. 2.5 B).

Table 2.1. Observed visitation rate by *Xylocopa* species (visits/flower/hour) to *Cyclopia*, including the number of hours of observations per species in both the wild and cultivated study sites.

	Wild			Cultivated		
	C. subternata	*C. genistoides*	*C. intermedia*	*C. subternata*	*C. genistoides*	*C. maculata*
Time (hrs)	6.03	1.8	10.22	18.55	17.58	11.05
X. capitata	0.014623	0.003283	-	0.004353	0.000748	0.010276
X. flavorufa	0.001246	-	-	0.000126	-	0.000007
X. caffra	-	0.049495	0.003179	0.000073	0.004415	0.000045
X. rufitarsis	0.000850	-	0.001794	0.000044	0.000150	0.000267
X. scioensis	0.000113	-	-	-	0.002844	0.000490
X. sicheli	-	0.002020	-	-	-	0.001271
Total	**0.016832**	**0.054798**	**0.004973**	**0.004596**	**0.008157**	**0.012356**

Table 2.2. Visitation rate by *Xylocopa* species (visits/flower/hour) to *Cyclopia* calculated from camera trap data, including the number of hours of observation time per species in both the wild and cultivated study sites.

	Wild			Cultivated	
	C. subternata	*C. genistoides*	*C. intermedia*	*C. subternata*	*C. maculata*
Time (hrs)	110.67	1544.41	178.17	20.16	130.58
X. capitata	0.0037857	-	0.0046711	0.0099206	0.0032729
X. caffra	-	-	-	-	0.0000091
X. rufitarsis	-	0.0000129	0.0003138	-	-
Total	**0.0037857**	**0.0000129**	**0.0049849**	**0.0099206**	**0.0032820**

Table 2.3. Visitation rate (visits/flower/hour) calculated from floral observation plots by non-pollinator visitors

| Visitor | Wild | | Cultivated | | Interaction |
	C. subternata	*C. intermedia*	*C. maculata*	*C. subternata*	
Diptera sp. 1	-	-	-	0.000019	Thieving tripped flowers
Diptera sp. 2	-	-	-	0.000015	Thieving tripped flowers
Apidae sp. 1	0.000170	-	-	0.000029	Attempted access
Apis mellifera	-	0.000094	-	0.000277	Attempted access/thieving
Apidae sp. 2	0.000170	-	-	0.000068	Thieving tripped flowers
Diptera sp. 3	0.000340	-	-	-	Attempted access
Apidae sp. 3	-	0.000031	-	-	Thieving tripped flowers
***Lema* sp. 1**	-	-	0.000007	-	Attempted access
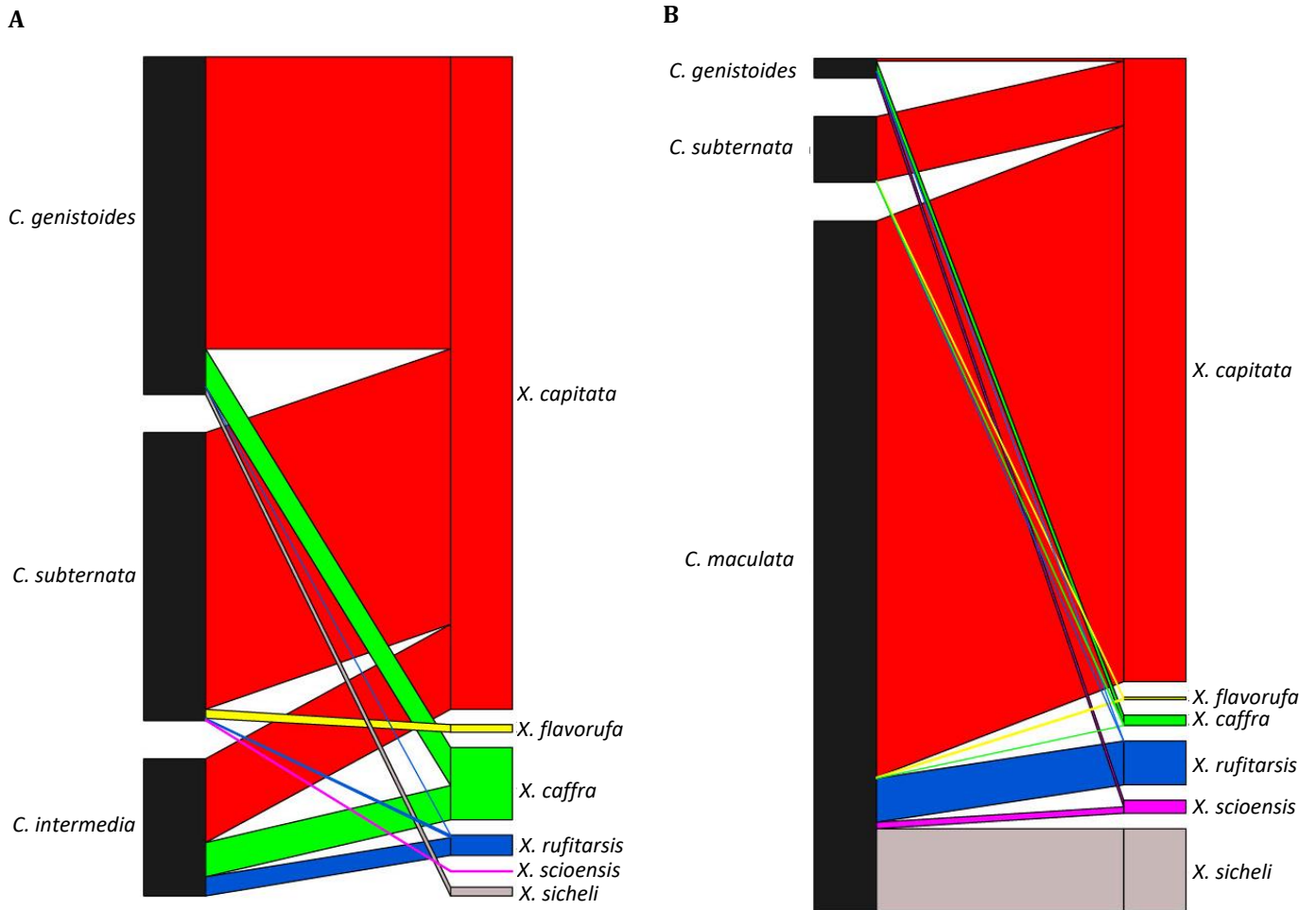
Fig. 2.3. Pollination networks visualizing pollinator sharing among *Cyclopia* species from (A) the wild and (B) cultivated study site. The visitor species are on the right side of the networks, each with unique colour (Red: *Xylocopa capitata*, Yellow: *X. flavorufa*, Green: *X. caffra*, Light blue: *X. rufitarsis*, Dark Blue: *X. scioensis*, Pink: *X. sicheli*). The *Cyclopia* study species are at the left of the networks. The relative abundance of each visitor is indicated by width of each rectangle alongside the species name. The link between each visitor species and plant study species indicates visitation interaction, the visitation rate indicated by the width of the lines between the pair (plant – pollinator). Visitation rate was calculated from direct observation and camera trapping.
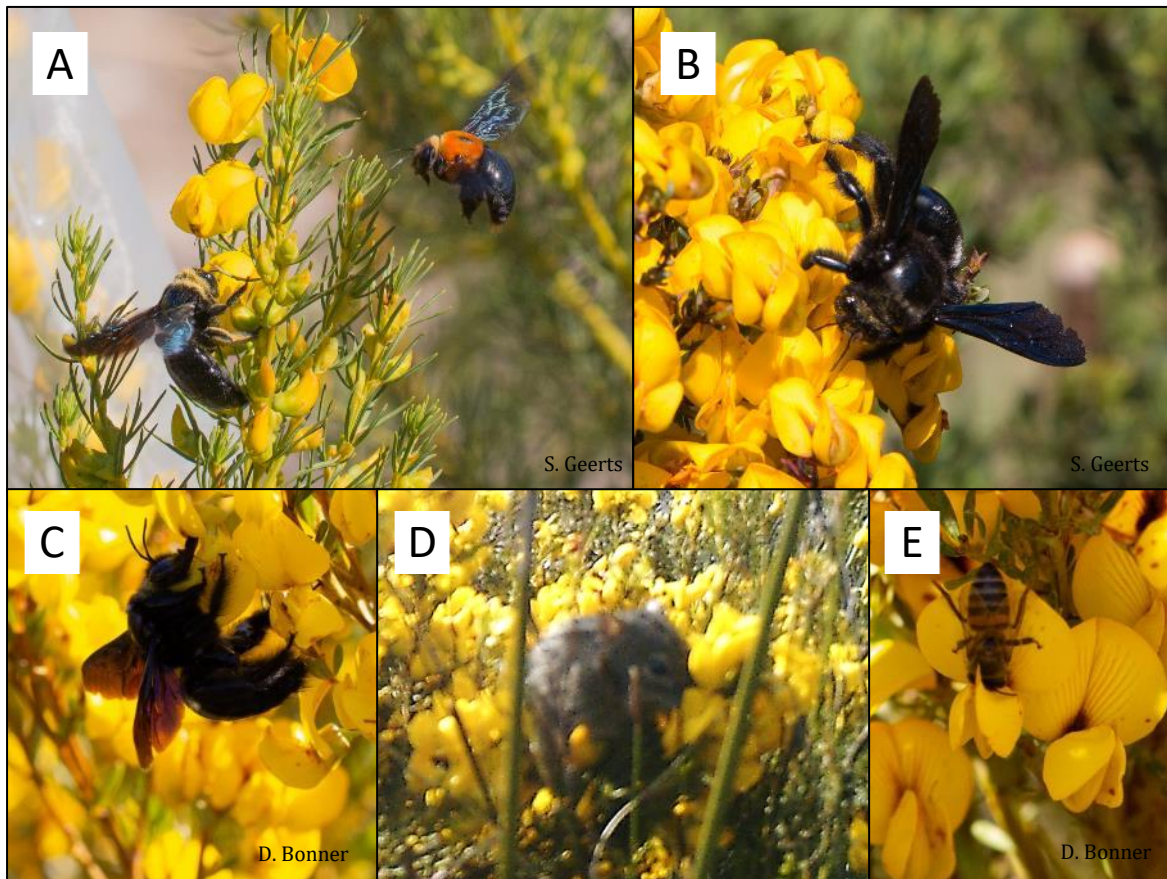
Fig. 2.4. (A) *Xylocopa capitata* individual visiting a *Cyclopia maculata* flower (left) and a *X. flavorufa* individual approaching the same *C. maculata* plant (right), (B) *Xylocopa capitata* visiting a *Cyclopia subternata* flower, (C) *Xylocopa capitata* successfully tripping and gaining access to a *Cyclopia* flower (*C. subternata*), (D) a striped field mouse caught by a camera trap foraging on *C. genistoides*, (E) honey bee (*Apis mellifera*) unsuccessfully attempting to access a *Cyclopia* flower.
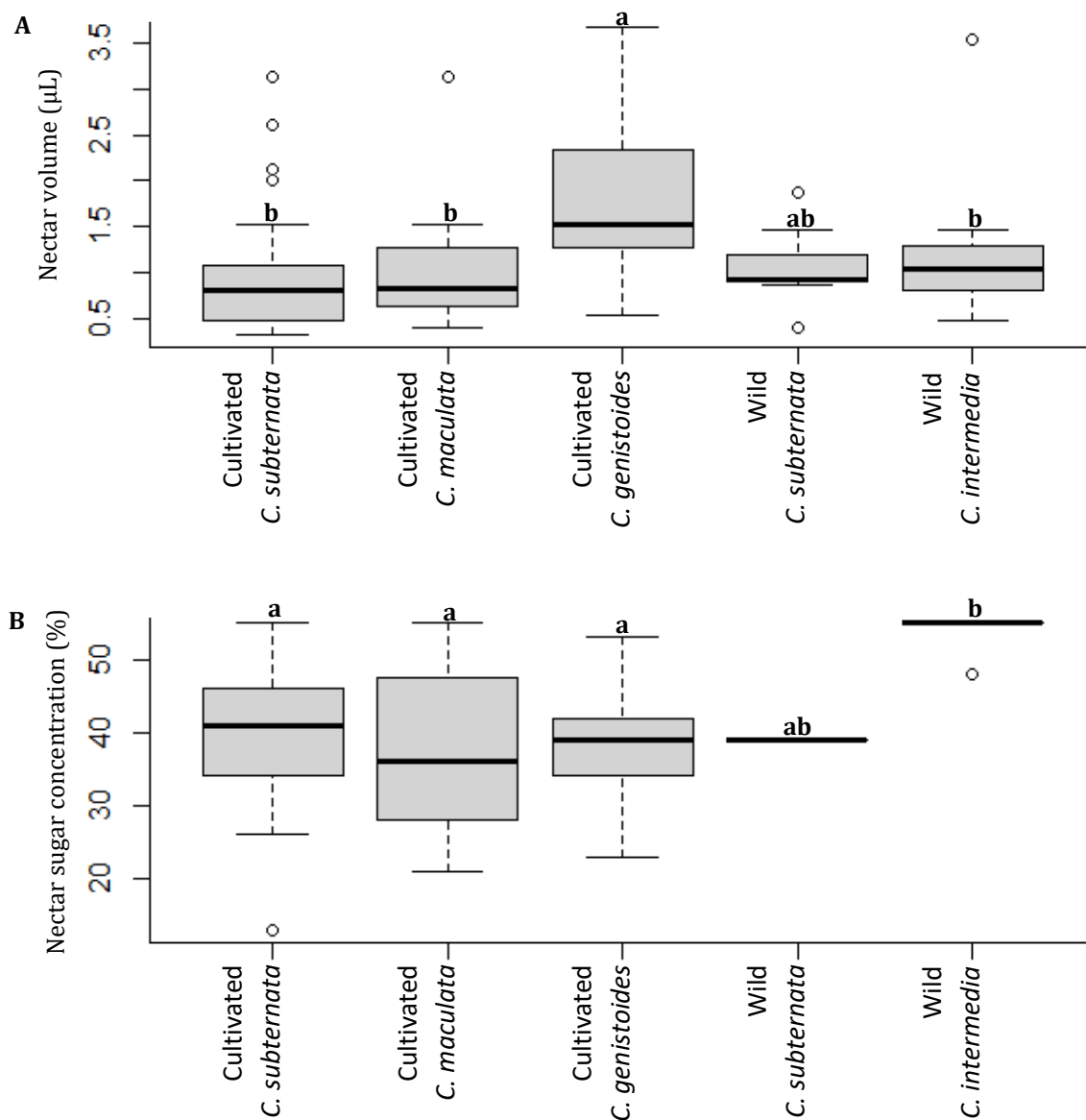
Fig. 2.5. (A) Nectar volume (μL) and (B) nectar sugar concentration (%) per flower for the *Cyclopia* spp. under study. Different letters indicate statistically significant differences. The thick line in each box represents the median, the box itself represents the interquartile range (IQR), and the whiskers extend the maximum and minimum values within 1.5 x IQR (white dots are the outliers).

2.4. Discussion

Carpenter bees (*Xylocopa* Latreille) were the only pollinators observed to access the reproductive parts of *Cyclopia* flowers. Xylocopinae have the ability to actively handle the moveable lateral and ventral petals donned by *Cyclopia* spp*.,* much like their papilionoid fabaceous relatives (Westerkamp, 1992; Etcheverry *et al.*, 2008; Grobler & Campbell, 2020). The ability of carpenter bees to trigger the "tripping" mechanism can be associated with their body size and therefore strength (Córdoba & Cocucci, 2011).

Generally, visitation rates were low, but slightly higher for all *Xylocopa* visitors, other than *X. scioensis*, in the wild versus the cultivated sites, despite cultivated and wild sites being in close proximity. There are many factors that influence the habitat preference of carpenter bees, including disturbance. Despite evidence of many pollinators being negatively affected by anthropogenic disturbance (Quintero *et al.,* 2009; Vanbergen, 2014; Geerts, 2011; Geerts & Pauw, 2011; Mnisi *et al.,* 2021), there are cases where species have thrived in altered landscapes such as *Xylocopa virginica* Linnaeus that nests in artificial wooden structures associated with human activity (Vickruck & Richards, 2017). The focus of pollination impacts of alien invasive tree species has been on resource competition (see for example Adedoja *et al.*, 2021), but impacts of alien invasive trees can also be unexpected, such as enhancing nesting sites for native pollinators in agricultural landscapes. In this study, the availability of nesting material (non-native *Pinus* spp.) may influence carpenter bee abundance. In addition, the abundance of forage in the cultivated site would be expected to attract more pollinators. However, in the wild sites, where fewer flowers are available owing to their spatial distribution, and are actively defended by males, each flower has a higher opportunity for visitation. Additionally, females are not territorial and therefore potentially disperse pollen more than males. Conversely, the abundance of plants and flowers in the territories defended in the cultivated sites will thus receive lower visitation if territories are similar in size. Therefore, the territorial behaviour displayed by males (Leys, 2000; Sugiura, 2008; Hefetz, 1983) may positively influence floral visitation in the wild study sites. As such, perhaps patterns of resource-use may be sex-specific in carpenter bees, like those recorded in hummingbirds (Maglianesi *et al.,* 2022), thus, resulting in lower visitation rates observed in the cultivated site in comparison to the wild site (Garibaldi *et al.,* 2011).

Plant-pollinator interactions, including flower visitation, may be affected by co-flowering plants through interspecific competition for pollinators, or at least plants which overlap in flowering phenology (Kehrberger & Holzschuh, 2019; Bergamo *et al.,* 2020). Furthermore, the low overall visitation rates of most Fabaceae species could be accounted for by the abundance of floral resources (pea plants have many flowers), resulting in competition between flowers for pollinator visits, similar to those recorded in Bergamo *et al.* (2020) with under 1 visit per flower

per hour on average. Grobler & Campbell (2020) studied the visitation rate of pollinators to *Cyclopia pubescens* at different distances from the roadside, and Córdoba & Cocucci (2017) obtained visitation rates for the pollinators of five papilionoid Fabaceae, with the low visitation rates observed here are similar to those studies, probably as a result of the highly abundant floral resources. Additionally, all *Cyclopia* species in this study had few links per species in both the wild and cultivated sites, indicating a specialised system with few pollinator species. Additionally, large flowering displays typical in cultivated patches are highly attractive and may likely attract bees from farther away (such as observed in Pasquet *et al.,* 2008).

It is proposed that the lack of honey bee (*Apis mellifera*) pollination, as well as other non-*Xylocopa* visitor pollination, in the genus *Cyclopia* (Table 2.3) is due to the floral morphology. The petals of *Cyclopia* flowers are alike to those present in all papilionoid Fabaceae; a keel-wing unit that requires active handling to expose the reproductive parts of the flower (Córdoba & Cocucci, 2011; Córdoba & Cocucci, 2017). When visitors are unable to exert the force required to open papilionoid flowers, they generally rob the flowers in search of floral rewards (Córdoba & Cocucci, 2011). *Cyclopia* flowers are likely too rigid to be opened by honey bees (Shaw pers. obs. 2021). Robbing may not necessarily affect the visitation rate of other pollinators, however, through flower mutation the overall reproductive ability may be reduced (de Souza *et al.,* 2019; Varma & Sinu, 2019). While signs of robbing were noted on cultivated *Cyclopia* flowers, no robbing was observed during floral observations. Whether honey bees can collect pollen on older tripped flowers, and thus contribute to pollination, needs to be explored. It is unlikely since honey bee visitation was low, all honey bee visits were to untripped flowers, and no contact with reproductive parts of the flowers were observed. Interactions observed by honey bees were likely in search of nectar rather than pollen.

The nectar volume of the *Cyclopia* flowers under study is low in comparison to other forage plants' flowers utilised by carpenter bees (Raju & Rao, 2006). Carpenter bees are generalist pollen and nectar foragers (Kaesar, 2010). However, carpenter bees (*X. micans*) have shown indifference to variability in nectar volume and sugar concentration in laboratory experiments (Perez & Waddington, 1996). Thus, the lower volume of nectar produced by *Cyclopia* flowers should have limited impact on carpenter bee foraging. The percentage sugar concentration of *Cyclopia* flowers is relatively similar to the 21 species reviewed by Raju & Rao (2006), further solidifying the notion that *Cyclopia* flowers are adapted to carpenter bee pollination. Furthermore, the higher sucrose – hexose ratio of usually higher than 9:1 in *Cyclopia* flowers allows for a source of higher energy value as opposed to other legumes with more balance sucrose – hexose ratios (see Van Wyk, 1993). With relatively large bees as the only pollinators, game camera traps, which have been identified as effective tools for insect monitoring that is comparable with human observation, could be used (Naqvi *et al.,* 2022).

The pollinator species richness observed from camera trapping was much lower than floral observations, with only *X. capitata, X. caffra* and *X. rufitarsis* observed. While *Xylocopa caffra* was identified as the most important pollinator for *Cyclopia intermedia* from floral observation visitation rates, only three *Xylocopa species* were observed visiting *C. intermedia* in a 10-hour camera trap period, none of which were *X. capitata* (the most important pollinator for *C. intermedia* according to visitation rates calculated from the camera trap data). This illustrates the importance of the blended approach in using both floral observations and camera traps to identify pollinators, as the observed interactions between the two methods were similar, however visitation rates were much lower likely owing to low detectability. In addition, the combined pollination network (drawn using data collected from both floral observations and camera trapping) (Fig. 2.3 A) is comparable to the pollination network from floral observation in the wild study sites (Appendix 2 B). This indicates that the camera trap data did not contribute any observable changes to the overall visitation rates, besides to *Xylocopa caffra* in the cultivated study site (cultivated *Cyclopia genistoides*), whereby camera traps picked up interaction which was not directly observed during floral observation. This could be due to the motion-sensing trap setting, which has been known to capture fewer insect visitors than scheduled camera trap settings (Naqvi *et al.*, 2022). Nevertheless, in this study camera trapping contributed to capturing pollinators on a indigenous crop.

The cultivation of indigenous plants is progressing, and their native pollinators can provide substantial increase in seed and fruit quantity and quality (Winfree *et al.,* 2007, Garibaldi *et al.,* 2013). Such as in the case of *Cyclopia*, an indigenous legume gaining traction in the global tea market and therefore in cultivation (Joubert *et al.*, 2011), which can only be effectively pollinated by one bee genus, *Xylocopa*. While autogamous selfing has previously produced fruit and seed set in *Cyclopia intermedia*, in the same study seed set was not produced for *C. maculata* or *C. subternata* (Koen, 2020). In addition, self-sterility in the *Cyclopia* genus was revealed through pollinator exclusion experiments (de Lange, 2010). Thus, *Cyclopia* may have varying degrees of selfing capabilities, but this requires further research (see Chapter 3). The role of *Cyclopia* pollinators is thus potentially critical for many *Cyclopia* species, in particular since seeds harvested from wild populations is a limited source.

Kaesar (2010) reviewed the potential for commercialisation of large carpenter bees as agricultural pollinators, arguing that their range of forage, activity in high tempered, ill-illuminated conditions and length of seasonal activity makes them ideal candidates for agricultural pollination. Although carpenter bees may reduce fruit and seed set significantly through nectar robbing of certain crop species such as rabbiteye blueberry *Vaccinium ashei* (Dedej & Delaplane, 2004; Sampson *et al.,* 2004), they have also been identified as efficient pollinators in other important crops such as passionfruit (Corbet & Willmer, 1980; Junqueira

et al., 2012). Here I show *Cyclopia* dependence on carpenter bees for successful reproduction, thus the value of these native pollinators is crucial for the honeybush tea industry.

The cultivation of indigenous plants in their natural range can pose challenges for conservation. While cultivation can be beneficial for the prevention of overharvesting in the wild (Bester, 2013), conservationists may argue that extensive cultivation of indigenous plants may threaten biodiversity (Van Wyk & Prinsloo, 2018). When indigenous plants are moved across their natural range for cultivation, the threat of genetic contamination through pollen transfer is substantial (Ford-Lloyd *et al.*, 2006). This may be a concern for the *Cyclopia* genus, where different species are moved across the range, sometimes beyond their natural distribution, for cultivation. This study provides baseline information on the pollination of commercially important *Cyclopia* species, useful for the management of cultivated plants and conservation of those in the wild. Now that the pollinators of *Cyclopia* have been identified, it is important to determine the distance that these pollinators can move and whether there is potential for movement between cultivated and wild sites and therefore pollen-flow. In addition, the time pollen grains remain viable under field conditions is important since other than pollinator travel distance, the length of pollen viability will influence the 'distance' pollen can travel (see Chapter 3).

2.5.   References

Adedoja, O., Erckie, L., Boatwright, J., Van Wyk, E. & Geerts, S. 2021. An invasive alien Proteaceae lures some, but not all nectar-feeding bird pollinators away from native Proteaceae in South African fynbos. *Plant Biology*, *23*, 915–922.

Barnado, N. 2013. *Sustainable harvesting of wild populations of Cyclopia intermedia in Kouga, Eastern Cape, South Africa* (M.Sc. Dissertation, Port Elizabeth: Nelson Mandela Metropolitan University).

Bergamo, P., Susin Streher, N., Traveset, A., Wolowski, M. & Sazima, M. 2019. Pollination outcomes reveal negative density-dependence coupled with interspecific facilitation among plants. *Ecology Letters, 23*, 129–139.

Bester, C. 2013. A model for commercialisation of honeybush tea, an indigenous crop. *Acta Horticulturae, 1007*, 889–894.

Biesmeijer, J., Roberts, S., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A., Potts, S., Kleukers, R., Thomas, C., Settele, J. & Kunin, W. 2006. Parallel Declines in

Pollinators and Insect-Pollinated Plants in Britain and the Netherlands. Science*, 313*, 351–354.

Corbet, S. & Willmer, P. 1980. Pollination of the yellow passionfruit: nectar, pollen and carpenter bees. *The Journal of Agricultural Science, 95*, 655–666.

Córdoba, S. & Cocucci, A. 2011. Flower power: its association with bee power and floral functional morphology in papilionate legumes. *Annals of Botany, 108*, 919–931.

Córdoba, S. & Cocucci, A. 2017. Does hardness make flower love less promiscuous? Effect of biomechanical floral traits on visitation rates and pollination assemblages. *Arthropod-Plant Interactions, 11*, 299–305.

Cousins, S. & Witkowski, E. 2012. African Aloe ecology: A review. *Journal of Arid Environments, 85*, 1–17.

Cowling, R., Rundel, P., Lamont, B., Kalin Arroyo, M. & Arianoutsou, M. 1996. Plant diversity in Mediterranean-climate regions. *Trends in Ecology and Evolution, 11*, 362–366.

Dedej, S. & Delaplane, K. 2004. Nectar-robbing carpenter bees reduce seed-setting capability of honey bees (Hymenoptera: Apidae) in Rabbiteye Blueberry, *Vaccinium ashei*, 'Climax'. *Environmental Entomology, 33*, 100–106.

de Lange, H. 2010. *Cyclopia* interspesie-teling. Oudtshoorn: S.n. Electronic copy.

de Souza, C., Salvador, M., Tunes, P., Di Stasi, L. & Guimarães, E. 2019. I've been robbed! – Can changes in floral traits discourage bee pollination? *PLOS ONE, 14*, 1–14.

Dormann, C., Frueund, J., Bluethgen, N. & Gruber, B. 2009. Indices, graphs and null models: analyzing bipartite ecological networks. *The Open Ecology Journal, 2*, 7–24.

Dunn, O. 1964. Multiple comparisons using rank sums. *Technometrics, 6*, 241–252.

Dunne, J., Williams, R. & Matinez, N. 2002. Food-web structure and network theory: role of connectance and size. *Proceedings of the National Academy of Sciences USA, 99*, 12917–12922.

Eardley, C. 1983. A taxonomic revision of the genus *Xylocopa* Latreille (Hymenoptera: Anthophoridae) in southern Africa. *Entomology Memoir, 58,* 1–67.

Etcheverry, A., Alemán, M. & Fleming, T. 2008. Flower morphology, pollination biology and mating system of the complex flower of *Vigna caracalla* (Fabaceae: Papilionoideae). *Annals of Botany, 102*, 305–316.

Ford-Lloyd, B., Dias, S. & Bettencourt, E. 2006. *Genetic erosion and pollution assessment methodologies.* Rome: Biodiversity International, 1–65.

Garibaldi, L., Sáez, A., Aizen, M., Fijen, T. & Bartomeus, I. 2020. Crop pollination management needs flower-visitor monitoring and target values. *Journal of Applied Ecology, 57*, 664–670.

Garibaldi, L., Steffan-Dewenter, I., Kremen, C., Morales, J., Bommarco, R., Cunningham, S. A., Carvalheiro, L., Chacoff, N., Dudenhoffer, J., Greenleaf, S., Holzschuh A., Isaacs, R. & Grace, O. 2011. Current perspectives on the economic botany of the genus *Aloe* L. (Xanthorrhoeaceae). *South African Journal of Botany, 77*, 980–987.

Garibaldi, L., Steffan-Dewenter, I., Winfree, R., Aizen, M., Bommarco, R., Cunningham, S., Kremen, C., Carvalheiro, L., Harder, L., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N., Dudenhoffer, J., Freitas, B., Ghazoul, J., Greenleaf, S., Hipolito, J., Holzschuh, A., Howlett, B., Isaacs, R., Javorek, S., Kennedy, C., Krewenka, K., Krishnan, S., Mandelik, Y., Mayfield, M., Motzke, I., Munyuli, T., Nault, B., Otieno, M., Petersen, J., Pisanty, G., Potts, S., Rader, R., Ricketts, T., Rundlof, M., Seymour, C., Schuepp, C., Szentgyorgyi, H., Taki, H., Tscharntke, T., Vergara, C., Viana, B., Wanger, T., Westphal, C., Williams, N. & Klein, A. 2013. Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science, 339*, 1608–1611.

Geerts, S. 2011. *Assembly and disassembly of bird pollination communities at the Cape of Africa* (Doctoral dissertation, Stellenbosch: Stellenbosch University).

Geerts, S. & Pauw, A. 2011. Farming with native bees (*Apis mellifera* subsp. *capensis* Esch.) has varied effects on nectar-feeding bird communities in South African fynbos vegetation. *Population ecology*, *53*, 333–339.

Genung, M., Fox, J., Williams, N., Kremen, C., Ascher, J., Gibbs, J. & Winfree, R. 2017. The relative importance of pollinator abundance and species richness for the temporal variance of pollination services. *Ecology, 98*, 1807–1816.

Gess, S. & Gess, F. 2014. *Wasps and bees of southern Africa.* South African National Biodiversity Institute, Pretoria. 1–320.Grobler, B. & Campbell, E. 2020. Pollinator activity and

the fecundity of a rare and highly threatened honeybush species along a highway in the Cape Floristic Region. *International Journal of Plant Sciences, 181*, 581–593.

Hefetz, A. 1983. Function of secretion of mandibular gland of male in territorial behavior of *Xylocopa sulcatipes* (Hymenoptera: Anthophoridae). *Journal of Chemical Ecology, 9*(7), 923–931.

Hung, K., Kingston, J., Albrecht, M., Holway, D. & Kohn, J. 2018. The worldwide importance of honey bees as pollinators in natural habitats. *Proceedings of the Royal Society B: Biological Sciences, 285*, 2017–2140.

Inouye, D. 1980. The terminology of floral larceny. *Ecology, 61*, 1251–1253.

IUCN Red List. 2021. *The IUCN Red List of Threatened Species.* <https://www.iucnredlist.org/> [9 March 2021].

Jordano P. 1987. Patterns of mutualistic interactions in pollination and seed dispersal: connectance, dependence asymmetries, and coevolution. *The American Naturalist. 129,* 657–77.

Joubert, E., Joubert, M., Bester, C., de Beer, D. & De Lange, J. 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets — The catalytic and supporting role of research. *South African Journal of Botany, 77*, 887–907.

Junqueira, C., Hogendoorn, K. & Augusto, S. 2012. The use of trap-nests to manage carpenter bees (Hymenoptera: Apidae: Xylocopini), pollinators of passion fruit (Passifloraceae: *Passiflora edulis* f. flavicarpa). *Annals of the Entomological Society of America, 105*, 884–889.

Keasar, T. 2010. Large carpenter bees as agricultural pollinators. *Psyche: A Journal of Entomology, 2010*, 1–7.

Kehrberger, S. & Holzschuh, A. 2019. How does timing of flowering affect competition for pollinators, flower visitation and seed set in an early spring grassland plant? *Scientific Reports, 9*.

Klein, A., Vaissière, B., Cane, J., Steffan-Dewenter, I., Cunningham, S., Kremen, C. & Tscharntke, T. 2007. Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences, 274*, 303–313.

Koen, J. 2020. *Reproductive anatomy, sexual compatibility, pollen viability and embryo rescue of* Cyclopia *species* (PhD thesis, Tshwane University of Technology).

Koen, J., Slabbert, M., Booyse, M. & Bester, C. 2019. Honeybush (*Cyclopia* spp.) pollen viability and surface morphology. *South African Journal of Botany, 128*, 167–173.

Leys, R. 2000. Mate locating strategies of the green carpenter bees *Xylocopa* (Lestis) *aeratus* and *X.* (L.) *bombylans. Journal of Zoology, 252*, 453–462.

Maglianesi, M., Maruyama, P., Temeles, E., Schleuning, M., Zanata, T., Sazima, M. & Dalsgaard, B. 2022. Behavioural and morphological traits influence sex-specific floral resource use by hummingbirds. *Journal of Animal Ecology*.

McKay, D. & Blumberg, J. 2006. A review of the bioactivity of South African herbal teas: rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*). *Phytotherapy Research, 21*, 1–16.

Melin, A., Rouget, M., Midgley, J. & Donaldson, J. 2014. Pollination ecosystem services in South African agricultural systems. *South African Journal of Science, 110*, 1–9.

Mnisi, B., Geerts, S., Smith, C. & Pauw, A. 2021. Nectar gardens on school grounds reconnect plants, birds and people. *Biological Conservation*, *257*, 109087.

Moolla, A. & Viljoen, A. 2008. 'buchu' – *Agathosma betulina* and *Agathosma crenulata* (Rutaceae): A Review. *Journal of Ethnopharmacology, 119*, 413–419.

Motsa, M., Slabbert, M., Bester, C. & Ngwenya, M. 2017. Phenology of honeybush (*Cyclopia genistoides* and *C. subternata*) genotypes. *South African Journal of Botany, 110*, 57–67.

Mucina, L. & Rutherford, M. 2011. *The vegetation of South Africa, Lesotho and Swaziland*. Pretoria: South African National Biodiversity Institute, 53–219.

Naqvi, Q., Wolff, P., Molano-Flores, B. & Sperry, J. 2022. Camera traps are an effective tool for monitoring insect–plant interactions. *Ecology and Evolution, 12*, 1–8.

Pasquet, R., Peltier, A., Hufford, M., Oudin, E., Saulnier, J., Paul, L., Knudsen, J., Herren, H. & Gepts, P. 2008. Long–distance pollen flow assessment through evaluation of pollinator foraging range suggests transgene escape distances. *Proceedings of the National Academy of Sciences, 105*, 13456–13461.

Perez, S. & Waddington, K. 1996. Carpenter bee (*Xylocopa micans*) risk indifference and a review of nectarivore risk-sensitivity studies. *American Zoology, 36*, 435–446.

Pettis, J. & Delaplane, K. 2010. Coordinated responses to honey bee decline in the USA. *Apidologie, 41*, 256–263.

Potts, S., Imperatriz-Fonseca, V., Ngo, H., Aizen, M., Biesmeijer, J., Breeze, T., Dicks, L., Garibaldi, L., Hill, R., Settele, J. & Vanbergen, A. 2016. Safeguarding pollinators and their values to human well-being. *Nature, 540*, 220–229.

Potts, S., Roberts, S., Dean, R., Marris, G., Brown, M., Jones, R., Neumann, P. & Settele, J. 2010. Declines of managed honey bees and beekeepers in Europe. *Journal of Apicultural Research, 49*, 15–22.

Quintero, C., Morales, C. and Aizen, M. 2009. Effects of anthropogenic habitat disturbance on local pollinator diversity and species turnover across a precipitation gradient. *Biodiversity and Conservation, 19*, 257–274.

Raju, A. & Rao, S. 2006. Nesting habits, floral resources and foraging ecology of large carpenter bees (*Xylocopa latipes* and *Xylocopa pubescens*) in India. *Current Science, 90*, 1210–1217.

R Core Team. 2021. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rodger, J., Bennett, J., Razanajatovo, M., Knight, T., Van Kleunen, M., Ashman, T., Steets, J., Hui, C., Burd, M., Burkle, L., Burns, J., Durka, W., Freitas, L., Kemp, J., Li, J., Pauw, A., Vamosi. J., Wolowski, M., Xia, J. & Ellis, A. 2021. Widespread vulnerability of flowering plant seed production to pollinator declines. *Science Advances, 7*, 1–10.

Sampson, B., Danka, R. & Stringer, S. 2004. Nectar robbery by bees *Xylocopa virginica* and *Apis mellifera* contributes to the pollination of rabbiteye blueberry. *Journal of Economic Entomology, 97*, 735–740.

Schutte, A. 1997. Systematics of the genus *Cyclopia* Vent. (Fabeacea, Podalyrieae). *Edinburgh Journal of Botany*, 54, 125–170.

Slabbert, E., Malgas, R., Veldtman, R. & Addison, P. 2019. Honeybush (*Cyclopia* spp.) phenology and associated arthropod diversity in the Overberg region, South Africa. *Bothalia, 49*, 1–13.

Slatkin, M. 1987. Gene flow in natural populations. *Annual Review of Ecology, Evolution, and Systematics, 16*, 393–430.

Smith, C. 1966. *Common names of South African plants*. Botanical Survey Memoir No. 35.Department of Agricultural Technical Services, Pretoria, South Africa, 247.

Somanathan, H., Saryan, P. & Balamurali, G. 2019. Foraging strategies and physiological adaptations in large carpenter bees. *Journal of Comparative Physiology A, 205*, 387–398.

Stokstad, E. 2007. The case of the empty hives. *Science, 316*(5827), 970–972.

Sugiura, S. 2008. Male territorial behaviour of the endemic large carpenter bee, *Xylocopa* (Koptortosoma) *ogasawarensis* (Hymenoptera: Apidae), on the oceanic Ogasawara Islands. *European Journal of Entomology, 105*, 153–157.

Vanbergen, A. 2014. Landscape alteration and habitat modification: Impacts on plant–pollinator systems. *Current Opinion in Insect Science, 5*, 44–49.

Van Wyk, B-E. 2008. A broad review of commercially important southern African medicinal plants. *Journal of Ethnopharmacology, 119*, 342–355.

Van Wyk, B.-E. 1993. Nectar sugar composition in southern African Papilionoideae (Fabaceae). Biochemical Systematics and Ecology, *21,* 271–277.

Van Wyk, B. & Gorelik, B. 2017. The history and ethnobotany of Cape Herbal Teas. *South African Journal of Botany, 110*, 18–38.

Van Wyk, A. & Prinsloo, G. 2018. Medicinal plant harvesting, sustainability and cultivation in South Africa. *Biological Conservation, 227*, 335–342.

Varma, S. & Sinu, P. 2019. Nectar robbing in bellflower (*Sesamum radiatum*) benefited pollinators but unaffected maternal function of plant reproduction. *Scientific Reports, 9*, 1–9.

Vickruck, J. & Richards, M. 2017. Nesting habits influence population genetic structure of a bee living in anthropogenic disturbance. *Molecular Ecology, 26*, 2674–2686.

Watanabe, M. 1994. Pollination worries rise as honey bees decline. *Science, 265*, 1170–1170.

Westerkamp, C. 1992. The co-operation between the asymmetric flower of *Lathyrus latifolius* (Fabaceae-Vicieae) and its visitors. *Phyton, 33,* 121–137.

Winfree, R., Williams, N., Dushoff, J. & Kremen, C. 2007. Native bees provide insurance against ongoing honey bee losses. *Ecology Letters, 10*, 1105–1113.

Chapter 3 : Pollen flow between wild and cultivated *Cyclopia* in Cape Fynbos

Abstract

In South Africa, there are several native plants with commercial value and potential. One such genus is *Cyclopia* Vent. (Fabaceae) or honeybush, native to the fynbos biome with significant commercial value in the tea industry of South Africa. While cultivation is necessary to prevent over-harvesting of the wild resource, the intensified cultivation of indigenous crops may result in genetic contamination – through pollen transfer – between distant, distinct populations and between different species. Here I examine the movements of the most abundant carpenter bee (Xylocopinae) pollinator, *Xylocopa capitata*, to determine whether pollinators move between flowers of cultivated and wild *Cyclopia*. Carpenter bee movement was investigated using mark-release-recapture and radio-tagging. Additionally, I performed hand-pollination experiments to determine whether crosses within and between species can produce viable seeds. Finally, pollen viability was quantified using Fluorochromatic Reaction tests to determine longevity under field conditions in order to identify the distance viable pollen can travel and thus initiate cross-pollination. Mark-release-recapture revealed travel distances up to 729 m, with regular movement between wild and cultivated plants. In addition, the maximum distance estimated in a single foraging bout by a radio-tagged carpenter bee was 1194 m, with a maximum daily home range size of 23 893 m². This indicates regular movement and utilisation of areas with both planted and wild *Cyclopia* plants. Owing to the indiscriminate foraging patterns displayed by carpenter bees, there is a high likelihood of utilization of both wild and cultivated *Cyclopia* during single foraging bouts. Under field conditions, *Cyclopia* pollen maintain more than 50% viability after 4.5 days. Furthermore, two between species crosses produced seeds (*C. subternata* x *C. genistoides* and *C. subternata* x *C. maculata*), illustrating the ability of hybrids to form. Pollinators move frequently between wild and cultivated plants when 1 km apart and are likely to do so between plants beyond this distance. Considering the extended longevity of *Cyclopia* pollen under field conditions, in addition to the ability of hybrid plants to form from between species crosses, the risk of gene flow through pollen-flow in *Cyclopia* is likely. To avoid genetic homogenization, a protocol guiding the planting of species within this genus is urgently needed.

3.1.   Introduction

The cultivation of indigenous plants, although necessary in many cases, may present challenges for their conservation (Van Wyk & Prinsloo, 2018). The movement of genes between wild and cultivated plant populations can have evolutionary consequences for local

conspecifics as well as their generic relatives (Delplancke *et al.*, 2011; Zhou *et al.*, 2020). Genetic contamination is evident between many indigenous cultivated species and their wild relatives, e.g. between beetroot species and *Beta vulgaris* ssp. *vulgaris* (Bartsch *et al.,* 1999), in *Medicago sativa* (Jencewski *et al.*, 1999), in *Helianthus annuus* (Burke *et al.*, 2002; Ureta *et al.*, 2008; Arias & Rieseberg, 1994), in *Oryza rufipogon* (Song *et al.*, 2003), between *Prunus dulcis* and *Prunus orientalis* (Delplancke *et al.*, 2011), in *Theobroma cacao* (De Schawe *et al.*, 2013), between cultivated *Malus domestica* and closest wild relatives (Cornille *et al.,* 2013), and in *Juglans regia* (Zhou *et al.,* 2020). Gene flow between wild and cultivated plant populations may result in the reduction of biodiversity and genetic fitness, and the distinctiveness between populations through outbreeding depression (Wolf *et al.,* 2001; Campbell & Waser, 2001). Hybridization can occur with the movement of closely related species beyond their normal home range and can contaminate the local gene pool.

The global export of indigenous crops is important for the economy of South Africa (Reinten & Coetzee, 2002). South Africa has a rich biodiversity, with many native plant species of commercial importance as indigenous vegetables, medicinal plants and floricultural crops (Reinten & Coetzee, 2002). The South African native *Cyclopia* genus has commercial value in terms of the honeybush tea industry (Joubert *et al.*, 2011). With the steady growth of the industry, it is clear that the genus is of economic value and importance in South Africa (McKay & Blumberg, 2006; Koen *et al.*, 2019). The expansion of cultivation is important to relieve the pressure from wild-harvested *Cyclopia* populations with the increased popularity of the product on the global tea market (Bester, 2013; McGregor, 2017). The shift in supply from wild-harvested material to cultivated biomass can be considered fundamental for the survival of wild *Cyclopia* populations. However, moving genetic material across the range for the cultivation of *Cyclopia* may pose an additional threat to the few localised, threatened *Cyclopia* species (Potts, 2017). As these species are confined to small areas, genetic contamination might have negative consequences for these species (Potts, 2017). Thus, determining gene flow through pollen contamination resulting from the introduction of *Cyclopia* species of economic importance into areas with threatened *Cyclopia* species is long overdue.

Genetic diversity is important for maintaining the quality, quantity and viability of crops (Hammer & Teklu, 2008). Unsampled traits and potentially rare alleles in *Cyclopia* may be valuable in terms of adaptation (Potts, 2017). The loss of these traits through genetic contamination and homogenisation pose a significant threat to the survival of some *Cyclopia* species. Phenotypic resilience is crucial to avoid local extinctions (Potts, 2017), which would have consequences for the honeybush tea industry as a whole. Genetic contamination may influence species resistance to environmental change, thus affecting the wild genetic material farmers utilize for growing honeybush. In addition, the potential for hybridization among

different species of *Cyclopia* will affect the seed source in cultivated areas on which honeybush farmers rely. Therefore, the conservation of the genetic material is important not only for biodiversity, but also for farming honeybush.

In order to determine whether genetic contamination is likely to occur, it is important to consider seed dispersal and pollination (Ellstrand, 1992). Seed dispersal is primarily by ants, and thus short distance (Schutte, 1997; Slabbert *et al.,* 2019). The primary pollinators of four of the six commercially important *Cyclopia* species (*Cyclopia subternata, C. maculata, C. intermedia* and *C. genistoides*) have been identified as *Xylocopa* species (see Chapter 2). Pollinator movement between habitat patches is important to prevent genetic and demographic erosion of isolated plant populations (Ellstrand & Elam, 1993). However, in this instance, the potential for cross-pollination between cultivated and wild populations poses a threat to the genetic distinctiveness of wild *Cyclopia* populations. Carpenter bees are generalist pollen and nectar foragers (Kaesar, 2010);ascribable to the larger body size of *Xylocopa* species, and their subsequent ability to fly longer distances, their likelihood to travel between habitat patches is increased (Cane, 1987; Greenleaf *et al.,* 2007). Members of the family *Xylocopa* are believed to be long-distance foragers (Cane, 1987) with disproportionately larger foraging ranges compared to smaller bee species (Greenleaf *et al.,* 2007; Gathmann & Tscharntke, 2002; Zurbuchen *et al.,* 2010). This is due to the large intertegular span of bees in this genus, which allows for significantly further flight distances (Cane, 1987; Greenleaf *et al.*, 2007). A study conducted by Pasquet *et al.* (2008) used radio-transmitters to track the movement of *Xylocopa flavorufa,* revealing foraging distances up to 6 km from nesting sites. Homing experiments revealed that large carpenter bees returned to their nesting sites after being displaced 10 km from their nests (Roubik, 1992). It is, however, proposed that movement of large carpenter bees is restricted to one third of the maximum flight distance recorded in homing tests (Roubik, 1992). Furthermore, site fidelity has been observed in carpenter bees where floral resources are in abundance and are renewable (Somanathan *et al.,* 2019). Information on the foraging ranges of *Xylocopa capitata,* the most abundant pollinator of *Cyclopia* (see Chapter 2), is lacking. It is important to determine the foraging distance of pollinators and compare this to the distance between wild and cultivated *Cyclopia* populations in order to establish whether cross-pollination is possible and at what distance.

There are different methods for tracking insect movement either directly or indirectly (Osborne *et al.*, 2008). It should be noted that most direct methods, that involve the tracking and marking of an individual, could alter the behaviour and movement (Osborne *et al.*, 2008). Numbered or coloured tags have been successfully used to track bumble bees – with similar weights to carpenter bees – in a direct method of monitoring, using mark-release-recapture (Osborne & Williams, 2001). Although the use of tracking devices has been argued to alter behaviour,

small, lightweight radio-transmitters are available and previous studies have shown that very small transmitters have negligible effect on carpenter bee flight and foraging ability (Pasquet *et al.,* 2008; Hagen *et al.,* 2011). Radio-tagging to track carpenter bee forage bouts can therefore aid in determining the typical foraging distance of a carpenter bee, and thus the ability to transfer pollen between wild and cultivated plants.

The survival time of *Cyclopia* pollen under field conditions is important in determining the potential for cross-pollination. If pollen has a limited survival time, shorter than the duration to complete a foraging trip between cultivated and wild populations, the risk of cross-pollination is reduced. Fortunately, extensive knowledge on *Cyclopia* pollen is available (Koen, 2020). Koen (2020) in an in vitro study tested *Cyclopia* pollen germination percentage (PGP) from pollen stored at -18°C from genotypes of *Cyclopia longifolia, C. maculata* and *C. subternata.* Pollen from these species remained viable for 540 days, besides one *C. subternata* genotype which remained viable for 180 days (with 12% pollen germination) (Koen, 2020). Pollen viability under field conditions are unknown, but will be much shorter due to higher temperatures and direct exposure to sunlight and wind (Dafni & Firmage, 2000). Factors that influence pollen survival in the wild include humidity, temperature, transport from anther to stigma (Dafni & Firmage, 2000), and UV-B radiation (Demchick & Day, 1996).

The pollen longevity of *Cyclopia* under field conditions will influence the ability of hybrid formation. There is very little literature available on between species crosses in *Cyclopia*, though species crosses are potentially possible (de Lange & von Mollendorf, 2006), but not well documented. Farmers harvest seeds to expand their plantations and may thus be affected by cross-pollination and the resulting formation of hybrid seed. Planting hybrid seed unknowingly will cause further genetic erosion of the *Cyclopia* under cultivation. More importantly, if hybridization occurs, there is the potential of genetic erosion of wild populations that grow in close proximity to cultivated sites (Potts, 2017). Within the tribe Podalyrieae, *Cyclopia* is one of two only known polyploids; in addition to *Virgilia* (Schutte, 1997). It has been proposed that the chromosome number of *Cyclopia* species may prevent hybridization between certain species (Schutte, 1997; Motsa *et al.*, 2018). However, attempting to predict the potential for hybridization based on ploidy levels is not clear-cut (Petit *et al.*, 1999), particularly since there are records of ploidy level variation between populations of the same *Cyclopia* species (Schutte, 1997). Additionally, how these species with varied ploidy levels are distributed across the landscape is unknown. Therefore, crossing experiments in the field are needed to determine the potential of hybridization in *Cyclopia.*

Therefore, here I aim to determine the potential for cross-pollination of *Cyclopia* species by investigating pollinator movement, pollen viability and the potential for hybridization in

Cyclopia. Specifically, I asked (1) whether pollinators move between flowers of cultivated and wild *Cyclopia*, (2) what is the distance travelled by *Cyclopia* pollinators? (3) what is the maximum survival time of *Cyclopia* pollen under field conditions? And finally, (4) whether crosses between wild and cultivated plants, within and between species, can produce viable seeds.

3.2.   Methods

3.2.1.   *Study area, site selection and study species*

The study was conducted on a honeybush farm in Twee Riviere (33˚52'08.6''S 23˚54'54.2''E), just outside of Joubertina in the Eastern Cape of South Africa (Appendix 3 A). The honeybush farm was located within the natural range of *Cyclopia*, thus where wild populations were naturally occurring. The study site was chosen according to its ideal elevation needed for radio-tracking (i.e., longer telemetry range) at the base of a valley, surrounded by natural Tsitsikamma sandstone fynbos vegetation (Mucina & Rutherford, 2011). This region is characterized by Mediterranean climate, with a mean annual precipitation of 575 mm, and annual temperature 15.7˚C (Cape Farm Mapper 2.6.15).

The *Cyclopia* species planted on the farm, as well as wild *Cyclopia* species that were growing naturally near the study site (within 1km), made up the study species. *Cyclopia* is endemic to the fynbos biome in the Eastern and Western Cape Provinces of South Africa (Schutte, 1997). There are 23 species in the genus *Cyclopia* (two of which are extinct; *C. filiformis* Kies and *C. laxiflora* Benth. (Hilton-Taylor, 1996)), all with varied distributions across the range. Cultivation has resulted in six commercially important honeybush species being moved across the range, in some cases beyond the natural distribution of the species (Joubert *et al.,* 2011). Four of the six commercially important honeybush species were selected as study species. The three main commercialized species are included, i.e., *Cyclopia genistoides* (L.) R.Br*, C. intermedia* E. Mey and *C. subternata* Vogel (Joubert *et al.,* 2011). Additionally, *Cyclopia maculata* (Andrews) Kies has potential for large scale commercialization and cultivation (Joubert *et al.,* 2011), and was therefore also selected.

At the Twee Riviere study site (Appendix 3 A), *Cyclopia subternata* and *C. maculata* were under cultivation, while populations of *C. subternata* and *C. intermedia* occurred naturally in the wild. These naturally occurring plants were growing near the planted honeybush, the closest at less than 50 m away. *Cyclopia subternata*, "vleitee", is a reseeder with ploidy of 2n = 6x = 54, and flowers between September and October (Motsa *et al.,* 2017). *Cyclopia maculata*, "needle-leaf honeybush", is a reseeder species with varied ploidy nature, but commonly 2n = 4x = 36, and flowers between September and November (Slabbert *et al.,*

2019). *Cyclopia intermedia,* "bergtee", is a resprouter species with ploidy variation, commonly 2n = 14x = 126, and also flowers between September and November (Barnado, 2013).

In order to quantify carpenter bee movement, one species of carpenter bee, *Xylocopa capitata* Smith*,* was monitored within an area of cultivated and neighboring wild-growing *Cyclopia. Xylocopa capitata* was used for the tracking aspect of the study owing to the robustness of the bee and therefore ability to carry the radio-transmitter without hinderance to flight and foraging. In addition, this species was the most abundant pollinator (Chapter 2). Radio transmitters were sourced from HOLOHIL, a Canadian electronics manufacturer. The abundance of *X. capitata* coupled with the size of the species (Appendix 2 A) and thus the potential for long-distance flight, meant that this species was also utilized for mark-release-recapture experiments. Ethics was granted under the Cape Peninsula University of Technology. The ethics permit reference number for this study is 216277566/06/2021.

Other than crossing experiments at the Twee Riviere study site, an additional site was identified for the hand-crossing aspect of the study. This site was useful to increase the number of commercially important species tested for hybridization and genetic contamination potential. This site was located coastally, at Pearly beach in the Overstrand of the Western Cape (34˚41'43.1''S 19˚36'15.5''E). The dominant vegetation in this landscape was Overberg sandstone fynbos, characterised by a mean-annual precipitation of 450 – 830 mm, and mean daily temperatures ranging between 6.3˚C and 25.6˚C (Mucina & Rutherford, 2011). The farm had 2.7 ha of cultivated *Cyclopia genistoides*, with a few cultivated *C. subternata* plants and a patch of wild growing *C. genistoides* in the native surrounding vegetation. *Cyclopia genistoides* is commonly known as coastal tea or "kustee", a resprouter species that has known variation in ploidy, commonly 2n = 10x = 90, and flowers between September and November (Motsa *et al.*, 2017; Slabbert *et al.*, 2019).

3.2.2. *Pollinator movement between cultivated and wild Cyclopia populations*

A mark-release-recapture method was used to track the movement of bees between the wild and cultivated study sites. In methods, similar to those described by Osbourne & Williams (2001), *Xylocopa capitata* individuals were caught using a heavy duty sweep net and marked using various colours of nail polish. The colours varied according to the site of capture and release, as well as the marking occasion (Table 3.1).

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Table 3.1. Dates of mark-release-recapture efforts of *Cyclopia* pollinator species *Xylocopa capitata*, in addition to the number of individuals marked in each site of capture at the Twee Riviere study site, and colours of nail polish used on different marking occasions.

Site of capture	Marking occasion	No. of individuals marked	Colour
Cultivated	25/09/2021 – 26/09/2021	28	Light blue
	05/10/2021 – 07/10/2021	48	Purple
	19/10/2021	27	White
Wild	06/09/2021 – 07/09/2021	16	Orange
	19/10/2021 – 20/09/2021	13	Red

Marked individuals were immediately released at designated locations within their area of capture (Appendix 3 A). Marking efforts were similar between the cultivated and wild sites (~7 and ~8 hrs, respectively), with slightly more time spent in the wild site to account for the rugged terrain and patchily distributed floral neighbourhood, and thus lower carpenter density (Shaw pers. obs. 2021). The carpenter bees were marked on the dorsal side of the thorax for ease in identification during observations. Marking took place between 10:00 and 16:00 in both the cultivated and the wild site. There were two points of release, one in the cultivated site and one in the wild site (Appendix 3 A). The options for the cultivated release point were, of course, limited to the cultivated site. The wild release point was chosen within a suitable distance of the cultivated site (~680 m) whilst remaining within a population of naturally occurring *Cyclopia*. Observation efforts (~41 and ~16 hrs in the cultivated and wild sites, respectively) were made to identify marked bees, recording the date, time, colour of the nail polish and GPS location. The skew in observation efforts was due to the rough terrain in the wild site, and the observer's ability to locate carpenter bees over an expansive area in comparison to the densely populated cultivated patch. Additionally, patchily distributed food and nectar resources were scattered across the landscape, reducing observation effectiveness. Thus, less time was spent in the wild observing marked bees. Observations took place from one hour after marking and release in both the cultivated and wild sites and were conducted on the same days in both sites. Fortunately, the visibility of marked bees was high as *Xylocopa capitata* is almost completely black allowing for a solid base for the colour to be clear from a distance (Fig. 3.1). Therefore, "recapture" was not necessary to identify marked bees and re-observations were sufficient. However, this increased the potential for marked individuals being counted more than once during the study period and could inflate recapture numbers. Though, the purpose of mark-release-recapture was to detect movement between the cultivated and wild sites which was unaffected by the duplicated recapture numbers. *Xylocopa capitata* individuals caught and marked in the cultivated site are termed "cultivated-caught", and individuals caught and marked in the wild site termed "wild-caught" throughout.

Fig. 3.1. A marked *X. capitata* (orange) caught on wild *Cyclopia* and a marked *X. capitata* (blue) caught within a cultivated stand of *Cyclopia.*

The number of cultivated-caught bees re-observed in the cultivated site and the wild site were counted, likewise for wild-caught bees. These re-observations in the non-capture site would indicate movement between the two sites, and thus illustrating the potential for pollen transfer. A map was created in QGIS version 3.16.4 using the GPS points taken from re-observations (QGIS, 2021). Measurements were completed in QGIS using the "Distance to nearest hub" tool to measure the distance between the sites of release and sites of re-observation. No measurements were conducted for cultivated-caught bees re-observed in the cultivated site, as GPS points were not taken due to the small area of the cultivated patch.

3.2.3. *Maximum foraging distance and daily home range*

Radio-transmitters were used to track the daily foraging distances of carpenter bees (*Xylocopa capitata*). Carpenter bee flight is relatively unimpeded while carrying a very small radio-transmitter (Pasquet *et al.,* 2008). The LB-2X radio-transmitters were selected for the study, as they are the smallest and most lightweight (0.27 g) 2-stage transmitters available commercially. Each transmitter had a small battery with a limited 12-day lifespan. The radio-transmitters were tracked using a TRX-48 receiver (Wildlife Materials, USA).

Xylocopa capitata bees were caught using a heavy duty sweep net and fitted with a radio-transmitter on the dorsal side of the thorax secured with a mixture of eyelash adhesive and superglue (recommendation by manufacturer, Edwards pers. comm.). Ethics was granted under the Cape Peninsula University of Technology. The ethics permit reference number for this study is 216277566/06/2021. The carpenter bees were then released and actively tracked on foot using radio telemetry. At each point that the carpenter bee with attached radio-transmitter was positively identified, the observer recorded the GPS location and noted the

behaviour. Behaviour included flying, foraging, territorial displays (indicated by a "criss-cross" flying pattern and chasing other individuals; Louw & Nicholson, 1983) and resting. These GPS points were used to plot the forage route of each individual tracked carpenter bee. The maximum range of signal from the tag in the study site, tested by steadily increasing distance of radio-transmitter from telemetry, was up to ~630 m. The maximum observed flight distance for each radio-tagged carpenter bee was determined and recorded during each radio-tracked foraging bout. While the focus was to determine the maximum forage distance of the carpenter bees (i.e., the maximum flight distance from the release point), it was beneficial to determine the home ranges of carpenter bees (i.e., the total area utilized by a radio-tagged bee during tracking), as this indicated the potential for regular movement between wild and cultivated *Cyclopia* populations. Radio-tagged bees were tracked for a period of one and four days, allowing for indication of daily home range. GPS points were plotted to form polygons indicating the forage route of each individual bee for two separate flowering seasons. The Minimum Convex Polygon (MCP) was used to estimate daily MCP home range sizes (Hagen *et al.,* 2011). The outer GPS point locations were connected, and the area of the resulting convex polygon was measured (Eddy, 1997). Measurements were completed in QGIS using the "Distance to nearest hub" tool to measure the distance between the site of release and furthest distance of each bee's flight path (i.e., maximum forage distance), and the "Convex hull" tool to measure the MCP home range size. In some instances, bees could only be tracked for a few hours before signal was lost, thus the daily home range size is likely a underestimation of the daily flight patterns.

3.2.4. *Cyclopia pollen viability*

The Fluorochromatic Reaction test (FCR) was used to quantify the survival time of *Cyclopia* pollen under field conditions. Flowering branches were collected from three species of *Cyclopia* and stored in fresh water to delay wilting. A subset of pollen was removed from *Cyclopia maculata, C. subternata* and *C. intermedia* flowers at various intervals between a half day and five-day period (*C. maculata n* = 9 flowers; *C. subternata n* = 5; *C. intermedia n* =6). The pollen subset was placed into an open petri dish and exposed to outdoor weather conditions to reproduce field conditions, including direct sunlight and wind. The diversely aged pollen samples were then subjected to FCR for analysis.

Pollen viability was determined using FCR as described and standardized by Pinillos & Cuevas (2008). The fluorescein diacetate was combined with acetone at 2 mgml^{-1} to form a stock solution. Drops of this solution were added to 2 ml of sucrose solution of a concentration indicated by "persistent cloudiness" (Heslop-Harrison *et al.*, 1984). Pollen grains were

distributed in a drop of the stock-sucrose-solution on a microscope slide whereby fluorochromatic reaction could commence. Incubation took place for 15 minutes before observation under the microscope to ensure optimal fluorescence (Heslop-Harrison & Heslop-Harrison, 1970; Pinillos & Cuevas, 2008). A fluorescent microscope was utilised for fluorescence detectability (Olympus BX 41). Photographs were taken through the microscope lens of each sample undergoing FCR. Using the resultant images, 200 pollen grains per species were demarcated at random before counting the number of viable and unviable pollen. Viable pollen grains were identifiable through bright fluorescence, indicating an intact plasmalemma of the pollen vegetative cell, within which esterases hydrolyse the fluorescein esters to release fluorescein, thus causing fluorescence (Shivanna & Heslop-Harrison, 1981).

3.2.5. *Within and between species crosses*

Hand-crossings were conducted to assess the potential for hybrid formation as a result of cross-pollination. These hand pollinations included within and between species crosses (Table 3.2 & 3.3). Widely used standard hand pollination procedures are available and were used to manipulate crossing (Kearns & Inouye, 1993). At bud stage, flowers were secured with a mesh bag to prevent pollinator visitation (see Motsa *et al.,* 2017 for *Cyclopia* phenology). Once flowers were open, two flowers were used per treatment per plant. In order to account for potential autonomous-autogamy in *Cyclopia* (Koen *et al.,* 2020), flowers were emasculated before hand pollination was conducted (Fig. 3.2).

Table 3.2. Hand-pollination treatments, motivation for the use of the treatment, and procedure followed.

Treatment	Motivation	Procedure
Control	Natural levels of pollination.	Flowers were not bagged to allow for natural pollination. No pollen was added to flowers. Buds were marked at the same stage as all other treatments.
Testing for pollen-limitation	To determine whether plants are pollen or resource limited.	Pollen was added by hand to flowers open to natural levels of pollination.
Testing for autonomous autogamy (selfing)	Anther dehiscence in *Cyclopia* takes place before a flower opens, increasing the potential for the deposition of self-pollen (Koen *et al.*, 2020).	Flowers were bagged to prevent pollinator interaction. No pollen was added to flowers.
Testing for self-compatibility	Genetic potential for some genotypes to self for reproductive assurance (Koen *et al.*, 2020).	Pollen was collected from flowers of the same plant (self-pollination), as well as from nearby (~5-10 m) plants of the same species (cross within) and added by hand to flowers which were bagged.
Testing for compatibility among *Cyclopia* spp.	Threat of hybridization between cultivars and species growing in nearby wild populations.	Pollen was collected from a pollen donor in the cultivated site (*C. subternata* or *C. maculata*) and added by hand to flowers which were bagged. Plants in the cultivated site were selected at random for pollen donation.

Table 3.3. Hand-pollination treatments applied to each *Cyclopia* study species, with two flowers used per treatment per plant, unless otherwise stated.

Species	Cultivated/wild (n = number of plants)	Treatments applied (n = total number of flowers treated/replications)
C. subternata	Wild (12)	Pollen limitation (23) Cross within sp. (24) Self-pollination (23) Between spp. (cult. *C. maculata* pollen) (24) Autonomous autogamy (24)
C. intermedia	Wild (15)	Pollen limitation (29) Cross within sp. (28) Self-pollination (29) Between spp. (cult. *C. maculata* pollen) (28) Between spp. (cult. *C. subternata* pollen) (29) Autonomous autogamy (29)
C. genistoides	Wild (16)	Pollen limitation (31) Cross within sp. (31) Self-pollination (31) Between spp. (cult. *C. subternata* pollen) (31) Autonomous autogamy (31)
C. subternata	Cultivated (25)	Pollen limitation (>60) Selfing (autonomous autogamy) (>100)
C. maculata	Cultivated (26)	Pollen limitation (90) Selfing (autonomous autogamy) (>100)
C. genistoides	Cultivated (25)	Pollen limitation (>80) Selfing (autonomous autogamy) (>100)

Fig. 3.2. (A) Intact *Cyclopia* flower, (B) *Cyclopia* flower with one removed wing and keel petal to expose thestamens and pistil, (C) *Cyclopia* flower with one removed wing petal, one removed keel petal, and removed stamens, exposing only the pistil.

The pollen from *Cyclopia* flowers in a cultivated population was collected from opened or partially opened flowers and brushed onto the pistil of *Cyclopia* flowers in a nearby wild population. After hand pollination, the flowers were secured in a mesh bag again to prevent pollination and the release of hybrid seeds. On each plant, a flower not chosen for pollination in the same stage of development as the flowers chosen for hand-pollination was marked and acted as control, i.e., natural pollination. The successful fertilisation of one flower results in the formation of one pod. The data from hand-crossings were represented as the proportion of pods produced in relation to the number of flowers hand-pollinated, and the proportion of seed production in relation to the maximum number of seeds that could have possibly been produced (indicated by underdeveloped ovules and seed scars).

The hybrid seeds that developed from the hand-crossings were harvested at pod-maturation, indicated by the colour change from grey to brown (Motsa *et al.*, 2017). This ensured collection before pods opened to allow for accurate seed counting. The harvested hybrid seeds were then scarified using hot water treatment with a short dipping period of approximately 15 seconds (Mbangcolo *et al.,* 2013). This was done because *Cyclopia* seeds will not germinate without scarification (Sutcliffe & Whitehead, 1995). To test seed viability (and have genetic material for a separate study), seeds produced from between species crosses were sowed in a medium mix in seedling trays (Bester pers. comm. 2021) at the ARC on the 9th of March 2022, after seed harvest in December 2021.

3.3. Results

Pollinator movement between cultivated and wild Cyclopia *populations*

A total of 103 carpenter bees (*Xylocopa capitata*) were caught and marked in the cultivated site (cultivated-caught) and 29 carpenter bees were caught and marked in the wild site (wild-caught). Out of the 85 re-observations, 72 occurred in the cultivated site (Appendix 3 B). All the cultivated-caught bees that were re-observed were re-observed in their site of initial capture, besides for one individual (Fig. 3.3 & Appendix 3 B). The one cultivated-caught bee re-observed in the wild site was seen approximately 729 m from the site of capture (Fig. 3.3; Appendix 3 B & Table 3.4). There were 12 wild-caught bees re-observed in the wild site (Fig. 3.3 & Table 3.4), and 10 wild-caught bees re-observed in the cultivated site which was 683 m from the wild release site (Fig. 3.3 & Table 3.4).

The wild-caught bees were re-observed 384±232 m (mean ± SD) from the point of release, while all cultivated-caught bees, besides one individual, remained in their site of capture. Most re-observations occurred within one or two days of release, however the longest period between marking and observation was 26 days where observation occurred outside the site of release (cultivated site) with the bee foraging at a wild *Cyclopia subternata* plant (at 729 m from the release site).

Maximum flight distance and daily foraging range

There were ten individual carpenter bees (*Xylocopa capitata*) successfully tracked using radio-tagging, five in both the 2021 and 2022 study periods. The furthest distance observed was 282.8 m flown in the 2021 study period (Table 3.5 & Fig. 3.4) and 1194.2 m flown in the 2022 study period (Table 3.5 & Fig. 3.4). The daily observed home range size for tagged carpenter bees, determined using the Minimum Convex Polygon (MCP) method, was 6160±9216 m² for 2021 and 7951±10 953 m² for 2022. MCP revealed the largest home range to be 22 431 m² in the 2021 flowering season (Table 3.5). In the 2022 flowering season, the bee with the largest home range size was 23 893 m² (Table 3.5). The bee with the largest home range in 2021 was released in the cultivated site of the study where it began foraging on cultivated *Cyclopia* flowers (Appendix 3 C). Following this, the bee travelled over the apple orchard into a patch of wild *C. intermedia* (Fig. 3.4) where it began foraging and showing territorial behaviour on an *Aspalathus* plant (Appendix 3 C). The bee with the largest home range in 2022 was released in the cultivated site and subsequently found at its the nesting site 1194.2 m away (Appendix 3 C).

Pollen viability remained high even at 4.5 days old for all study species; *C. maculata* (56.5%), *C. subternata* (72.5%), and *C. intermedia* (68.5% at 4 days old, 80.5% at 5.5 days old; no 4.5 day samples) (Fig. 3.5). Pollen viability for all aged samples remained over 50% throughout.

Within and between species crosses

The flowers of all *Cyclopia* study species produced pods naturally, and at similar levels to open flowers to which pollen was added (Fig. 3.6 A). None of the *Cyclopia* species showed signs of autonomous autogamy (selfing), with zero pods produced in the absence of pollinators (Fig. 3.6 A). In *Cyclopia genistoides* and *C. intermedia* no pods or seeds were produced from self-compatibility testing, both within plant (self-pollination) and between conspecific plants (cross within) (Fig. 3.6 A&B). Additionally, no self-pollination pods were produced from *C. subternata* hand-pollinations, although one pod with seeds was produced from a cross between conspecific plants (cross within) (Fig. 3.6 A&B). The between species crosses that successfully produced pods was cultivated *C. maculata* x wild *C. subternata* and cultivated *C. subternata* x wild *C. genistoides* (Fig. 3.6 A).

Seed production varied greatly between the study species. *Cyclopia subternata* produced twice the ratio of seeds from natural pollination and pollen limitation compared to *C. intermedia*, with *C. genistoides* producing half that of *C. intermedia* (Fig. 3.6 B). *Cyclopia subternata* produced 50% of the maximum possible seed production through within conspecific crosses, while *C. intermedia* produced 30% and *C. genistoides* produced no seeds (Fig. 3.6 B). Seed production from within-plant (self-pollination) crosses was observed in *C. subternata* and *C. intermedia,* while *C. genistoides* produced no seeds (Fig. 3.6 B). A total of ten seeds were produced from between species crosses; four from two pods of maternal wild *C. subternata* and cultivated *C. maculata* pollen, and six from two pods of maternal wild *C. genistoides* and cultivated *C. subternata* pollen (Fig. 3.6 B). This indicates a between species pod production success rate of ~4%.

All seeds produced from *Cyclopia subternata* x *C. maculata* crosses germinated successfully (Fig. 3.7 A), while only 50% (3/6 seeds) of seeds from *C. genistoides* x *C. subternata* germinated successfully (Fig. 3.7 B).

Table 3.4. Numbers of bees marked for the mark-recapture experiment. Marked bee individuals were re-observed in either the natural area (wild re-observation) or in the cultivated area (cultivated re-observation). Individuals were re-observed rather than re-captured, increasing the opportunity for marked individuals to be counted more than once during the study period. Total re-observations are the total number of re-observations in each respective site (cultivated or wild).

Marking occasion	Colour	Capture site	Bees marked (female: male)	Wild re-observations	Cultivated re-observations	Total re-observations
25-26 Sep. 2021	Blue	Cultivated	28 (17: 11)	1	9	
5-7 Oct. 2021	Purple	Cultivated	48 (42: 6)	0	9	
19 Oct. 2021	White	Cultivated	27 (25: 2)	0	44	63
6-7 Oct. 2021	Orange	Wild	16 (1: 15)	7	10	
19-20 Oct 2021	Red	Wild	13 (4: 9)	5	0	22
Total			132	12	72	85
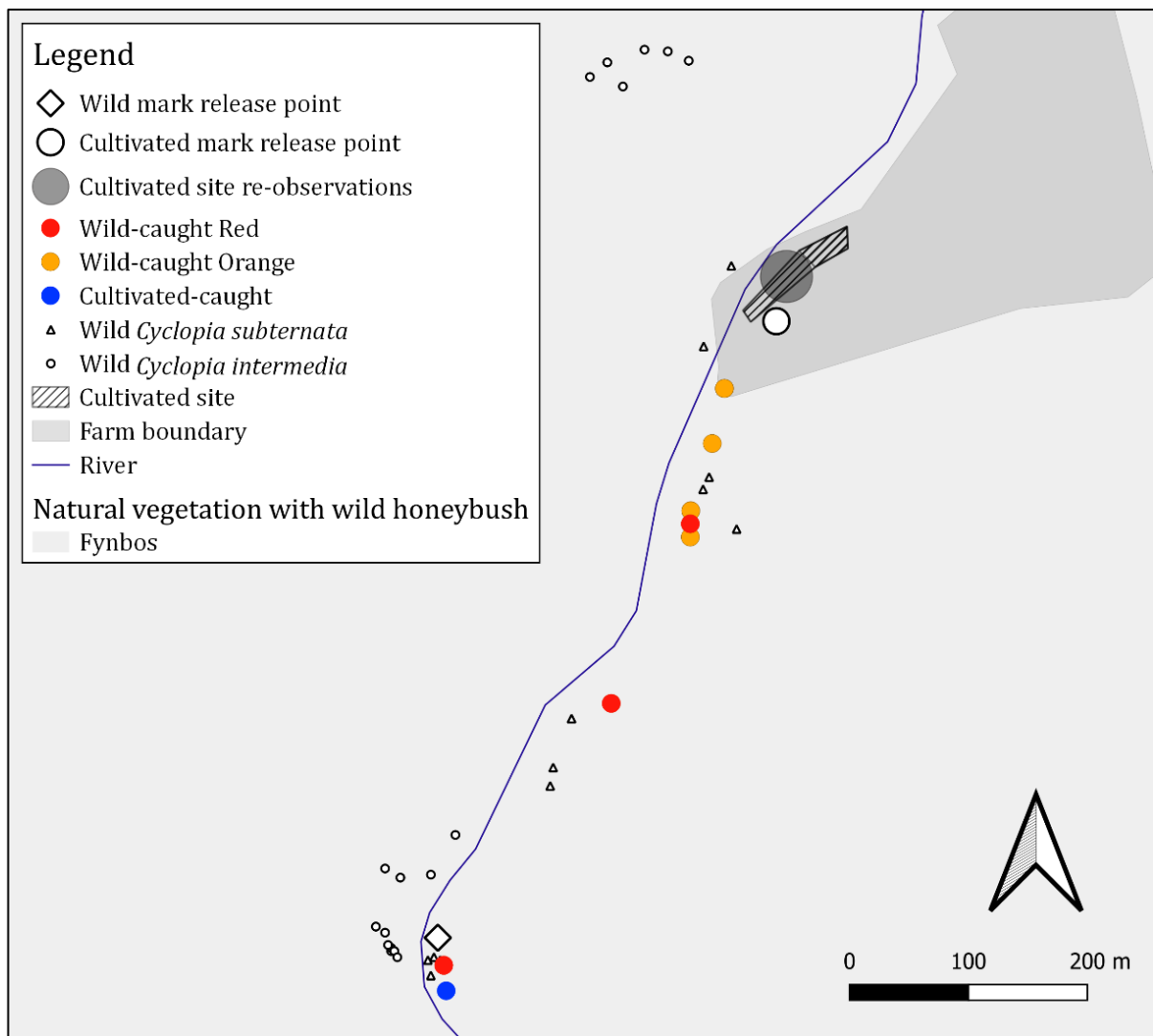
Fig. 3.3. Observation locations of the marked *Xylocopa capitata* re-observed in the wild site as well as in the cultivated site (represented by the enlarged grey dot labelled "Cultivated site re-observations" indicating wild-caught bee re-observations and cultivated-caught bee re-observations, where n = 10 and 62, respectively). Wild populations are indicated along and near the river, adjacent to the footpath utilized by observers.
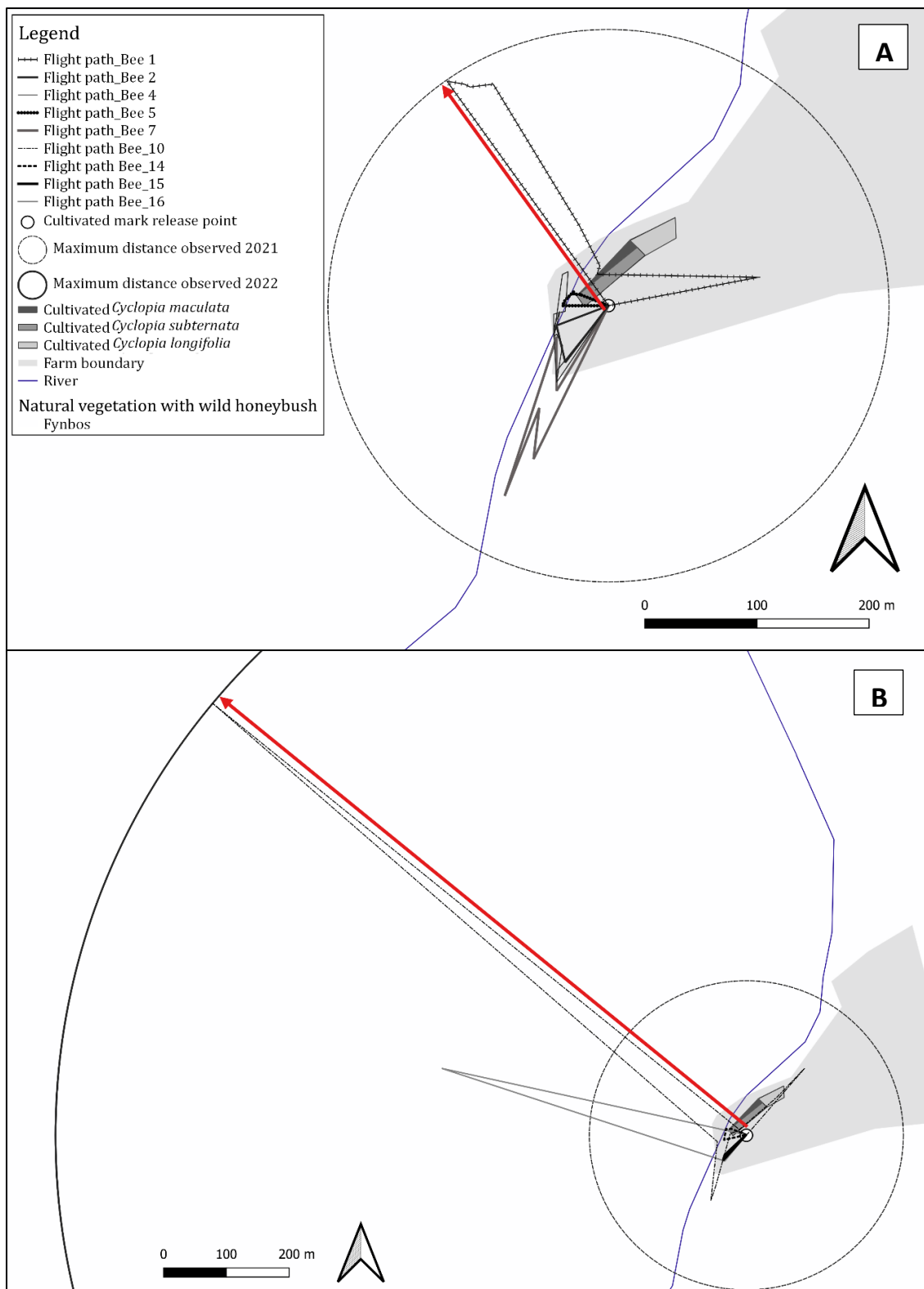
Fig. 3.4. Flight paths of radio-tagged carpenter bees (*Xylocopa capitata*) during the (A) 2021 flowering season and (B) 2022 flowering season in Twee Riviere. The red arrows indicate the longest distance flown away from the cultivated site by a radio-tagged carpenter bee, (A) 283 m and (B) 1194 m.

Table 3.5. Maximum foraging distance travelled from site of release, and home range size (calculated using the MCP method) for each radio-tagged carpenter bee in the 2021 and 2022 flowering seasons.

Bee ID	Maximum foraging distance (m)	Home range size (m²)
Bee_1	283	22431
Bee_2	73	1010
Bee_4	95	3017
Bee_5	41	329
Bee_7	226	4012
Bee_10	1194	23893
Bee_12	43	115
Bee_14	39	557
Bee_15	62	200
Bee_16	501	14991
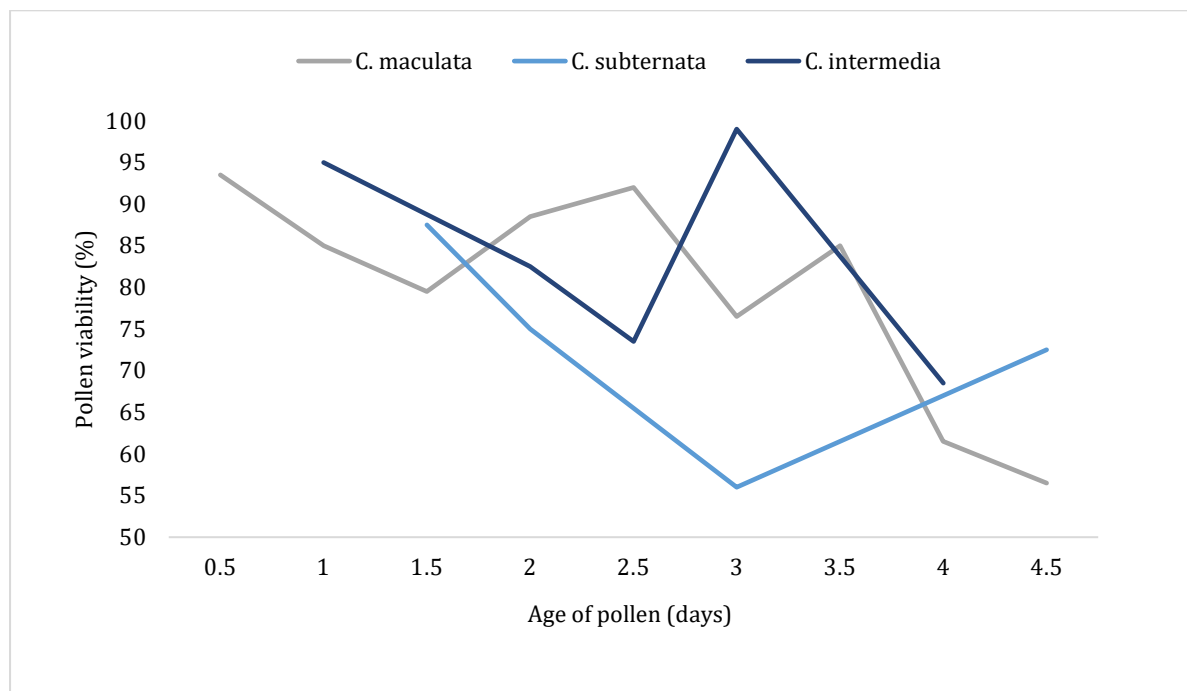
Fig. 3.5. Pollen viability of *Cyclopia maculata, C. subternata* and *C. intermedia* at various ages between half a day and 4.5 days old.
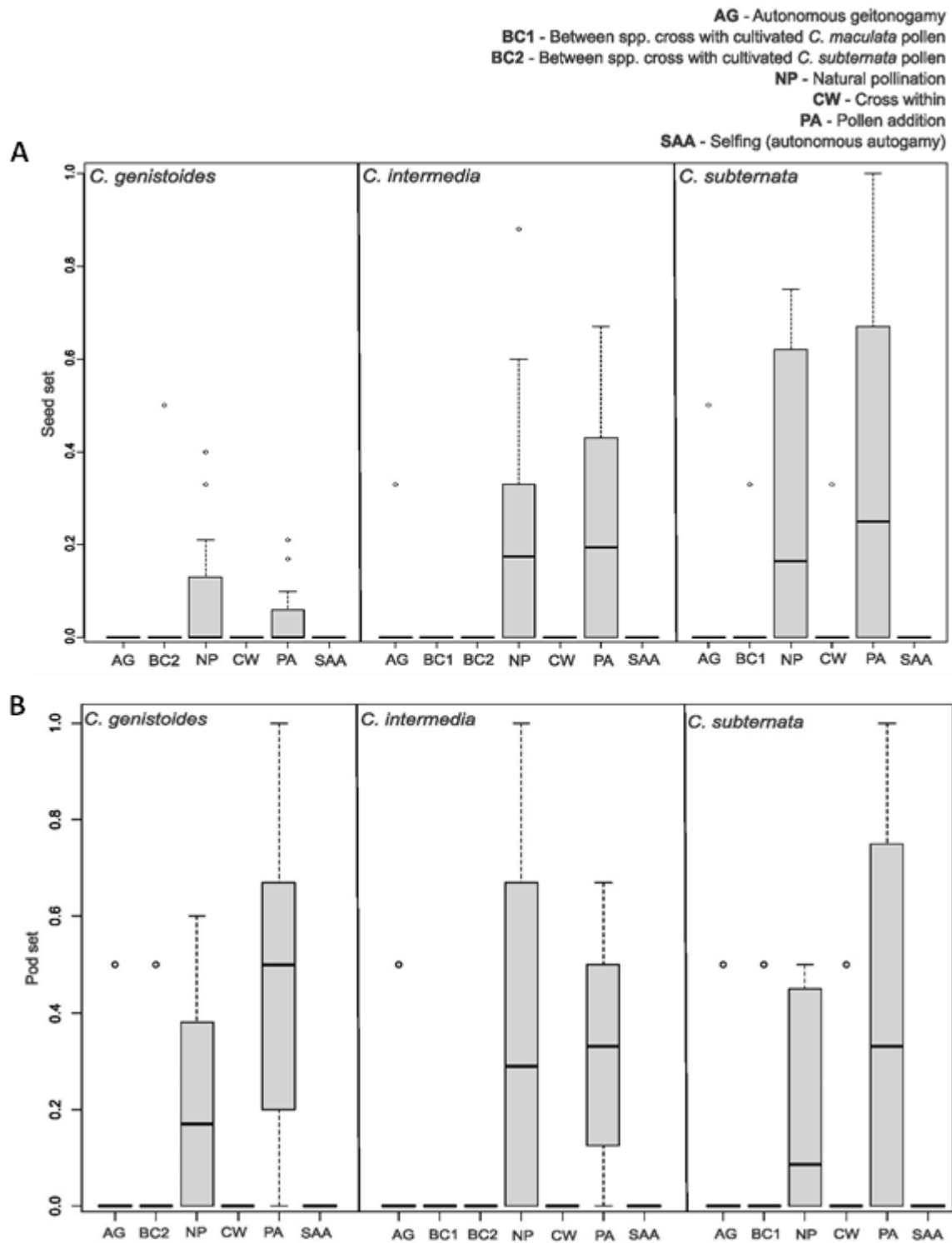
Fig. 3.6. (A) Proportion of seeds produced per pod from hand-pollination experiments, and (B) Proportion of pods produced from hand-pollinated flowers, in natural populations of three *Cyclopia* species. The thick line in each box represents the median, the box itself represents the interquartile range (IQR), and the whiskers extend the maximum and minimum values within 1.5 x IQR (white dots are the outliers).
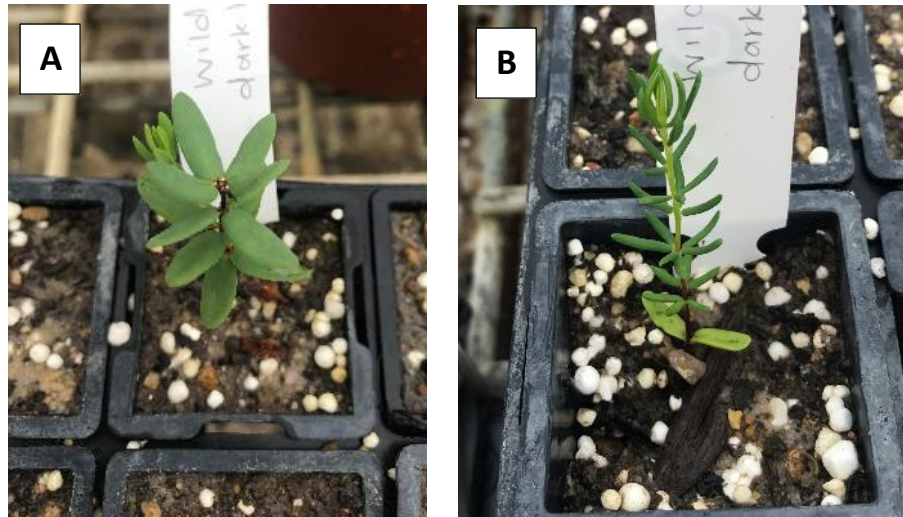
Fig. 3.7. (A) Seedling produced from maternal wild *C. subternata* x cultivated *C. maculata* cross, and (B) seedling produced from maternal wild *C. genistoides* x cultivated *C. subternata* cross.

3.4.   Discussion

Using mark-release-reobservation experiments I was able to determine regular movement of carpenter bee pollinators between the wild and cultivated *Cyclopia* sites, with up to 729 m travelled from the initial site of release. Movement between the wild and cultivated sites was also confirmed with radio-tagging and tracking efforts, to determine the daily home ranges of carpenter bees. A maximum daily home range of 23 893 m² was recorded, indicating utilization of both cultivated and wild sites. These patterns demonstrate between-site carpenter bee foraging of wild and cultivated *Cyclopia* plants, therefore necessitating the need to determine pollen longevity. Under field conditions, pollen remained viable for at least 4.5 days in all *Cyclopia* species under study, thus illustrating the potential for regular transfer of viable pollen between wild and cultivated *Cyclopia*. Hand-pollinations including within and between species crosses revealed the potential for hybrids to form.

Mark-release-reobservation efforts confirm the ability of carpenter bees to move between patches of wild and cultivated *Cyclopia* (between site movement was observed in 11 marked carpenter bees, i.e., 13%). Of the 11 re-observations of marked bees that travelled between the cultivated and wild sites, eight were male. Male solitary bees are suggested to have a higher potential for cross-pollination owing to their long-distance movements in search of mates (as observed in Dorchin *et al.,* 2012; Ne'eman *et al.*, 2006). The remainder of the re-observations (n=74; 53 female), occurred in the initial site of capture and release. Interestingly, this indicates high site fidelity particularly among female carpenter bees. Although, the one cultivated-caught bee re-observed in the wild site was female, therefore there is potential for

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between site movement from cultivated to wild that is not limited to male carpenter bees. During captures, no preference was given toward a gender, however 83 of the 103 bees captured and marked in the cultivated site were female, with 24 of the 29 bees captured and marked in the wild site being male. However, rather than assuming patterns of resource-use are sex-specific in carpenter bees, similarly to those recorded in hummingbirds (Maglianesi *et al.,* 2022), it is recommended that high between site movement from wild to cultivated is as a result of resource availability. Additionally, rather than relating limited between site movement observed in cultivated-caught bees (mostly female) to site fidelity, this was likely also as a result of forage availability in the cultivated site.

The smaller foraging ranges (256±362 m) observed in this study are an indication of daily movement rather than seasonal movement. Additionally, some radio-tagged bees were only tracked for a few hours following the loss of telemetry signal and thus only reflect a small part of their home range. Ten individual carpenter bees were successfully radio-tagged and tracked using radio telemetry. Of these, the mean daily home ranges of 0.6 ha and 0.8 ha in 2021 and 2022, respectively (6160 m² and 7951 m²), were similar to the daily home range size recorded of two radio-tagged *Bombus hortorum,* of 0.25 ha and 1.37 ha (Hagen *et al.,* 2011). In this study, six additional carpenter bees were successfully fitted with radio-transmitters, however, subsequently flew out of telemetry range following their release. Thus, it is important to note that the distances obtained are not the maximum distances of daily flight in *Xylocopa* spp., but rather the observed maximum distances. Maximum forage distances under field conditions is almost impossible to determine, since the rare long distance dispersal events are hard to find measure, thus observed maximum distances were calculated to present a more accurate illustration of spatial use. Nevertheless, the optimal forage theory, whereby bee foragers are predicted to remain in areas with high floral resources to meet their energy demands (Pyke, 1978), must be considered in this context. Honeybush farms provide a wealth of forage resources in a confined area, thus the likelihood of foragers expending more energy in search of additional forage, is unlikely. Site fidelity and flower constancy has been previously recorded in large solitary bees during mark-release-recapture experiments (Dorchin *et al.,* 2012; Bhattacharya *et al.,* 2003). Even so, I found that most radio-tagged individuals flew between wild and cultivated sites, foraging indiscriminately on *Cyclopia* species in both sites.

Carpenter bee pollinators may move short distances per day as shown through radio-tracking in this study, but over time can move much further, and duration of pollen viability is thus important. I show that *Cyclopia* pollen is viable for multiple days under field conditions, likely even beyond the period used in this study (4.5 days). This is important, since pollen longevity of many plant species is typically a few hours to a few days (Dafni & Firmage, 2000). Considering the low pollinator visitation rates previously observed in *Cyclopia* (see Chapter

2), the longevity of pollen may aid in ensuring effective pollination (Dafni & Firmage, 2000; Beardsell *et al.,* 1993), but also increases the probability of long-distance pollen-flow. Other factors influencing pollen longevity have been hypothesized, including pollen travel distance (Proctor, 1998), pollen competitive ability (Harder & Wilson*.,* 1994), and frequency of pollinator activity (James & Knox, 1993) which may all drive the long period pollen remains viable in *Cyclopia.* By relating the time pollen remains viable to the distance of pollinator movement, we can determine the distance at which hybridization is likely. Somanathan *et al.* (2019) found that large bees when displaced as far as 10 km from the nesting sites do return, although this distance is not expected to be achieved under natural circumstances. The data collected from mark-release-recapture in this study indicates movement of up to 680 m daily, and from radio-tagging indicates up to ~1.2 km daily. The data collected from radio-tagging does provide some guidance in determining the minimum distance that can be travelled with viable pollen. The movement of bees between sites as observed in the mark-release and radio-tagging aspect of the study, enables significant potential for pollen transfer and the initiation of gene flow in the genus *Cyclopia*. And while daily foraging ranges may be small in this study, the close proximity of wild *Cyclopia* to cultivated *Cyclopia* does enhance the likelihood of regular pollen flow at these short distances with a diminishing likelihood and frequency as the distance increases. And indeed, hand pollination did produce hybrids between species that have very minimal overlap in natural range.

All *Cyclopia* species studied here were unable to produce seed autogamously. Dichogamy, a common condition in angiosperms whereby the male and female reproductive organs do not reach sexual maturity simultaneously, thereby intercepting self-pollination (Lloyd & Web, 1986), may be present in *Cyclopia* to prevent self-autogamy. The lack of strong pollen limitation was an interesting result, particularly since many pollinators were available, in addition to the accessibility of compatible pollen with an abundance floral resources. Thus, this may be a result of inadequate pollen receipt, perhaps as the technique used for hand-pollination was not as abrasive as with pollinator interaction, stimulating pollen-reception. Importantly, between species crosses produced hybrid seeds, which were subsequently planted and had a 70% germination rate. This illustrates the potential of *Cyclopia* to form hybrids as a result of pollinator mediated pollen movement between cultivated and wild sites. A very low pod production success rate of ~4% was obtained from between-species crosses, similar to that of de Lange & von Mollendorf (2006) who obtained a ~2% success rate. This indicates a low possibility of hybrid production in the wild between the species in this study and those of de Lange & von Mollendorf (2006), particularly without human intervention. However, there are 23 species of *Cyclopia,* between which certain cross compatibilities may be much higher, increasing the likelihood and threat of genetic contamination. Further

research into the ploidy levels of *Cyclopia* in addition to crossing experiments between different species and population pairs, and therefore compatibility between and within species, is urgently needed to produce a detailed planting protocol.

This study provides important baseline information regarding the pollinator movement of the commercially important and native *Cyclopia* species in South Africa. The pollinator tracking efforts provide clear evidence of regular movement across sites of planted and wild *Cyclopia*. This further illustrates the ability of pollinators to move between different species of *Cyclopia* as well as between those distant and distinct populations, thus demonstrating the potential for genetic contamination through pollen transfer in *Cyclopia.* Although the risk of genetic contamination between cultivated sites and wild *Cyclopia* populations will never be completely zero, due to occasional long-distance dispersal events, a protocol aimed at minimizing the risk should be considered. This should take into account pollinator movement, flowering phenology, seed dispersal, ploidy, and the origin of cultivated material. Pollen-flow is key, since the transfer of pollen between two sites will initiate cross-contamination. The maximum distance of 10 km identified in previous studies is not a practical "safe-distance" guideline due to the expansive native range of *Cyclopia*, covering a large portion of the fynbos biome in the Western and Eastern Cape provinces (Schutte, 1997), where *Cyclopia* is also cultivated. Distances travelled by bees as observed in this study can aid to inform the risks of planting at different distances from wild populations. The results of this study indicate that carpenter bees rarely travel beyond ~1.5 km daily, however owing to the potential for long-dispersal events, such as in the case of young bees that have been expelled from their natal nests (Scholtz *et al.,* 2021), travel distances are likely much further and the ~1.5 km potential daily distance should be applied very conservatively. Furthermore, the longevity of *Cyclopia* pollen grains creates a high risk of pollen transfer between *Cyclopia* planted within a few kilometres from wild populations, except if locally indigenous material is used. Factors such as these should be explored in the development of a safe-planting guideline not only for *Cyclopia*, but for all indigenous species with commercial value.

3.5.   References

Arias, D. & Rieseberg, L. 1994. Gene flow between cultivated and wild sunflowers. *Theoretical and Applied Genetics*, *89*, 655–660.

Barnado, N. 2013. *Sustainable harvesting of wild populations of Cyclopia intermedia in Kouga, Eastern Cape, South Africa* (M.Sc. Dissertation, Port Elizabeth: Nelson Mandela Metropolitan University).

Bartsch, D., Lehnen, M., Clegg, J., Pohl-Orf, M., Schuphan, I. & Ellstrand, N. 1999. Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. *Molecular Ecology*, 8, 1733–1741.

Beardsell, D., Knox, R., & Williams, E. 1993. Breeding system and reproductive success of *Thryptomene calycina* (Myrtaceae). *Australian Journal of Botany, 41*, 333–353.

Bester, C. 2013. A model for commercialisation of honeybush tea, an indigenous crop. *Acta Horticulturae, 1007*, 889–894.

Bhattacharya, M., Primack, R. & Gerwein, J. 2003. Are roads and railroads barriers to bumblebee movement in a temperate suburban conservation area? *Biological Conservation, 109*, 37–45.

Burke, J., Gardner, K. & Rieseberg, L. 2002. The potential for gene flow between cultivated and wild sunflower (*Helianthus annuus*) in the United States. *American Journal of Botany*, 89, 1550–1552.

Campbell, D. & Waser, N. 2001. Genotype-by-environment interaction and the fitness of plant hybrids in the wild. *Evolution, 55*, 669–676.

Cane, J. 1987. Estimation of bee size using intertegular span (Apoidea). *Journal of the Kansas Entomological Society, 60*, 145–147.

Cornille, A., Gladieux, P. & Giraud, T. 2013. Crop-to-wild gene flow and spatial genetic structure in the closest wild relatives of the cultivated apple. *Evolutionary Applications, 6*, 737–748.

de Lange, H. & Von Mollendorf, L. 2006. Current position of plant improvement in the honeybush tea industry. *South African Honeybush Tea Association Newsletter, 14*, 8–15.

Delplancke, M., Alvarez, N., Espíndola, A., Joly, H., Benoit, L., Brouck, E. & Arrigo, N. 2011. Gene flow among wild and domesticated almond species: insights from chloroplast and nuclear markers. *Evolutionary Applications, 5*, 317–329.

Demchik, S. & Day, T. 1996. Effect of enhanced UV_B radiation on pollen quantity, quality, and seed yield in *Brassica rapa* (Brassicaceae). *American Journal of Botany, 83,* 573–579.

De Schawe, C., Durka, W., Tscharntke, T., Hensen, I. & Kessler, M. 2013. Gene flow and genetic diversity in cultivated and wild cacao (*Theobroma cacao*) in Bolivia. *American Journal of Botany, 100*, 2271–2279.

Dafni, A., & Firmage, D. 2000. Pollen viability and longevity: Practical, ecological and evolutionary implications. *Pollen and Pollination,* 113–132.

Dorchin, A., Filin, I., Izhaki, I. & Dafni, A. 2012. Movement patterns of solitary bees in a threatened fragmented habitat. *Apidologie, 44*, 90–99.

Eardley, C. 1983. A taxonomic revision of the genus *Xylocopa* Latreille (Hymenoptera: Anthophoridae) in southern Africa. *Entomology Memoir, 58,* 1–67.

Eddy, W. 1977. A new convex hull algorithm for planar sets. *ACM Transactions on Mathematical Software, 3*, 398–403.

Ellstrand, N. 1992. Gene flow by pollen: implications for plant conservation genetics. *Oikos, 63*, 77.

Ellstrand, N. & Elam, D. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics, 24*, 217–242.

Gathmann, A. & Tscharntke, T. 2002. Foraging ranges of solitary bees. *Journal of Animal Ecology, 71*, 757–764.

Greenleaf, S., Williams, N., Winfree, R. & Kremen, C. 2007. Bee foraging ranges and their relationship to body size. *Oecologia, 153*, 589–596.

Grobler, B. & Campbell, E. 2020. Pollinator activity and the fecundity of a rare and highly threatened honeybush species along a highway in the Cape Floristic Region. *International Journal of Plant Sciences, 181*, 581–593.

Hagen, M., Wikelski, M. & Kissling, W. 2011. Space use of bumblebees (*Bombus* spp.) revealed by radio-tracking. *PLoS ONE, 6*, e19997.

Hammer, K. & Teklu, Y. 2008. Plant genetic resources: selected issues from genetic erosion to genetic engineering. *Journal of Agriculture and Rural Development in the Tropics and Subtropics, 109*, 15–50.

Harder, L. & Wilson, W. 1994. Floral evolution and male reproductive success: Optimal dispensing schedules for pollen dispersal by animal-pollinated plants. *Evolutionary Ecology, 8*, 542–559.

Heslop-Harrison, J. & Heslop-Harrison, Y. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. *Stain Technology, 45*, 115–120.

Heslop-Harrison, J., Heslop-Harrison, Y. & Shivanna, K. 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. *Theoretical and Applied Genetics, 67*, 367–375.

Hilton-Taylor, C. 1996. Red Data List of southern African plants. 1. corrections and additions. *Bothalia, 26*, 177–182.

James, E. & Knox, R. 1993. Reproductive-biology of the Australian species of the genus *Pandorea* (Bignoniaceae). *Australian Journal of Botany, 41*, 611.

Jenczewski, E., Prosperi, J. & Ronfort, J. 1999. Evidence for gene flow between wild and cultivated *Medicago sativa* (Leguminosae) based on allozyme markers and quantitative traits. *American Journal of Botany, 86*, 677–687.

Joubert, E., Joubert, M., Bester, C., de Beer, D. & De Lange, J. 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets — The catalytic and supporting role of research. *South African Journal of Botany, 77*, 887–907.

Keasar, T. 2010. Large carpenter bees as agricultural pollinators. *Psyche: A Journal of Entomology, 2010*, 1–7.

Kearns, C. & Inouye, D. 1993. *Techniques for pollination biologists*. University Press of Colorado, Niwot.

Koen, J. 2020. *Reproductive anatomy, sexual compatibility, pollen viability and embryo rescue of* Cyclopia *species* (PhD thesis, Tshwane University of Technology).

Koen, J., Slabbert, M., Booyse, M. & Bester, C. 2020. Honeybush (*Cyclopia* spp.) anther-stigma distance and intraspecies cross compatibilities. *South African Journal of Plant and Soil.*

Koen, J., Slabbert, M., Booyse, M. & Bester, C. 2019. Honeybush (*Cyclopia* spp.) pollen viability and surface morphology. *South African Journal of Botany, 128*, 167–173.

Lloyd, D. & Webb, C. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand Journal of Botany, 24*, 135–162.

Louw, G. & Nicolson, S. 1983. Thermal, energetic and nutritional considerations in foraging and reproduction of the carpenter bee *Xylocopa capitata*. *Journal of the Entomological Society of South Africa, 46,* 227–240.

Maglianesi, M., Maruyama, P., Temeles, E., Schleuning, M., Zanata, T., Sazima, M. & Dalsgaard, B. 2022. Behavioural and morphological traits influence sex-specific floral resource use by hummingbirds. *Journal of Animal Ecology.*

Mbangcolo, M., Reinten, E. & Agenbag, G. 2013. Effect of different seed treatments on germination of *Cyclopia* spp. seeds. *AGRIPROBE Elsenburg Journal, 10*, 22–24.

McGregor, G. 2017. Industry review: an overview of the honeybush industry. Department of Environmental Affairs and Development Planning, Cape Town.

McKay, D. & Blumberg, J. 2006. A review of the bioactivity of South African herbal teas: rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*). *Phytotherapy Research, 21*, 1–16.

Motsa, M., Bester, C., Slabbert, M., Hannweg, K. & Booyse, M. 2018. Flow cytometry: a quick method to determine ploidy levels in honeybush (*Cyclopia* spp.). *Genetic Resources and Crop Evolution, 65*, 1711–1724.

Motsa, M., Slabbert, M., Bester, C. & Ngwenya, M. 2017. Phenology of honeybush (*Cyclopia genistoides* and *C. subternata*) genotypes. *South African Journal of Botany, 110*, 57–67.

Mucina, L. & Rutherford, M. 2011. *The vegetation of South Africa, Lesotho and Swaziland*. Pretoria: South African National Biodiversity Institute, 53–219.

Ne'eman, G., Shavit, O., Shaltiel, L. & Shmida, A. 2006. Foraging by male and female solitary bees with implications for pollination. *Journal of Insect Behaviour, 19*, 383–401.

Osborne, J., Loxdale, H. & Woiwod, I. 2008. *Chapter 2 Monitoring insect dispersal: methods and approaches in Dispersal ecology*. Cambridge: Cambridge University Press, 24–49.

Osborne, J. & Williams, I. 2001. Site constancy of bumble bees in an experimentally patchy habitat. *Agriculture, Ecosystems & Environment, 83*, 129–141.

Pasquet, R., Peltier, A., Hufford, M., Oudin, E., Saulnier, J., Paul, L., Knudsen, J., Herren, H. & Gepts, P. 2008. Long-distance pollen flow assessment through evaluation of pollinator foraging range suggests transgene escape distances. *Proceedings of the National Academy of Sciences, 105*, 13456–13461.

Petit, C., Bretagnolle, F. & Felber, F. 1999. Evolutionary consequences of diploid–polyploid hybrid zones in wild species. *Trends in Ecology & Evolution, 14*, 306–311.

Pinillos, V. & Cuevas, J. 2008. Standardization of the fluorochromatic reaction test to assess pollen viability. *Biotechnic & Histochemistry, 83*, 15–21.

Potts, A. 2017. Genetic risk and the transition to cultivation in Cape endemic crops—The example of honeybush (*Cyclopia*)? *South African Journal of Botany, 110*, 52–56.

Proctor, H. 1998. Effect of pollen age on fruit set, fruit weight, and seed set in three orchid species. *Canadian Journal of Botany, 76*, 420–427.

Pyke, G. 1978. Optimal foraging: Movement patterns of bumblebees between inflorescences. *Theoretical Population Biology, 13*, 72–98.

QGIS Development Team. 2021. *QGIS Geographic Information System*. Open Source Geospatial Foundation Project. http://qgis.osgeo.org.

Reinten, E. & Coetzee, J. 2002. *Commercialization of South African indigenous crops: aspects of research and cultivation of products*. In: Janick, J., Whipkey, A. (Eds.), Trends in New Crops and New Uses. ASHS Press, Alexandria, VA.

Roubik, D. 1992. *Ecology and natural history of tropical bees*. Cambridge: Cambridge University Press.

Scholtz, C., Scholtz, J. & Klerk, D. 2021. *Pollinators, Predators & Parasites: The ecological roles of insects in Southern Africa*. Struik Nature, an imprint of Penguin Random House South Africa, Cape Town.

Schutte, A. 1997. Systematics of the genus *Cyclopia* Vent. (Fabaceae, Podalyrieae). *Edinburgh Journal of Botany, 54*, 125–170.

Shivanna, K. & Heslop-Harrison, J. 1981. Membrane state and pollen viability. *Annals of Botany, 47*, 759–770.

Slabbert, E., Malgas, R., Veldtman, R. & Addison, P. 2019. Honeybush (*Cyclopia* spp.) phenology and associated arthropod diversity in the Overberg region, South Africa. *Bothalia, 49*, 1–13.

Somanathan, H., Saryan, P. & Balamurali, G. 2019. Foraging strategies and physiological adaptations in large carpenter bees. *Journal of Comparative Physiology A, 205*, 387–398.

Song, Z., Lu, B., Zhu, Y. & Chen, J. 2003. Gene flow from cultivated rice to the wild species *Oryza rufipogon* under experimental field conditions. *New Phytologist, 157*, 657–665.

Sutcliffe, M. & Whitehead, C. 1995. Role of ethylene and short-chain saturated fatty acids in the smoke-stimulated germination of *Cyclopia* seed. *Journal of Plant Physiology, 145*, 271–276.

Ureta, M., Carrera, A., Cantamutto, M. & Poverene, M. 2008. Gene flow among wild and cultivated sunflower, *Helianthus annuus* in Argentina. *Agriculture, Ecosystems & Environment, 123*, 343–349.

Van Wyk, A. & Prinsloo, G. 2018. Medicinal plant harvesting, sustainability and cultivation in South Africa. *Biological Conservation, 227*, 335–342.

Wolf, D., Takebayashi, N. & Rieseberg, L. 2001. Predicting the risk of extinction through hybridization. *Conservation Biology, 15*, 1039–1053.

Zhou, H., Zhao, P. & Woeste, K. & Zhang, S. 2020. Gene flow among wild and cultivated common walnut (*Juglans regia*) trees in the Qinling Mountains revealed by microsatellite markers. *Journal of Forestry Research, 32,* 2189–2201.

Zurbuchen, A., Landert, L., Klaiber, J., Müller, A., Hein, S. & Dorn, S. 2010. Maximum foraging ranges in solitary bees: only few individuals have the capability to cover long foraging distances. *Biological Conservation, 143*, 669–676.

Chapter 4 : A protocol to guide planting of indigenous plant species within their native range: South African honeybush as a case study

Abstract

Indigenous plants with commercial value are often moved within their native range for horticulture, floriculture, and agriculture. This creates the potential for gene flow between different species and genetic lineages within species, which could have negative consequences for wild populations. Gene flow between cultivated populations and their wild relatives through cross-pollination has been widely studied, illustrating the potential for genetic contamination of native isolated plant populations in addition to hybridization between species. Honeybush, *Cyclopia* Vent.*,* is an endemic genus in the fynbos biome with commercial value in the tea industry of South Africa at risk of genetic contamination. There are six *Cyclopia* species recognized for their economic value, which are moved across their native range in the Western and Eastern Cape provinces of South Africa for cultivation. I present a planting protocol to ensure the genetic integrity and conservation of indigenous flora using *Cyclopia* as a case study. This protocol considers the barriers of the native range, flowering phenology, ploidy levels, pollinator movement, seed dispersal, harvest time and the origin of cultivated genetic material. These barriers were identified and refined through workshops, facilitating participative discussion between local and international academics, researchers and managers in addition to industry members. Four barriers were incorporated into the final protocol, including range, ploidy, seed dispersal and pollen-flow distances, and seed source, while the remaining barriers were omitted with sufficient rationale. These barriers covered risk assessment and management, with the level of risk quantified into three categories: low, moderate and high. This protocol provides an important guideline for planting not only *Cyclopia,* but also other indigenous crops planted within the native range in other parts of the world.

4.1. Introduction

Gene flow between cultivated populations and their wild relatives has been widely studied, revealing the potential for genetic contamination in many economically valuable species (e.g., Bartsch *et al.,* 1999; Jenczewski *et al.,* 1999; Burke *et al.,* 2002; Song *et al.,* 2003; Ureta *et al.,* 2008; Delplancke *et al.,* 2011; De Schawe *et al.,* 2013; Cornille *et al.,* 2013; Zhou *et al.,* 2020). Additionally, of the world's thirteen most important crops, 12 have shown evidence of hybridization with wild relatives (Ellstrand *et al.*, 1999). Since the increase in cultivation of

transgenic crops, gene flow between crops and their wild relatives is receiving increased attention (Snow & Morán-Palma, 1997; Ellstrand, 2001).

The consequences of gene flow, and the resulting genetic contamination, between cultivated and wild plants provide challenges for conservation. Pollen-transfer between agricultural crops and naturally co-occurring plants can result in hybridization when the species are closely related (Hancock *et al.,* 1996; Ellstrand *et al.,* 1999). Hybridization may result in reduced genetic distinctiveness between populations (Wolf *et al.,* 2001; Campbell & Waser, 2001). Furthermore, genetic assimilation occurs when genetic material from crop plants replaces those in the wild (Wolf *et al.,* 2001), a direct consequence of gene flow causing reduced genetic diversity of wild populations. Moreover, genetic contamination through hybridization (and possibly followed by introgression) is not only problematic in transgenic crops, but between indigenous cultivated species and their naturally occurring wild relatives.

There are many plant species cultivated in their native range for commercial profit. In South Africa, there is a wealth of biodiversity with an abundance of indigenous vegetables, medicinal plants and floricultural crop species of commercial importance (Reinten & Coetzee, 2002; Jansen Van Rensburg *et al.,* 2007). The global export of these indigenous crops is important for the economy (Reinten & Coetzee, 2002). However, cultivation of indigenous plants might be controversial as the effects of extensive cultivation of indigenous plants may outweigh the benefits (Van Wyk & Prinsloo, 2018). One of the challenges of indigenous plant cultivation is the potential for genetic contamination of local conspecifics and congenerics through gene flow (Delplancke *et al.*, 2011; Zhou *et al.*, 2020). This would be particularly detrimental for distant and distinct local populations.

Genetic contamination has been evident in indigenous plant species in South Africa (Hawkins *et al.*, 2011; Macqueen & Potts, 2018; Bello *et al.*, 2018). Macqueen & Potts (2018) confirmed hybridization between two fynbos species, the locally-native *Protea eximia* and a species, *P. susannae* moved outside the natural range. Additionally, the effects of genetic contamination have been identified in *Aspalathus linearis* "rooibos", a species harvested for tea (Malgas *et al.*, 2010; Hawkins *et al.*, 2011). These studies demonstrate the threat that gene flow between different species, and gene flow within species between genetically distinct populations, poses on the genetic integrity of local plant populations.

Gene flow may pose a threat to honeybush *Cyclopia* Vent., cultivated in its native range in South Africa. *Cyclopia* is an endemic genus in the fynbos biome with commercial value in the tea industry of South Africa (Joubert *et al.,* 2011). There are 23 species in the genus *Cyclopia* of which two are extinct (Hilton-Taylor, 1996). Six *Cyclopia* species are recognized for their economic value, these include *Cyclopia genistoides* (L.) R. Br*, C. intermedia* E. Mey*, C.*

maculata (Andrews) Kies*, C. subternata* Vogel*, C. sessiliflora* Eckl. & Zeyh.*,* and *C. longifolia* Vogel (Joubert *et al.*, 2011). *Cyclopia* is native to the Western and Eastern Cape provinces, in the fynbos biome of South Africa, where it is also cultivated (Schutte, 1997). The tea is marketed both locally and internationally on an increasing scale (Bester, 2013). Cultivation has resulted in the six commercially important species being moved across the range, in some cases beyond the natural distribution of the species (Joubert *et al.,* 2011). This enables the potential for gene flow between species, potentially resulting in hybridization (Wolf *et al.,* 2001), as well as within species of localised genotypes, potentially resulting in genetic erosion and loss of distinctiveness (Campbell & Waser, 2001).

There is a need to develop guidelines to limit gene flow, and the resulting consequences arising from genetic contamination, between indigenous cultivated plant species and their wild relatives. This can be done using a risk analysis, taking into consideration (1) risk assessment, (2) risk management, and (3) risk communication (Kumschick *et al.,* 2020; Datta *et al.,* 2020). These key components guide the development of efficient management strategies (Kumshick *et al.,* 2020). By identifying and implementing barriers to gene flow, the safe planting of indigenous agricultural flora can commence with minimized threat of genetic contamination. Temporal isolation by utilising flowering phenology can be used as a barrier to prevent gene flow. Indeed, pollen-mediated gene flow was significantly reduced in maize with increased temporal isolation (Halsey *et al.,* 2005; Palaudelmas *et al.,* 2008). However, these studies consider temporally distant sowing times in a crop that is harvested on an annual basis. Cultivated *Cyclopia* plants live for approximately 7–10 years (Joubert *et al.,* 2011) and temporal isolation is therefore unlikely to be a practical barrier. Additionally, spatial, or distance isolation, is a gene flow barrier commonly used to reduce unwanted pollen-transfer. This simply refers to the increased distance between genetically modified crops, herbicide-resistant crops, cultivars or cultivated plants and their wild relatives. This facilitates the reduction of transgene escape and hybridization (Scheffler *et al,* 1993; Scheffler *et al.,* 1995; Rong *et al.,* 2006). In this sense, certain safe distances or "isolation distances" have been identified for sunflowers (beyond 1000 m (Arias & Rieseberg, 1994)), and maize (50 m (Sanvido *et al.,* 2008)). In terms of the potential for genetic contamination in *Cyclopia*, spatial isolation may present a feasible gene flow barrier. Moreover, other factors including the native range of the genus, the compatibility of species crosses within the genus by considering ploidy levels, the harvest time of the commercially important species, and the origin of cultivated plants need to be considered.

Cyclopia is an ideal study system owing to the expansive range of the genus, and the increasing commercial importance (Joubert *et al.,* 2011), and since *Cyclopia* is cultivated in the native range, the potential for gene flow between and within species is highly probable

(Potts, 2017). Furthermore, as honeybush tea is still an emerging product which has not yet reached its full potential, there is still time to implement preventative measures against extensive genetic contamination. Therefore, here I aim to develop a protocol guiding the planting of *Cyclopia* to minimize the potential for gene flow between cultivated and native *Cyclopia* populations.

4.2.   Methods

4.2.1. *Preliminary planting protocol*

A preliminary planting protocol was designed in the form of a decision tree, intended for the use by honeybush farmers, aimed at reducing the potential for gene flow and genetic contamination of *Cyclopia.* This protocol was based on the data collected from pollinator observations, pollinator tracking and hand-pollination experiments (see Chapter 2 & 3). Based on this data, risk assessment and management barriers of genetic contamination were identified and scrutinised in relation to their efficacy in a planting protocol. The risk assessment barriers included the (1) natural distribution (i.e., range), (2) flowering phenology, (3) ploidy levels and (4) seed dispersal, while the risk management barriers considered; (5) the distance at which *Cyclopia* can be safely planted in relation to wild *Cyclopia* populations according to gene flow through pollen transfer, as well as (6) harvesting time and (7) seed source. After identifying potential barriers, the level of risk of genetic contamination could be determined based on the answer to a question designed for each of the barriers (Appendix 4 A).

4.2.2. *Workshop with academics and conservation practitioners*

The preliminary protocol was presented through a workshop at an international conference (International Mediterranean Ecosystems Conference / Fynbos Forum 2022) for refinement. The aim of the protocol was to minimize the potential for gene flow between cultivated and native *Cyclopia* populations. Participative discussion was facilitated in order to ensure each barrier was effectively discussed. Participants ($n = 25$) consisted of local and international academics, researchers, and managers, working in different capacities with varied experience.

After a brief introductory presentation entailing background information and motivation for the planting protocol, the preliminary protocol was presented. The participants were encouraged to scrutinise each barrier and suggest additional barriers. Round table discussions were held based on the seven barriers identified prior to the workshop. The discussions were taped by

the conference organisers, shared with online participants and later made available to the conference attendees through a file-sharing portal. The input was used to refine the protocol.

4.2.3. _Workshop with the honeybush tea industry_

The refined protocol was then presented to industry represented by the members of the South African Honeybush Tea Association (SAHTA) Annual General Meeting. The meeting attendees ($n = 27$) consisted of honeybush farmers, processors, researchers, packagers and Western Cape Department of Agriculture government officials. A brief introduction on the background and motivation for the planting protocol was presented, followed by a detailed breakdown of each of the selected barriers. The attendees were then invited to share their opinions on the viability of each barrier, and of the planting protocol in its entirety. Round table discussions were facilitated and used to refine the protocol.

4.3.   Results and discussion

Seven barriers were considered for the protocol to limit genetic outcrossing between wild and cultivated _Cyclopia_ (Appendix 4 A). Of these, five barriers are included in the protocol and their risks quantified (Fig. 4.1). These barriers align with a risk analysis including the basic steps thereof, i.e., risk assessment, risk management, and risk communication (Kumschick _et al.,_ 2020). Risk assessment involves the likelihood and consequence of gene flow occurring, while risk management provides solutions for minimizing genetic outcrossing (Kumschick _et al.,_ 2020). Thus, risk assessment barriers identified for minimizing gene flow between cultivated and wild populations include (1) the native range of the species concerned, (2) the flowering phenology, and (3) the ploidy levels. In terms of risk management, the following is included; the planting distance in relation to (4) seed dispersal distances and (5) pollen-transfer distances, (6) the time of harvest, and (7) the seed source. These barriers are discussed below. Two of these are unlikely to act as barriers (flowering phenology and harvest time) and were therefore not included in the planting protocol, and the reasons for this are discussed below.
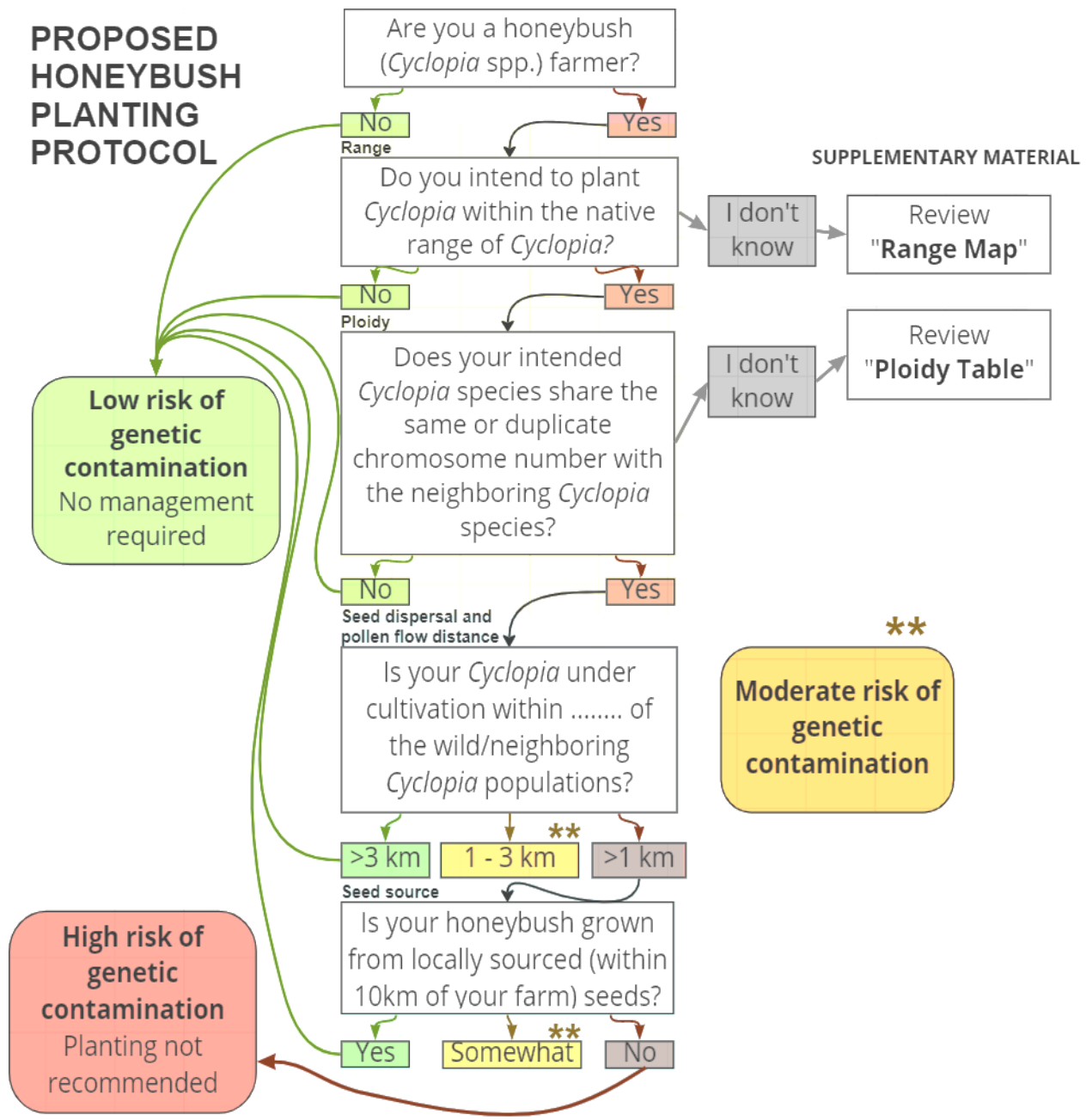
Fig. 4.1. Protocol guiding the planting of indigenous agricultural flora to minimize genetic outcrossing to nearby wild populations.

4.3.1. Range

Whether the farmer is intending to plant *Cyclopia* in the native range is important to consider, since if not, the protocol does not apply. Thus, the risk of genetic contamination will be low (Fig. 4.1). However, *Cyclopia* is mostly cultivated in the native range (Joubert *et al.,* 2011), where the climate and soil are most suitable for optimal growth, thus creating a higher risk for genetic contamination to occur (Fig. 4.1). Additionally, while six *Cyclopia* species hold the highest commercial value (Joubert *et al.,* 2011), there is potential for other species to become commercialized. This increases the potential for gene flow to occur, with species being moved across the range for cultivation. Furthermore, range sizes differ between *Cyclopia* species, from one population for the threatened *C. longifolia* and *C. pubescens,* to *C. intermedia* that occurs throughout most of the Cape Floristic Kingdom (Schutte, 1997). The highly localised species should be carefully considered. There are numerous threatened *Cyclopia* species (Raimondo *et al.,* 2009), and planting alongside these is an important aspect to consider with the consequences of gene flow from cultivated plants being potentially detrimental.

There is significant overlap between *Cyclopia* species across the range (Fig. 4.2), and extensive mapping of wild *Cyclopia* populations is of importance to ensure the minimization of genetic contamination. INaturalist and GBIF data points are readily available (Fig. 4.2), however these only consider the populations accessible by people (i.e., along hiking paths, in public areas, etc.). Nevertheless, this does aid to delineate species ranges to some extent and thus outlining the areas where native populations are likely to occur. These areas will be of higher genetic-contamination risk, thus validating range as a barrier. Furthermore, it was agreed in both workshops that range made an efficient barrier. An "I don't know" option is included in the protocol for farmers who are not aware of the native range of *Cyclopia* species and will thus be referred to a range map (Fig. 4.1 & Fig. 4.2).
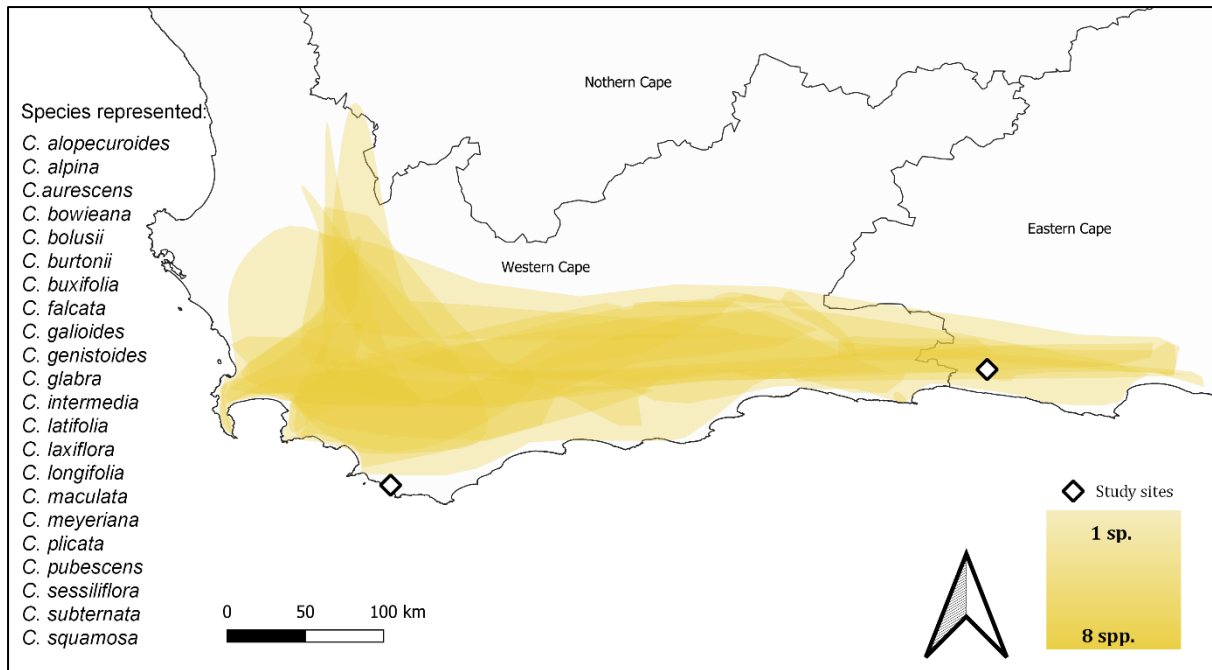
Fig. 4.2. Wild *Cyclopia* population localities from research grade INaturalist and GBIF identifications in the Western and Eastern Cape, South Africa. Non-coloured sections of the map indicate no record of *Cyclopia*, the lightest yellow indicates one species recorded in the area, and the darkest yellow indicates eight species recorded. *Cyclopia* is cultivated across the range, mostly in areas between the indicated study sites located inland, and with some cultivation along the coast.

4.3.2. Ploidy

Ploidy is a potentially important barrier and thus appears early in the planting protocol (Fig. 4.1). Polyploidization is recognized as a driver of plant evolution through its effects on diversification and speciation (Otto & Whitton, 2000; Soltis *et al.,* 2009). *Cyclopia* species are polyploid, with a basic chromosome number of x = 9 (Niemandt *et al.,* 2018). The chromosome number of *Cyclopia* species may prevent hybridization to produce viable seeds with incompatible chromosome numbers (Schutte, 1997; Motsa *et al.*, 2018; Bester pers. comm. 2021). Therefore, in this way, ploidy could be used as a barrier to reduce gene flow by selecting incompatible species which can be safely planted alongside one another indicated by the "no" answer in the protocol. Whereas, conversely, "yes" would indicate greater potential for hybridization opportunity. There are six *Cyclopia* species with published data on their chromosome numbers: *C. maculata* (Schutte, 1997), *C. subternata* (Schutte, 1997), *C. intermedia* (Schutte, 1997), *C. meyeriana* (Schutte, 1997), *C. genistoides* (Motsa, 2016), and *C. longifolia* (Motsa, 2016) (Table 4.1). The lack of information on the remaining *Cyclopia* species is represented by an "I don't know" option in the protocol (Fig. 4.1). The polyploid nature of the genus may affect which species can form viable hybrids produced through cross-

pollination, as observed in *Lachenalia* (Kleynhans *et al.,* 2009). However, there are some *Cyclopia* species that exhibit variation in chromosome number (Table 4.1). Therefore, the variation in chromosome number within some *Cyclopia* species makes the prediction of possible hybrids difficult. Consequently, there is a need to determine chromosome numbers for all other *Cyclopia* species but also across the range for widespread species, which could be developed through a consumer pay approach to build a database for the entire genus. This would increase the efficacy of this barrier in the planting protocol.

Table 4.1. *Cyclopia* species with known chromosome numbers, including five species of commercial value as well as *C. meyeriana.*

Species	Chromosome number (2n)
C. genistoides*	10x = 90
C. subternata	6x = 54
C. maculata*	4x = 36
C. intermedia*	14x = 146
C. longifolia	6x = 54
C. meyeriana	14x = ±126

*Species with variable chromosome numbers

4.3.3. Planting distance

This barrier considers "safe" planting distances, or spatial isolation, at which gene flow through seed dispersal and pollen-transfer is unlikely to occur. *Cyclopia* has dissilient seed pods upon ripening, causing the seeds to be dispersed (Schutte, 1997; Slabbert *et al.,* 2019). In addition, ant dispersal, or myrmecochory, is strongly suggested by the presence of a fleshy aril on *Cyclopia* seed (Schutte, 1997), an advantageous and common method that protects seed from predators in fynbos (Cowling *et al.,* 1994). Furthermore, a study investigating the movement of *C. pubescens* seeds found a significant increase of seedlings within close proximity to ant hills (du Toit & Campbell, 1999), another indication of myrmecochory. Seed dispersal distances through myrmecochory are relatively low (mean under 2 m) (Bond & Slingsby, 1983), but up to 180 m (Gómez & Espadaler, 2013). However, the potential for alternate means of dispersal has not yet been explored. Animals such as baboons (Shaw pers. obs.) may present opportunity for dispersal, though the survivability of seeds through the digestive tract provides an additional aspect which needs to be considered (Kreitschitz *et al.,* 2020). Nevertheless, seed dispersal was considered an integral part of the planting protocol, and thus was included as a barrier using known dispersal distances by myrmecochory.

Additionally, seed dispersal through water is an important aspect to consider with a few *Cyclopia* species growing along waterways (i.e., *C. subternata*). Plants that occur higher up in elevation may be at risk of seed transport further down mountainsides. However, this is of less concern with plantations typically on lower slopes, therefore seeds from wild populations may disperse into cultivated sites, however the opposite is unlikely.

Genetic contamination through pollen transfer is important to consider as pollen-flow influences the potential for cross-contamination. The pollinators of four of the six commercially important *Cyclopia* species have been identified; *Xylocopa capitata* Smith*, X. flavorufa* De Geer*, X. rufitarsis* Lepeletier*, X. caffra* Linnaeus*, X. scioensis* Gribodo and *X. sicheli* Vachal (see Chapter 2). Carpenter bee (Xylocopinae) flight distances are generally thought to be significantly further than smaller bees due to a positive correlation in body size and flight distance (Greenleaf *et al.*, 2007). Mark-release-recapture experiments revealed carpenter bee movement up to 729 m from the site of release, with regular distances of 683 m travelled between a cultivated site and wild patches (see Chapter 3). In addition, radio-tracking of carpenter bees revealed a daily flight distance of up to 1.2 km from the site of capture, where the individuals' nesting site was located (see Chapter 3). With *Cyclopia* pollen showing viability beyond 4.5 days, and between species crosses producing hybrids (*C. subternata* x *C. genistoides* and *C. subternata* x *C. maculata*) (see Chapter 3), the likelihood of pollinators initiating genetic contamination within a 1.2 km distance is highly likely. In literature, a "safe" planting distance has been advised as a result of homing tests producing up to 10 km flight distances (Potts, 2017; Pasquet *et al.,* 2008). Although, carpenter bee movement is thought to be restricted to one third the maximum flight distance as recorded from homing tests (Roubik, 1989). Therefore, a low risk distance of 3 km is advised considering homing tests and the one-third restriction theory (Fig. 4.1). While the risk of genetic contamination is lower at this distance, the risk is never completely absent, owing to rare long-dispersal events of both seeds and carpenter bees. The 1.2 km daily forage distance which was observed in *Cyclopia* pollinators was used to determine the high risk distance of 1 km, within which genetic contamination risk is at the highest (Fig. 4.1). Seed dispersal distances through myrmecochory are much shorter and thus incorporated into this high risk pollen-flow distance.

4.3.4. Seed source

The source of seed can influence the potential for genetic contamination, therefore seed source was deemed an important barrier in the planting protocol. Sourcing and collecting seeds from local populations will prevent the introduction of new genetic material into an area, and thus potential for genetic contamination to occur. This risk management barrier considers

genetic contamination between species, as well as within species with localised genetic structure. Haplotype screening of the chloroplast genetic diversity in *Cyclopia subternata* has revealed highly spatially structured diversity in some populations (Galuszynski & Potts, 2020). The "local" (i.e., low risk) distance of 10 km (Fig. 4.1) advised in this protocol needs to be considered in combination with other factors (see Potts, 2017). Firstly, seeds should not be moved over watersheds or between mountain ranges (Potts, 2017; Britton *et al.,* 2014). Secondly, seeds should not be transported over drastic altitudinal gradients (Potts, 2017). Farmers who do not source local seeds for planting will therefore be at high risk of genetic contamination (Fig 4.1), with partial use ("somewhat") of local material indicating a moderate risk of genetic contamination (Fig. 4.1). If a farmer sources seeds locally, the other barriers will not apply and therefore *Cyclopia* can be planted within 3 km from wild populations. However, the gene pool should not be influenced by breeding programmes or through trait-selection. The risk of selecting for traits favourable for production or taste is unknown and therefore requires further quantification.

4.3.5. Harvest time

The time of the year that farmers choose to harvest honeybush can significantly influence the risk of genetic contamination. Choosing to harvest before the flowering season (i.e., when plants are in bud) will reduce the risk of gene flow. Since farmers rely on seeds for planting (Karsen *et al.,* 2022), a few branches are left during harvesting to ensure seed production. Additionally, annual harvesting is sustainable for species such as *Cyclopia subternata* and *C. genistoides*, although harvest date is optimised through recovery, yield and quality consideration (North *et al.,* 2017),whilst other species are not harvested annually. In addition, in *C. subternata* the leaf phenolic content, sensory profile and thus quality of the tea product produced was affected by the harvest season (Mabizela *et al.,* 2020). Therefore, farmers may be restricted to harvest during certain times of the year, regardless of flowering phenology, in order to produce a profitable harvest. At the planting protocol workshop, many farmers agreed that harvest seasons are altered according to a number of factors, which include variable effects such as current market demand. For example, as a result of COVID-19, many honeybush farmers suffered decreased sales (Kritzinger pers. comm. 2022), and subsequently did not harvest for one or more years. These variabilities decrease the efficacy of harvest time as a barrier of genetic contamination; thus, harvest time was omitted from the protocol.

4.3.6. Flowering phenology

Flowering phenology is not included in the protocol, as it is not a reliable barrier. Flowering varies between *Cyclopia* species (Schutte, 1997), and the pollen-transfer potential is significantly reduced with disparate flowering times, thus the potential for genetic contamination through cross-pollination is potential reduced (Halsey *et al.,* 2005). Even though the flowering phenology of the six commercially important *Cyclopia* species is available in literature (Table 4.2), these represent peak flowering times, and typically some flowers are produced before and after these flowering periods (Shaw pers. obs.; Motsa *et al.,* 2017). Importantly, abiotic factors, such as temperature, water and nutrient availability, and light intensity, affect the flowering time in plants (Rathcke & Lacey, 1985). Furthermore, pollinator interactions and competition between co-flowering plants may also impact flowering time (Elzinga *et al.,* 2007). There has been record of plants elongating their flowering period in response to low pollinator visitation (Yasaka *et al.,* 1998), likely from competition among co-flowering plants (Mosquin, 1971). Additionally, the recently studied effects of climate change on flowering phenology are of increasing relevance, and may well affect the efficacy of this barrier (Inouye, 2022). Because of this, and the subsequent large variation in flowering phenology annually and across localities, this barrier was omitted from the planting protocol.

Table 4.2. Flowering phenology of commercially important *Cyclopia* species

Species	Flowering time								Reference
	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	
C. genistoides					■	■	■		Motsa *et al.,* 2017; Slabbert *et al.,* 2019
C. subternata					■	■			Motsa *et al.,* 2017
C. maculata					■	■	■		Slabbert *et al.,* 2019
C. intermedia					■	■	■		Barnado, 2013
C. longifolia					■	■	■		Grobler & Campbell, 2020
C. sessiliflora	■	■							Joubert *et al.,* 2011

4.4.   Conclusion and recommendations

The risk categories in the planting protocol are defined as low, moderate and high risk of genetic contamination, where farms at low risk require no management, while farms at high risk are recommended to avoid planting honeybush. Importantly, these risk categories should be conservatively applied when planting in proximity to a threatened *Cyclopia* species. In terms of moderate risk, more work is required to establish mitigation measures to lower the risk of genetic contamination. It is suggested that mitigation measures are developed in

supplementation to this protocol, particularly considering ploidy level research across the *Cyclopia* genus which would provide a baseline of information on incompatibility between *Cyclopia* species. In addition, physical barriers such as planting under shade-cloth netting or in a green-house could be explored to reduce gene flow through pollinator exclusion.

Gene flow barriers have been studied in relation to genetically modified (GM) and herbicide-resistant crop management. Pollen-trapping, or sowing conventional crops around GM crops, is a method used to reduce gene flow between GM and conventional crops (Morris *et al.,* 1994; Vrbničanin *et al.,* 2017). Additionally, certain biological barriers have been utilised with great success, including male sterility (Hvarleva *et al.,* 2009), maternal inheritance (Daniell *et al,* 1998), and the unreliable seed sterility method using Gene Use Restriction Technology (GURT) (Eastham & Sweet, 2002). However, these barriers are unlikely to reduce gene flow in genetically unaltered indigenous agricultural flora. Instead, as in the case of *Cyclopia*, farmers occasionally rely exclusively on wild populations to source genetic material for propagation. This further solidifies the importance of conserving gene pools of wild populations, not just for conservation but for the benefit of industry. Though, studies have suggested that gene flow is much higher in some cases from cultivated crops to wild populations (Papa & Gepts, 2003; Jencewski *et al.,* 1999). This promotes asymmetric introgression, ultimately leading to the displacement of wild alleles (Papa, 2005). Nevertheless, the effects of outcrossing between cultivated and wild populations are undesirable, and this protocol provides a practical tool to reduce genetic contamination between native species under cultivation and their wild relatives.

It is important for farmers to evaluate the unique set of conditions that will affect gene flow on their land. Therefore, this planting protocol is designed taking into consideration the most effective barriers to minimize the risk of genetic contamination in honeybush farming. This protocol will be valuable for the conservation of the *Cyclopia* genus, an endemic genus in the fynbos biome (Schutte, 1997), as well as for the honeybush tea industry in safeguarding the genetic material needed for cultivation. For other threats, such as invasive alien species, impact classifications have been developed (Hawkins *et al.,* 2015), however these are lacking for planting locally indigenous crops and need to be developed. In terms of risk communication, appropriate stakeholder engagement should be conducted to refine risk categories, but more importantly, to raise awareness of potential risks outlined here. These communications should be industry-based involving parties affected by planting protocolling (see Novoa *et al.,* 2018 for 12-step framework for engaging stakeholders). Additionally, these engagements should aim to actively reduce conflict that may arise between affected parties through facilitated engagement (Novoa *et al.,* 2018); without which, environmental management strategies may be hindered (Cole, 1993; de Wit *et al.,* 2001).

4.5.   References

Arias, D. & Rieseberg, L. 1994. Gene flow between cultivated and wild sunflowers. *Theoretical and Applied Genetics*, 89, 655–660.

Barnado, N. 2013. *Sustainable harvesting of wild populations of Cyclopia intermedia in Kouga, Eastern Cape, South Africa* (M.Sc. Dissertation, Port Elizabeth: Nelson Mandela Metropolitan University).

Bartsch, D., Lehnen, M., Clegg, J., Pohl-Orf, M., Schuphan, I. & Ellstrand, N. 1999. Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. *Molecular Ecology*, *8*, 1733–1741.

Bello, A., Stirton, C., Chimphango, S. & Muasya, A. 2018. Morphological evidence for introgressive hybridization in the genus *Psoralea* L. (Psoraleeae, Fabaceae). *South African Journal of Botany, 118*, 321–328.

Bester, C. 2013. A model for commercialisation of honeybush tea, an indigenous crop. *Acta Horticulturae*, *1007*, 889–894.

Bester, C., Blomerus, L., & Kleynhans, R. 2009. Development of new floriculture crops in South Africa. *Acta Horticulturae, 813*, 67–72.

Bond, W. & Slingsby, P. 1983. Seed dispersal by ants in shrublands of the Cape Province and its evolutionary implications. *South African Journal of Science, 79*, 231–233.

Britton, M., Hedderson, T., & Verboom, G. 2014. Topography as a driver of cryptic speciation in the high-elevation Cape Sedge *Tetraria triangularis* (Boeck.) C. B. Clarke (*Cyperaceae: Schoeneae). Molecular Phylogenetics and Evolution, 77*, 96–109.

Burke, J., Gardner, K. & Rieseberg, L. 2002. The potential for gene flow between cultivated and wild sunflower (*Helianthus annuus*) in the United States. *American Journal of Botany*, *89*, 1550–1552.

Campbell, D. & Waser, N. 2001. Genotype-by-environment interaction and the fitness of plant hybrids in the wild. *Evolution*, *55*, 669–676.

Cole, D. 1993. Minimizing conflict between recreation and nature conservation. In: Smith, D.S., Hellmund, P.C. (Eds.), *Ecology of Greeways: Design and Function of Linear Conservation Areas.* University of Minnesota Press, Minneapolis, 105–122.

Cornille, A., Gladieux, P. & Giraud, T. 2013. Crop-to-wild gene flow and spatial genetic structure in the closest wild relatives of the cultivated apple. *Evolutionary Applications, 6*, 737–748.

Cowling, R., Pierce, S., Stock, W., & Cocks, M. 1994. Why are there so many myrmecochorous species in the Cape Fynbos? *Tasks for Vegetation Science,* 159–168.

Daniell, H., Datta, R., Varma, S., Gray, S. & Lee, S. 1998. Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nature Biotechnology, 16*, 345–348.

Datta, A., Kumschick, S., Geerts, S. & Wilson, J. 2020. Identifying safe cultivars of invasive plants: six questions for risk assessment, management, and communication. *NeoBiota, 62*, 81–97.

Delplancke, M., Alvarez, N., Espíndola, A., Joly, H., Benoit, L., Brouck, E. & Arrigo, N. 2011. Gene flow among wild and domesticated almond species: insights from chloroplast and nuclear markers. *Evolutionary Applications*, *5*, 317–329.

De Schawe, C., Durka, W., Tscharntke, T., Hensen, I. & Kessler, M. 2013. Gene flow and genetic diversity in cultivated and wild cacao (*Theobroma cacao*) in Bolivia. *American Journal of Botany*, *100*, 2271–2279.

de Wit, M., Crookes, D. & Van Wilgen, B. 2001. Conflicts of interest in environmental management: estimating the costs and benefits of a tree invasion. *Biological Invasions, 3*, 167–178.

du Toit, S. & Campbell, E. 1999. The effect of fire on two eastern Cape *Cyclopia* species (Fabaceae). *South African Journal of Botany, 65*, 203–207.

Eastham, K. & Sweet, J. 2002. *Genetically modified organisms (GMOs): the significance of gene flow through pollen transfer*. European Environment Agency, Copenhagen. Environmental Issue Report no. 28.

Ellstrand, N. 2001. When transgenes wander, should we worry? *Plant Physiology, 125*, 1543–1545.

Ellstrand, N., Prentice, H. & Hancock, J. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology, Evolution, and Systematics*, *30*, 539–563.

Elzinga, J., Atlan, A., Biere, A., Gigord, L., Weis, A., & Bernasconi, G. 2007. Time after time: Flowering phenology and biotic interactions. *Trends in Ecology & Evolution, 22*, 432–439.

Galuszynski, N. & Potts, A. 2020. Application of High Resolution Melt analysis (HRM) for screening haplotype variation in a non-model plant genus: *Cyclopia* (Honeybush). *PeerJ*, 8, e9187.

Gómez, C. & Espadaler, X. 2013. An update of the world survey of myrmecochorous dispersal distances. *Ecography*, *36*, 1193–1201.

Greenleaf, S., Williams, N., Winfree, R. & Kremen, C. 2007. Bee foraging ranges and their relationship to body size. *Oecologia*, *153*, 589–596.

Grobler, B. & Campbell, E. 2020. Pollinator activity and the fecundity of a rare and highly threatened honeybush species along a highway in the Cape Floristic Region. *International Journal of Plant Sciences*, *181*, 581–593.

Halsey, M., Remund, K., Davis, C., Qualls, M., Eppard, P., & Berberich, S. 2005. Isolation of maize from pollen-mediated gene flow by time and distance. *Crop Science, 45*, 2172–2185.

Hancock, J., Grumet, R. & Hokanson, S. 1996.The opportunity for escape of engineered genes from transgenic crops. *Hortscience, 31*, 1080–1085.

Hawkins, C., Bacher, S., Essl, F., Hulme, P., Jeschke, J., Kühn, I., Kumschick, S., Nentwig, W., Pergl, J., Pyšek, P., Rabitsch, W., Richardson, D., Vilà, M., Wilson, J., Genovesi, P. & Blackburn, T. 2015. Framework and guidelines for implementing the proposed IUCN Environmental Impact Classification for alien taxa (EICAT). *Diversity and Distributions, 21*, 1360–1363.

Hawkins, H., Malgas, R. & Biénabe, E. 2011. Ecotypes of wild rooibos (*Aspalathus linearis* (Burm. F) Dahlg., Fabaceae) are ecologically distinct. *South African Journal of Botany*, *77*, 360–370.

Hilton-Taylor, C. 1996. Red Data List of southern African plants. 1. corrections and additions. *Bothalia*, 26, 177–182.

Hvarleva, T., Hristova, M., Bakalova, A., Hristov, M., Atanassov, I. & Atanassov, A. 2009. CMS lines for evaluation of pollen flow in sunflower relevance for transgene flow mitigation*. Biotechnology and Biotechnological Equipment, 23*, 1309–1315.

Inouye, D. 2022. Climate change and phenology. *WIREs Climate Change, 13*, e764.

Jansen Van Rensburg, W., Van Averbeke, W., Slabbert, R., Faber, M., Van Jaarsveld, P., Van Heerden, I., Wenhold, F. & Oelofse, A. 2009. African leafy vegetables in South Africa. *Water SA, 33*, 317–326.

Jenczewski, E., Prosperi, J. & Ronfort, J. 1999. Evidence for gene flow between wild and cultivated *Medicago sativa* (Leguminosae) based on allozyme markers and quantitative traits. *American Journal of Botany*, *86*, 677–687.

Joubert, E., Joubert, M., Bester, C., de Beer, D. & De Lange, J. 2011. Honeybush (*Cyclopia* spp.): From local cottage industry to global markets — The catalytic and supporting role of research. *South African Journal of Botany*, 77, 887–907.

Kleynhans, R., Spies, J., & Spies, P. 2009. Cross-ability in the genus *Lachenalia. Acta Horticulturae, 813*, 385–392.

Karsen, P., Lötze, E., Valentine, A. & Hoffman, E. 2022. Propagation and cultivation practices of Honeybush (*Cyclopia* spp.) for the sustainable production of an export quality indigenous South African tea. *Crop Science, 62*, 1702–1733.

Kreitschitz, A., Haase, E., & Gorb, S. 2020. The role of mucilage envelope in the endozoochory of Selected Plant Taxa. *The Science of Nature, 108*(1).

Kumschick, S., Wilson, J. & Foxcroft, L. 2020. A framework to support alien species regulation: The Risk Analysis for Alien Taxa (RAAT). *NeoBiota, 62*, 213–239.

Mabizela, G., Muller, M., De Beer, D., Van der Rijst, M., Slabbert, M., Joubert, E., & Bester, C. 2020. Effect of genotype and harvest season on quality characteristics of *Cyclopia subternata:* Phenolic content and sensory profile. *South African Journal of Botany, 132*, 491–501.

Macqueen, T. & Potts, A. 2018. Re-opening the case of Frankenflora: Evidence of hybridisation between local and introduced *Protea* species at Van Stadens Wildflower Reserve. *South African Journal of Botany*, *118*, 315–320.

Malgas, R., Potts, A., Oettlé, N., Koelle, B., Todd, S., Verboom, G. & Hoffman, M. 2010. Distribution, quantitative morphological variation and preliminary molecular analysis of different growth forms of wild rooibos (*Aspalathus linearis*) in the northern Cederberg and on the Bokkeveld Plateau. *South African Journal of Botany*, *76*, 72–81.

Morris, W., Kareiva, P. & Raymer, P. 1994. Do barren zones and pollen traps reduce gene escape from transgenic crops? *Ecological Applications, 4*, 157–165.

Mosquin, T. 1971. Competition for pollinators as a stimulus for the evolution of flowering time. *Oikos, 22*, 398.

Motsa, M. 2016. Natural phenology, fecundity, genetic variation and seed dormancy of *Cyclopia* species (Honeybush). Unpublished D.Tech. thesis, Tshwane University of Technology, Pretoria, 159–163.

Motsa, M., Bester, C., Slabbert, M., Hannweg, K. & Booyse, M. 2018. Flow cytometry: a quick method to determine ploidy levels in honeybush (*Cyclopia* spp.). *Genetic Resources and Crop Evolution*, *65*, 1711–1724.

Motsa, M., Slabbert, M., Bester, C. & Ngwenya, M. 2017. Phenology of honeybush (*Cyclopia genistoides* and *C. subternata*) genotypes. *South African Journal of Botany*, *110*, 57–67.

Niemandt, M., Roodt-Wilding, R., Tobutt, K. & Bester, C. 2018. Microsatellite marker applications in *Cyclopia* (Fabaceae) species. *South African Journal of Botany*, *116*, 52–60.

North, M., Joubert, E., De Beer, D., De Kock, K., & Joubert, M. 2017. Effect of harvest date on growth, production and quality of Honeybush (*Cyclopia genistoides* and *C. subternata*). *South African Journal of Botany, 110*, 132–137.

Novoa, A., Shackleton, R., Canavan, S., Cybèle, C., Davies, S., Dehnen-Schmutz, K., Fried, J., Gaertner, M., Geerts, S., Griffiths, C., Kaplan, H., Kumschick, S., Le Maitre, D., Maesey, G., Nunes, A., Richardson, D., Robinson, T., Touza, J. & Wilson, J. 2018. A framework for engaging stakeholders on the management of alien species. *Journal of Environmental Management, 205*, 286–297.

Otto, S. & Whitton, J. 2000. Polyploid incidence and evolution. *Annual Review of Genetics, 34*, 401–437.

Palaudelmas, M., Mele, E., Penas, G., Pla, M., Nadal, A., Serra, J., Salvia, J. & Messeguer, J. 2008. Sowing and flowering delays can be an efficient strategy to improve coexistence of genetically modified and conventional maize. *Crop Science*, *48*, 2404–2413.

Papa, R. 2005. Gene flow and introgression between domesticated crops and their wild relatives. *Role of Biotechnology*, 71–76.

Papa, R. & Gepts, P. 2003. Asymmetry of gene flow and differential geographical structure of molecular diversity in wild and domesticated common bean (*Phaseolus vulgaris* L.) from Mesoamerica. *Theoretical and Applied Genetics, 106*, 239–250.

Pasquet, R., Peltier, A., Hufford, M., Oudin, E., Saulnier, J., Paul, L., Knudsen, J., Herren, H. & Gepts, P. 2008. Long-distance pollen flow assessment through evaluation of pollinator foraging range suggests transgene escape distances. *Proceedings of the National Academy of Sciences, 105*, 13456–13461.

Potts, A. 2017. Genetic risk and the transition to cultivation in Cape endemic crops—The example of honeybush (*Cyclopia*)? *South African Journal of Botany*, *110*, 52–56.

Raimondo, D., von Staden, L., Foden, W., Victor, J., Helme, N., Turner, R., Kamundi, D. & Manyama, P. 2009. *Red List of South African Plants.* Strelitzia 25. South African National Biodiversity Institute, Pretoria.

Rathcke, B. & Lacey, E. 1985. Phenological patterns of terrestrial plants. *Annual Review of Ecology and Systematics, 16*, 179–214.

Reinten, E. & Coetzee, J. 2002. Commercialization of South African indigenous crops: aspects of research and cultivation of products. In: Janick, J., Whipkey, A. (Eds.), Trends in New Crops and New Uses. ASHS Press, Alexandria, VA.

Rong, J., Lu, B., Song, Z., Su, J., Snow, A., Zhang, X., Sun, S., Chen, R. & Wang, F. 2006. Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields. *New Phytologist, 173*, 346–353.

Roubik, D. 1989. *Ecology and natural history of tropical bees.* Cambridge: Cambridge University Press.

Sanvido, O., Widmer, F., Winzeler, M., Streit, B., Szerencsits, E. & Bigler, F. 2008. Definition and feasibility of isolation distances for transgenic maize. *Transgenic Research*, *17,* 317–355.

Scheffler, J., Parkingston, R. & Dale, P. 1993. Frequency and distance of pollen dispersal from transgenic oilseed rape (*Brassica napus*). *Transgenic Research, 2*, 356–364.

Scheffler, J., Parkingston, R. & Dale, P. 1995. Evaluating the effectiveness of isolation distances for field plots of oilseed rape (*Brassica napus*) using a herbicide-resistance transgene as a selectable marker. *Plant Breeding, 114*, 317–321.

Schutte, A. 1997. Systematics of the genus *Cyclopia* Vent. (Fabaceae, Podalyrieae). *Edinburgh Journal of Botany, 54*, 125–170.

Slabbert, E., Malgas, R., Veldtman, R. & Addison, P. 2019. Honeybush (*Cyclopia* spp.) phenology and associated arthropod diversity in the Overberg region, South Africa. *Bothalia*, *49*, 1–13.

Snow, A. & Morán-Palma, P. 1997. Commercialization of transgenic plants: potential ecological risks. *Bioscience, 47*, 86–96.

Soltis, D., Albert, V., Leebens-Mack, J., Bell, C., Paterson, A., Zheng, C., Sankoff, D., de Pamphilis, C., Kerr Wall, P. & Soltis, P. 2009. Polyploidy and angiosperm diversification. *American Journal of Botany, 96*, 336–348.

Song, Z., Lu, B., Zhu, Y. & Chen, J. 2003. Gene flow from cultivated rice to the wild species *Oryza rufipogon* under experimental field conditions. *New Phytologist, 157*(3), 657–665.

Ureta, M., Carrera, A., Cantamutto, M. & Poverene, M. 2008. Gene flow among wild and cultivated sunflower, *Helianthus annuus* in Argentina. *Agriculture, Ecosystems & Environment, 123*, 343–349.

Van Wyk, A. & Prinsloo, G. 2018. Medicinal plant harvesting, sustainability and cultivation in South Africa. *Biological Conservation*, 227, 335–342.

Vrbničanin, S., Božić, D. & Pavlović, D. 2017. Gene flow from herbicide-resistant crops to wild relatives. *Herbicide Resistance in Weeds and Crops*.

Wolf, D., Takebayashi, N. & Rieseberg, L. 2001. Predicting the risk of extinction through hybridization. *Conservation Biology, 15*, 1039–1053.

Yasaka, M., Nishiwaki, Y., & Konno, Y. 1998. Plasticity of flower longevity in *Corydalis ambigua. Ecological Research, 13*, 211–216.

Zhou, H., Zhao, P., Woeste, K. & Zhang, S. 2020. Gene flow among wild and cultivated common walnut (*Juglans regia*) trees in the Qinling Mountains revealed by microsatellite markers. *Journal of Forestry Research,* DOI: 10.1007/s11676-020-01254-z

Chapter 5: Conclusion and recommendations

Gene flow is important to maintain the genetic integrity of plant populations, particularly those in isolation (Ellstrand & Elam, 1993). Gene flow between cultivated populations and their co-occurring wild relatives can result in genetic contamination (Ellstrand *et al.,* 1999), a condition which has been widely studied (e.g. Arias & Rieseberg, 1994; Ellstrand *et al.,* 1999; Burke *et al.,* 2002; Cornille *et al.,* 2013). This introgression between cultivated plants and wild populations can have significant consequences, including the loss of genetic resources through homogenisation (Millennium Ecosystem Assessment, 2005), possible extinction of locally adapted variations (Ellstrand *et al.,* 1999; Wolf *et al.,* 2001; Gepts & Papa, 2003), and hybridization coupled with hybrid vigour (Uwimana *et al.,* 2012). The risk of introgression from native agricultural flora moved across the range for cultivation needs to be considered. With *Cyclopia* Vent., or honeybush, still emerging into global markets and not yet reaching its full potential, preventative measures can still be applied to minimize genetic-contamination risks. Therefore, this study aimed to determine the risk of cross-pollination and thus genetic contamination between wild and cultivated *Cyclopia* species and populations. This was achieved through identifying the primary pollinators, determining their movement through tracking efforts, and finally identifying the potential for hybrid plants to form as a result of cross-pollination.

Carpenter bees (*Xylocopa* Latreille) were the only observed pollinators of four commercially important *Cyclopia* species (see Chapter 2). The few links per species indicate a specialised system with few pollinator species. This illustrates the dependency of *Cyclopia* on these native pollinators for successful reproduction, and in turn the dependency of the honeybush tea community, particularly since seeds harvested from wild populations is a limited source. Regular movement between the wild and cultivated sites was observed using mark-release-recapture and radio-tagging (see Chapter 3). These daily movements may be short distance, however, carpenter bees are likely to travel further than the observed distances in this study, thus the duration of pollen viability is important. The longevity of *Cyclopia* pollen coupled with the ability of hybrids to form illustrate the potential for genetic contamination and introgression in this genus. A preliminary planting protocol was developed for the *Cyclopia* genus (see Chapter 4). This planting protocol is valuable to the honeybush tea industry, allowing farmers to evaluate the unique set of conditions affecting the risk of gene flow on their land. This protocol is important for the genetic integrity and conservation of indigenous flora, focussing on *Cyclopia* as a case study. Though, with occasional long-distance dispersal events, the risk of genetic contamination will never be absent. There are impact classifications that have been developed for other threats such as invasive alien species (Hawkins *et al.,* 2015), and can be

potentially developed for introgression in native agricultural flora. This presents a future research opportunity to be explored in more detail not only in *Cyclopia,* but all indigenous plants of commercial value. Additionally, further mitigation measures should be investigated to supplement this study. The next step, risk communication, relies on facilitated stakeholder engagement to relay the results and consequences of this study (Novoa *et al.,* 2018).

This planting protocol provides a valuable tool for identifying the risk of genetic contamination at individual plantations. With many cultivated-to-wild introgression studies focussing on genetic contamination from herbicide-resistant, genetically modified and transgenic crops (e.g., Snow & Morán-Palma, 1997; Eastham & Sweet, 2002; Arias & Rieseberg, 1994; Burke *et al.,* 2002; Cornille *et al.,* 2013; Sohn *et al.,* 2022), this protocol provides an important baseline of information beneficial not only to maintain the genetic integrity of *Cyclopia,* but all native agricultural flora. Isolation distances are a practical measure used in other crops (Scheffler *et al,* 1993; Scheffler *et al.,* 1995; Rong *et al.,* 2006) which provide value in this instance. The information on *Cyclopia* pollination and the observed maximum distances determined in this study can inform the risk of planting at different distances from naturally occurring wild populations. With the honeybush industry relying heavily on seed (rather than cuttings), a wide range of genetic material will be present in the plantation throughout its lifecycle (Eastham & Sweet, 2002), illustrating the necessity for the minimization of cultivated-to-wild gene flow in its entirety for the conservation of the genetic integrity of the genus.

From this thesis, there are a number of unanswered questions that provide valuable research opportunities to enhance the conservation of indigenous agricultural flora. The polyploid nature of the *Cyclopia* genus (Schutte, 1997), and ploidy variation within a few species, will affect the compatibility between *Cyclopia* species and thus success of hybrid formations. Thus, further research on the ploidy levels of *Cyclopia* coupled with additional hand-crossing experiments may act as a valuable tool for efficient protocolling, and possibly introgression prevention (i.e. species with different chromosome numbers could be planted alongside, without the risk of genetic contamination). Moreover, the ploidy level of all *Cyclopia* populations across the landscape should be determined, owing to the ploidy variability not only across, but also within certain species. Additionally, it is important to develop and up-to-date network of all honeybush farms, their location and the species under cultivation. This will aid to determine the current threat of introgression, as well as identify the native populations at risk of genetic contamination. In particular, the red-listed species (i.e., *C. longifoli, C. pubescens, C. squamosa, C. aurescens, C. falcata, C. glabra, C. bolusii, C. burtonii, C. alopecuroides* and *C. plicata*; Raimondo *et al.,* 2009) highly localised species (i.e., *Cyclopia pubescens* and *C. longifolia*; Schutte, 1997) at threat of introgression should receive special attention, with perhaps a buffer zone where planting is prohibited. In fact, extensive mapping

of the wild and cultivated populations of *Cyclopia* is desperately needed for the implementation of efficient conservation management strategies. This is not unrealistic considering the widely available mapping tools and projects such as those on INaturalist. It is important to note, that while this protocol focuses on new plantings there is already a few hundred ha of planted honeybush, which is an aspect that has not been covered in detail in this thesis. The protocol and risk management measures should thus be adapted to manage these areas going forward. Furthermore, other than commercial farming, the risk of introgression from current community honeybush farming initiatives also needs to be quantified. While these projects have had successes (Bester pers. comm. 2022; Horn, 2019), they need to be scaled up and expanded to fully utilise the benefits of farming honeybush as an indigenous crop, but in a sustainable manner. As an additional aspect, it is important to question the shortfalls in these initiatives, including the lack of current profitability. Nevertheless, it is imperative to ensure the community and commercial farmers are aware of the risks of introgression on the wild resource through knowledge-sharing and education. Furthermore, the implementation of management strategies to minimize the threat of genetic contamination using certain horticultural practices (Karsen *et al.,* 2022) and the planting protocol can be incentivised through green certification, an important future aspect of this industry.

References

Arias, D. & Rieseberg, L. 1994. Gene flow between cultivated and wild sunflowers. *Theoretical and Applied Genetics*, *89*, 655–660.

Burke, J., Gardner, K. & Rieseberg, L. 2002. The potential for gene flow between cultivated and wild sunflower (*Helianthus annuus*) in the United States. *American Journal of Botany*, *89*, 1550–1552.

Cornille, A., Gladieux, P. & Giraud, T. 2013. Crop-to-wild gene flow and spatial genetic structure in the closest wild relatives of the cultivated apple. *Evolutionary Applications, 6*, 737–748.

Duraiappah, A., Naeem, S., Agardy, T., Ash, N., Cooper, H., Diaz, S., Faith, D., Mace, G., McNeely, J., Mooney, H., Oteng-Yeboah, A., Pereira, H., Polasky, S., Prip, C., Reid, W., Samper, C., Schei, P., Scholes, R., Schutyser, F. & Van Jaarsveld, A. 2005. *Ecosystems and human well-being: biodiversity synthesis; a report of the Millennium Ecosystem Assessment.* World Resources Institute.

Eastham, K. & Sweet, J. 2002. *Genetically modified organisms (GMOs): the significance of gene flow through pollen transfer*. European Environment Agency, Copenhagen. Environmental Issue Report no. 28.

Ellstrand, N. & Elam, D. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics, 24*, 217–242.

Ellstrand, N., Prentice, H. & Hancock, J. 1999. Gene flow and introgression from domesticated plants into their wild relatives. *Annual Review of Ecology, Evolution, and Systematics*, *30*, 539–563.

Gepts, P. & Papa, R. 2003. Possible effects of (trans)gene flow from crops on the genetic diversity from landraces and wild relatives. *Environmental Biosafety Research, 2*, 89–103.

Horn, A. 2019. Honeybush tea: Small industry, big potential. [online] Farmer's Weekly. Available at: [Accessed 22 September 2022].

Karsen, P., Lötze, E., Valentine, A. & Hoffman, E. 2022. Propagation and cultivation practices of Honeybush (*Cyclopia* spp.) for the sustainable production of an export quality indigenous South African tea. *Crop Science, 62*, 1702–1733.

Novoa, A., Shackleton, R., Canavan, S., Cybèle, C., Davies, S., Dehnen-Schmutz, K., Fried, J., Gaertner, M., Geerts, S., Griffiths, C., Kaplan, H., Kumschick, S., Le Maitre, D., Maesey, G., Nunes, A., Richardson, D., Robinson, T., Touza, J. & Wilson, J. 2018. A framework for engaging stakeholders on the management of alien species. *Journal of Environmental Management, 205*, 286–297.

Raimondo, D., von Staden, L., Foden, W., Victor, J., Helme, N., Turner, R., Kamundi, D. & Manyama, P. 2009. *Red List of South African Plants.* Strelitzia 25. South African National Biodiversity Institute, Pretoria.

Rong, J., Lu, B., Song, Z., Su, J., Snow, A., Zhang, X., Sun, S., Chen, R. & Wang, F. 2006. Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields. *New Phytologist, 173*, 346–353.

Scheffler, J., Parkingston, R. & Dale, P. 1993. Frequency and distance of pollen dispersal from transgenic oilseed rape (*Brassica napus*). *Transgenic Research, 2*, 356–364.

Scheffler, J., Parkingston, R. & Dale, P. 1995. Evaluating the effectiveness of isolation distances for field plots of oilseed rape (*Brassica napus*) using a herbicide-resistance transgene as a selectable marker. *Plant Breeding, 114*, 317–321.

Schutte, A. 1997. Systematics of the genus *Cyclopia* Vent. (Fabaceae, Podalyrieae). *Edinburgh Journal of Botany, 54*, 125–170.

Snow, A. & Morán-Palma, P. 1997. Commercialization of transgenic plants: potential ecological risks. *Bioscience, 47*, 86–96.

Sohn, S., Thamilarasan, S., Pandian, S., Oh, Y., Ryu, T., Lee, G. & Shin, E. 2022. Interspecific hybridization of transgenic *Brassica napus* and *Brassica rapa*—an overview. *Genes, 13*, 1–13.

Uwimana, B., Smulders, M., Hooftman, D., Hartman, Y., van Tienderen, P., Jansen, J., McHale, L., Michelmore, R. van de Wiel, C. & Visser, R. 2012. Hybridization between crops and wild relatives: The contribution of cultivated lettuce to the vigour of crop–wild hybrids under drought, salinity and nutrient deficiency conditions. *Theoretical and Applied Genetics, 125*, 1097–1111.

Wolf, D., Takebayashi, N. & Rieseberg, L. 2001. Predicting the risk of extinction through hybridization. *Conservation Biology, 15*, 1039–1053.

Appendices

Appendix 2.A. Accession numbers for specimen accessioned into the Iziko South Africa Museum. The specimen included representatives of flower visitors observed accessing *Cyclopia* flowers.

Accession no.	Species	Notes
SAM-HYM-B027144	*Xylocopa scioensis*	
SAM-HYM-B027145	*Xylocopa scioensis*	
SAM-HYM-B027146	*Xylocopa flavorufa*	
SAM-HYM-B027147	*Xylocopa albifrons*	Collected species after pollinator observation period (thus was not included in pollination networks). Collected in the wild study site during mark-release-recapture and radio-tagging efforts.
SAM-HYM-B027148	*Xylocopa albifrons*	
SAM-HYM-B027149	*Xylocopa albifrons*	
SAM-HYM-B027150	*Xylocopa albifrons*	
SAM-HYM-B027213	*Xylocopa caffra*	
SAM-HYM-B027214	*Xylocopa caffra*	
SAM-HYM-B027215	*Xylocopa rufitarsis*	
SAM-HYM-B027216	*Xylocopa sicheli*	
SAM-HYM-B027217	*Xylocopa capitata*	

Appendix 2.B. Pollination network for wild *Cyclopia* spp. from the visitation rates calculated from: (A) the floral observations data, (B) from the camera trap data.

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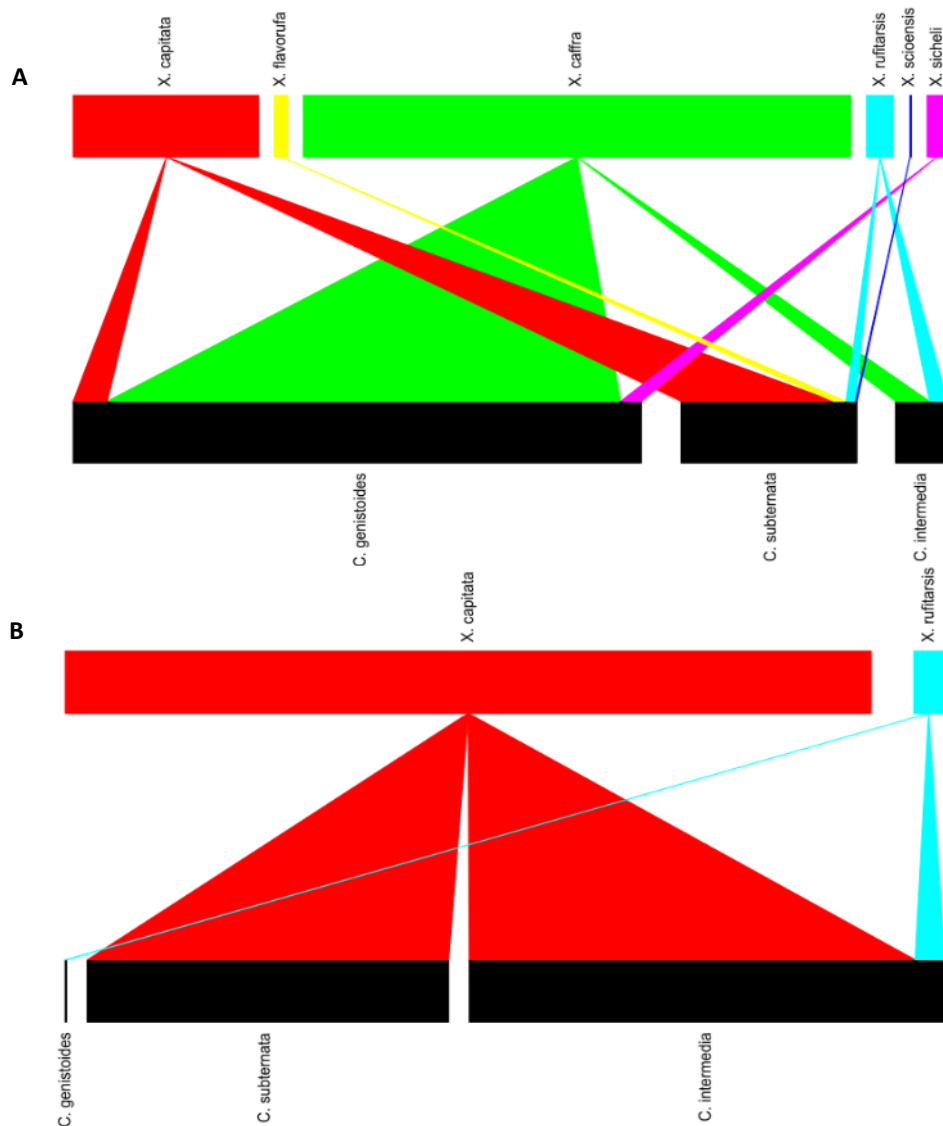
Appendix 2.C. Pollination network for cultivated *Cyclopia* spp. from the visitation rates calculated from: (A) the floral observations data, (B) from the camera trap data.
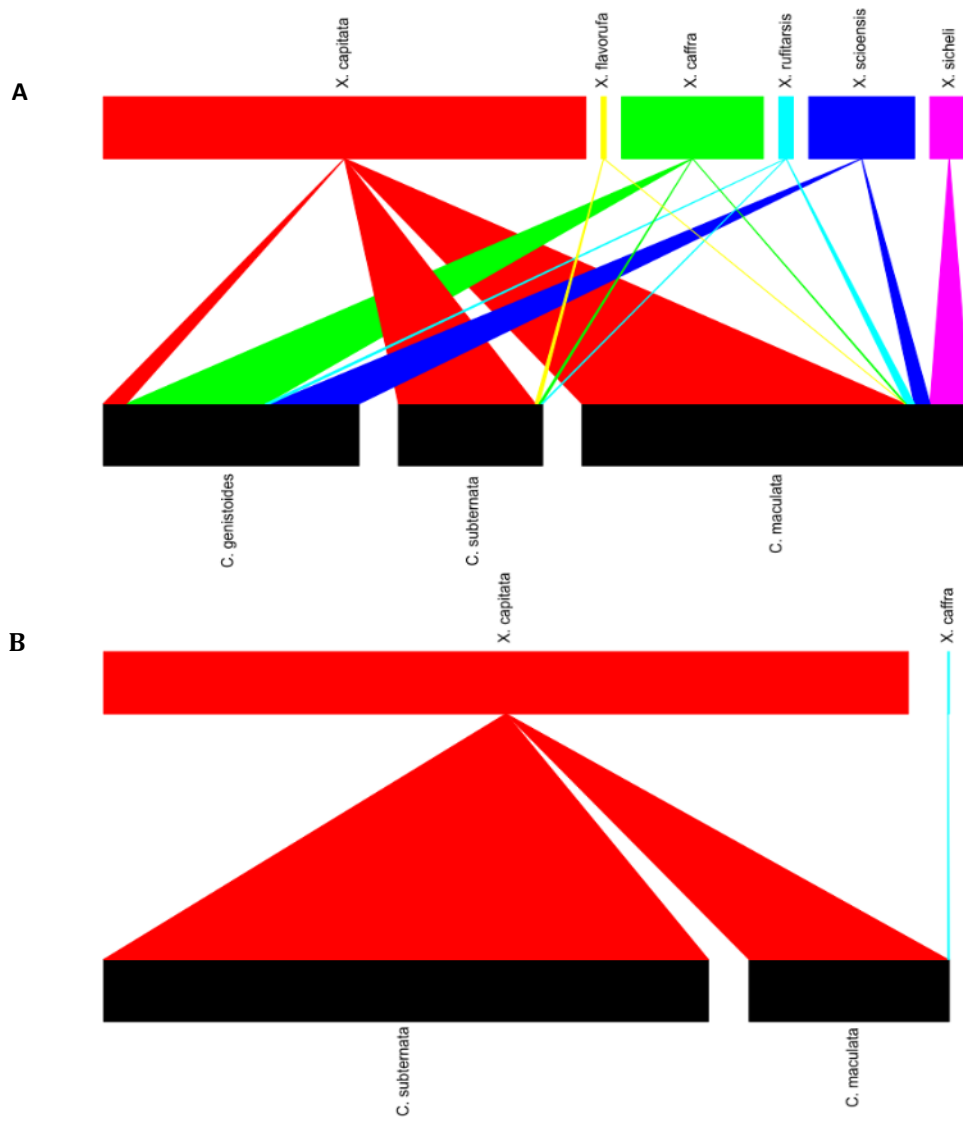
Appendix 3.D. Farm boundary, cultivated site, wild honeybush populations, and wild and cultivated release points of the study site in the Eastern Cape, South Africa.

Appendix 3.E. Date, colour and location of re-observations for the mark-release-recapture experiment

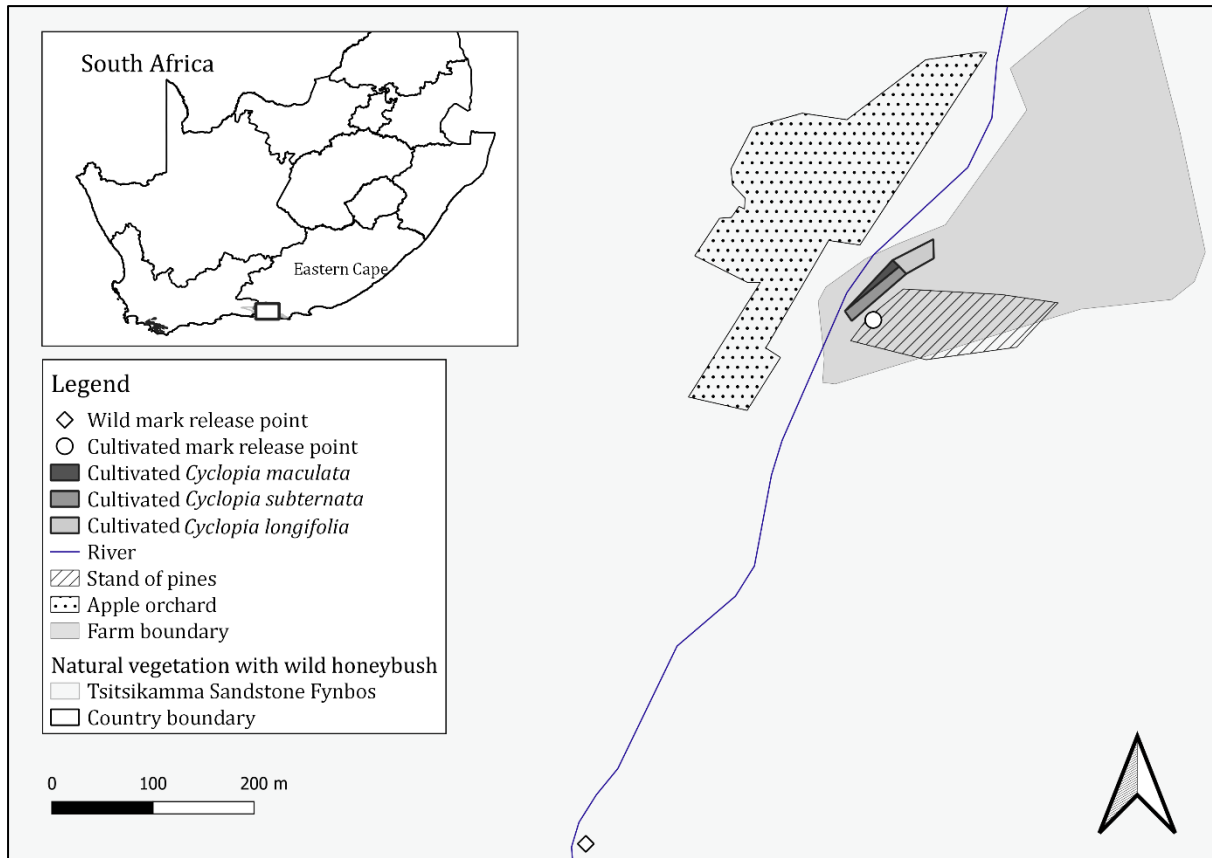| No.	Date	Colour	Location	No.	Date	Colour	Location
1	24/09/2021	Light blue	Cultivated	44	19/10/2021	White	Cultivated
2	25/09/2021	Light blue	Cultivated	45	19/10/2021	White	Cultivated
3	05/10/2021	Purple	Cultivated	46	19/10/2021	White	Cultivated
4	06/10/2021	Purple	Cultivated	47	19/10/2021	White	Cultivated
5	06/10/2021	Orange	Cultivated	48	19/10/2021	White	Cultivated
6	06/10/2021	Purple	Cultivated	49	19/10/2021	White	Cultivated
7	07/10/2021	Orange	Cultivated	50	19/10/2021	White	Cultivated
8	07/10/2021	Orange	Cultivated	51	19/10/2021	White	Cultivated
9	07/10/2021	Orange	Cultivated	52	19/10/2021	White	Cultivated
10	07/10/2021	Purple	Cultivated	53	19/10/2021	White	Cultivated
11	07/10/2021	Orange	33˚52'09.3"S 23˚54'54.0"E	54	19/10/2021	Red	33˚52'28.164"S 23˚54'44.82"E
12	07/10/2021	Orange	33˚52'14.16"S 23˚54'52.884"E	55	19/10/2021	Red	33˚52'28.164"S 23˚54'44.82"E
13	07/10/2021	Orange	33˚52'11.1"S 23˚54'53.6"E	56	19/10/2021	Red	33˚52'28.164"S 23˚54'44.82"E
14	07/10/2021	Orange	Cultivated	57	19/10/2021	Orange	33˚52'09.3"S 23˚54'54.0"E
15	07/10/2021	Purple	Cultivated	58	20/10/2021	White	Cultivated
16	07/10/2021	Purple	Cultivated	59	20/10/2021	Light blue	Cultivated
17	07/10/2021	Orange	Cultivated	60	20/10/2021	White	Cultivated
18	07/10/2021	Orange	Cultivated	61	20/10/2021	White	Cultivated
19	08/10/2021	Orange	Cultivated	62	20/10/2021	Orange	33˚52'13.3"S 23˚54'52.9"E
20	08/10/2021	Purple	Cultivated	63	20/10/2021	Red	33˚52'13.728"S 23˚54'52.884"E
21	08/10/2021	Purple	Cultivated	64	20/10/2021	Red	33˚52'19.6"S 23˚54'50.3"E
22	08/10/2021	Light blue	Cultivated	65	20/10/2021	Orange	33˚52'09.3"S 23˚54'54.0"E
23	08/10/2021	Orange	Cultivated	66	20/10/2021	White	Cultivated
24	08/10/2021	Purple	Cultivated	67	20/10/2021	White	Cultivated
25	08/10/2021	Light blue	33˚52'29.0"S 23˚54'44.9"E	68	20/10/2021	White	Cultivated
26	08/10/2021	Orange	33˚52'13.3"S 23˚54'52.9"E	69	20/10/2021	White	Cultivated
27	19/10/2021	Light blue	Cultivated	70	20/10/2021	White	Cultivated
28	19/10/2021	White	Cultivated	71	20/10/2021	White	Cultivated
29	19/10/2021	White	Cultivated	72	20/10/2021	White	Cultivated
30	19/10/2021	White	Cultivated	73	20/10/2021	White	Cultivated
31	19/10/2021	White	Cultivated	74	20/10/2021	White	Cultivated
32	19/10/2021	White	Cultivated	75	20/10/2021	White	Cultivated
33	19/10/2021	White	Cultivated	76	20/10/2021	White	Cultivated
34	19/10/2021	White	Cultivated	77	20/10/2021	White	Cultivated
35	19/10/2021	White	Cultivated	78	20/10/2021	White	Cultivated
36	19/10/2021	White	Cultivated	79	20/10/2021	Light blue	Cultivated
37	19/10/2021	White	Cultivated	80	20/10/2021	White	Cultivated
38	19/10/2021	White	Cultivated	81	20/10/2021	Light blue	Cultivated
39	19/10/2021	White	Cultivated	82	20/10/2021	White	Cultivated
40	19/10/2021	Light blue	Cultivated	83	20/10/2021	White	Cultivated
41	19/10/2021	Light blue	Cultivated	84	20/10/2021	White	Cultivated
42	19/10/2021	Orange	Cultivated	85	20/10/2021	White	Cultivated
43	19/10/2021	White	Cultivated				

Appendix 3.F. GPS points marked during radio-tagging of carpenter bee individuals

GPS name	GPS points	Time	Behaviour
Bee No. 1 – Male (7/10/2021 10:09 – 8/10/2021 10:09)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	10:09	Flew off from site of release
002	33˚52'06.0"S 23˚54'55.3"E		Foraging in cultivated site
003	33˚52'05.7"S 23˚54'55.4"E	12:20	Flying over apple farm
004	33˚52'04.3"S 23˚54'54.7"E	12:27	Foraging at wild Aspalathus
005	33˚51'59.3"S 23˚54'51.7"E	12:36	Foraging at wild Aspalathus
006	33˚51'59.4"S 23˚54'50.9"E	12:41	Foraging at wild Aspalathus
007	33˚51'59.3"S 23˚54'50.7"E	13:47	Foraging and defending at wild Aspalathus
008	33˚51'59.2"S 23˚54'50.1"E	13:51	Foraging at wild Aspalathus
011	33˚52'06.1"S 23˚55'00.9"E	10:09	Transmitter found off bee @ nest site
Bee No. 2 – Male (7/10/2021 09:27 – 8/10/2021 09:25)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	09:27	Flew off from site of release
009	33˚52'09.1"S 23˚54'54.2"E	14:15	Foraging at Asteraceae
010	33˚52'07.8"S 23˚54'53.9"E	09:25	Foraging at cultivated maculata, removed tag
Bee No. 3 – Male (19/10/2021 14:18 – 20/10/2021 14:40)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	14:18	Release point
032	33˚52'09.4"S 23˚54'53.9"E	14:32	On keurboom
033	33˚52'09.8"S 23˚54'53.9"E	14:33	Fighting cap. male @ keurboom
034	33˚52'07.9"S 23˚54'53.8"E	14:45	Foraging on C. mac
035	33˚52'07.3"S 23˚54'54.0"E	14:50	Fighting and foraging on C. mac
036	33˚52'07.4"S 23˚54'54.0"E	14:51	Foraging on C. mac
037	33˚52'08.2"S 23˚54'53.8"E	14:55	On keurboom
038	33˚52'07.4"S 23˚54'53.8"E	15:00	Foraging on C. mac
039	33˚52'07.3"S 23˚54'54.2"E	15:04	Foraging on C. mac
040	33˚52'05.9"S 23˚54'54.3"E	15:09	Fighting and foraging @ keurboom
041	33˚52'06.0"S 23˚54'54.1"E	15:48	Territorial around Asteraceae
042	33˚52'08.2"S 23˚54'53.8"E	09:18	On keurboom
043	33˚52'08.1"S 23˚54'53.9"E	09:55	Foraging @ keurboom
044	33˚52'08.5"S 23˚54'54.1"E	12:35	Foraging @ keurboom
033	33˚52'09.8"S 23˚54'53.9"E	14:40	Captured on keurboom
Bee No. 4 – Female (19/10/2021 13:10 – 19/10/2021 13:37)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	13:19	Release point
Mark release point	33˚52'07.1"S 23˚54'55.7"E	13:19	Tree above release point resting
027	33˚52'06.7"S 23˚54'54.7"E	13:33	Foraging on C. mac
028	33˚52'06.7"S 23˚54'54.5"E	13:33	Foraging on C. mac
029	33˚52'06.6"S 23˚54'54.5"E	13:34	Foraging on C. mac
030	33˚52'06.9"S 23˚54'54.2"E	13:35	Foraging on C. mac
031	33˚52'07.1"S 23˚54'54.1"E	13:37	Foraging on C. mac
Bee No. 5 – Female (19/10/2021 14:08 – 19/10/2021)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	14:08	Release point
Bee No. 6 – Male (12/11/2021 11:00 – 13/11/2021 14:23)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	11:00	Release point
046	33˚52'12.5"S 23˚54'53.1"E	12:50	Foraging @ keurboom (cult.)
047	33˚52'10.7"S 23˚54'53.3"E	12:56	Foraging @ keurboom
048	33˚52'13.8"S 23˚54'52.1"E	13:01	Foraging @ keurboom
047	33˚52'10.7"S 23˚54'53.3"E	10:00	Foraging @ keurboom
049	33˚52'08.2"S 23˚54'53.9"E	14:19	Foraging @ keurboom (cult.)
050	33˚52'10.1"S 23˚54'53.9"E	14:23	Caught foraging on keurboom (cult.)
Bee No. 7 – Male (12/11/2021 10:28 – 12/11/2021)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	10:28	Release point
Bee No. 8 – Male (12/11/2021 10:57 – 12/11/2021)			
Mark release point	33˚52'07.1"S 23˚54'55.7"E	10:57	Release point
Bee No. 10 – Male (17/09/2022 13:17 – 27/09/2022)			
Mark release point	33.86863° 023.91547°	13:17	Release point
080	33.86748° 023.91646°	13:26	Strong signal @ keurboom
081	33.86796° 023.91599°	13:32	on ferns
082	33.86856° 023.91533°	13:36	Foraging on cult C. sub
083	33.86861° 023.91520°	13:38	Foraging on cult C. sub
084	33.86952° 023.91493°	13:47	Flying over river
093	33.86875° 023.91496°	13:56	Strong signal @ nesting site
094	33.86108° 023.90627°		Retrieved @ nesting site
Bee No. 11 – Female (17/09/2022 12:04 – 17/09/2022)			
Mark release point	33.86863° 023.91547°	12:04	Release point
Bee No. 12 – Male (17/09/2022 12:10 – 17/09/2022)			
Mark release point	33.86863° 023.91547°	12:10	Release point
079	33.86876° 023.91502°	12:54	Flying over cultivated site
Bee No. 13 – Female (26/09/2022 16:13 – 26/09/2022)			

Mark release point	33.86863° 023.91547°	16:21	Release point
Bee No. 14 – Male (26/09/2022 16:13 – 29/09/2022)			
Mark release point	33.86863° 023.91547°	16:13	Release point
095	33.86853° 023.91522°	11:03	Foraging on C. mac
096	33.86853° 023.91512°	12:26	Foraging on C. mac
101	33.86872° 023.91509°	14:45	Foraging on C. mac
101	33.86872° 023.91509°	14:53	Retrieved
Bee No. 15 – Male (27/09/2022 12:54 – 27/09/2022)			
Mark release point	33.86863° 023.91547°	12:57	Release point
097	33.86908° 023.91507°	13:05	Foraging on keurboom
099	33.86898° 023.91510°	13:25	Foraging on keurboom
Bee No. 16 – Male (27/09/2022 12:57 – 27/09/2022)			
Mark release point	33.86863° 023.91547°	13:01	Release point
098	33.86909° 023.91510°	13:09	Foraging on keurboom
100	33.86747° 023.91023°	14:07	Foraging on senecio
098	33.86909° 023.91510°	14:43	Foraging on keurboom

Appendix 4.A. Identified barriers to genetic contamination, related protocol questions and risk of genetic contamination determined from answer to the protocol question. The rationale behind the amendment or omission of each barrier is outlined.

	Barrier	Protocol question	Answer	Risk of genetic contamination	Amendment/Omission rationale
Risk Assessment	1. Range	Do you intend planting *Cyclopia* within the native range of *Cyclopia*?	Yes - No	High Moderate Low	None
	2. Flowering	Does your intended *Cyclopia* species overlap in flowering with the neighbouring *Cyclopia* species?	Yes - No	High Moderate Low	Omit from protocol. Too much variation in flowering phenology between different localities and between years.
	3. Ploidy	Does your intended *Cyclopia* species share similar chromosome no. with the neighbouring *Cyclopia* species?	Yes - No	High Moderate Low	None
	4. Distance (seed dispersal)	Is *Cyclopia* under cultivation within:	<180 m >180 m – 2 km > 2 km	High Moderate Low	Combine barrier number 4 & 5. This will prevent confusion when navigating the protocol. Change high-risk distance from 180 m to 200 m. Allows for easier reading and retention.
Risk Management	5. Distance (pollen-flow)	Is *Cyclopia* under cultivation within:	<1 km 3 km 10 km	High Moderate Low	
	6. Harvest time	Do you ensure harvest takes place before the flowering season? (How often do you harvest -once, twice/year?)	No Sometimes Yes	High Moderate Low	Harvest seasons are changed according to a number of factors such as the state of the market. In addition, harvest season influences the tea quality.
	7. Seed source	Is your honeybush grown from locally sourced seeds?	No Somewhat Yes	High Moderate Low	None