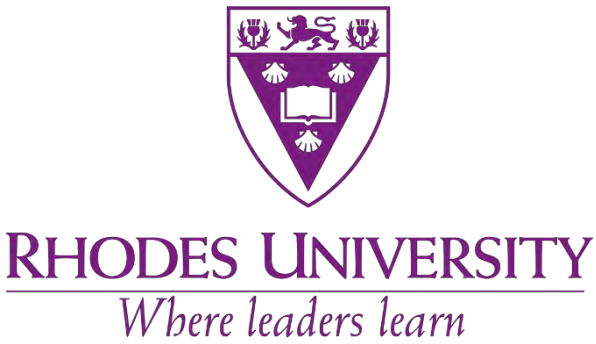
Prioritization of potential biocontrol agents for the invasive alien weed, *Cylindropuntia pallida* (Cactaceae) in South Africa and insights into the use of cochineal in future biological control programmes.



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MASTER OF SCIENCE

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By

Wade Terence Sexton

Abstract

Cylindropuntia pallida F.M. Knuth (Cactaceae) is an invasive alien species in Australia, Saudi Arabia, Spain, Namibia, and South Africa. Like other invasive *Cylindropuntia* species, it has no commercial value and has had numerous negative impacts on the environment and the economy. Since the biocontrol of invasive cactus weeds using cochineal insects has been successful in the past, it is likely that it will be the best form of control against *C. pallida*. In this thesis, the aims were to prioritize potential biocontrol agents for *C. pallida* in South Africa as well as to gain insights into how to select the most damaging agent for other cactus weeds in the future.

Two lineages of the cochineal insect *Dactylopius tomentosus* Lamark, were considered as potential biocontrol agents for *C. pallida* in South Africa. One lineage, referred to as the 'californica var. parkeri' lineage had already been released against *C. pallida* in Australia, where it is an effective agent. The second lineage was collected off *C. pallida* in Mexico and was imported into quarantine in South Africa and is referred to as the 'pallida' lineage. The compatibility of both cochineal lineages with invasive *C. pallida* from South Africa was tested by comparing cochineal fitness parameters when reared on South African *C. pallida*. Surprisingly, the 'californica var. parkeri' lineage had much lower fitness on South African *C. pallida* plants compared with Australian plants, whilst the 'pallida' lineage had higher fitness on South African plants. These results indicate that the 'pallida' lineage is the preferred candidate and should be considered further as a potential agent but also raises interesting questions as to whether 'old associations' or 'new associations' are better for cactus biocontrol with cochineal lineages.

A 'new association' in biocontrol is defined as a biocontrol agent that attacks a target weed which it has not co-evolved with (like the 'californica var. parkeri' lineage on *C. pallida,* which was collected off *C. bernardina* outside of the indigenous distribution of *C. pallida*). 'Old associations', are when the biocontrol agent and weed have a long-standing evolutionary relationship in the indigenous distribution (like the 'pallida' lineage on *C. pallida,* which was collected off *C. pallida* in its indigenous distribution). To test whether 'new' or 'old associations' are more likely to be effective in controlling

invasive cactus with cochineal, the fitness and performance of three *D. tomentosus* cochineal lineages on seven different invasive *Cylindropuntia* species was tested with the aim to assess whether fitness and performance decrease with more genetically distant plant species from the primary host plant. For every fitness performance metric tested in this study, there was a correlation between phylogenetic distance and performance, with fitness measurements decreasing for each cochineal lineage on less closely related species. This study provides evidence that 'old associations' should be prioritized for Cactaceae biocontrol agent selection.

Fitness assessments like those present in this thesis contribute to predicting the efficacy of an agent as well as help in the agent selection process. This thesis should contribute towards the successful biocontrol of *C. pallida* in South Africa and improve the selection process for finding new biological control agents for other cactus weeds. Since *C. pallida* is a high priority weed in South Africa, the benefits of managing this weed will have numerous positive impacts, such as restoring native biodiversity and helping return profits to landowners and communities.

Publications arising from this study

CHAPTER 2: SEXTON, W.T, MUSKETT, P.C., MCCONNACHIE, A.J., PORTILLO, L., VIGUERAS, A.L. AND PATERSON, I.D. Biocontrol of thistle cholla cactus, *Cylindropuntia pallida*: a successful agent in Australia is incompatible with the invasive populations in South Africa. *Biocontrol Science and Technology*. (In review)

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Chapter 1

General introduction

1.1 Invasive alien plants

Invasive plants are one of the greatest threats to ecosystems globally (Vitousek et al. 1997, Pimentel et al. 2000, Pyšek et al. 2020). The negative impacts of invasive plants include damaging the economy, reducing agricultural productivity, and can lead to a reduction in diversity of native species, increasing the risk of ecosystem collapse (Richardson & van Wilgen 2004, van Wilgen et al. 2004). These ecological and economic impacts have made invasive plants an area of great concern, and because of this, invasion biology has become a fully-fledged field in science, with many scientists studying the biology and ecology of invasive species as well as finding methods of control (Simberloff et al. 2005).

1.1.1 What are their impacts?

The negative impacts of invasive plants are numerous, being both direct and indirect (Linders et al. 2019). Direct effects include outcompeting native species for resources such as water, space, and nutrients (Richardson et al. 2000). This can place a strain on native species, reducing their numbers and, in some cases, even cause extinctions (Gurevitch & Padilla 2004). Indirectly, there is substantial evidence that invasive alien plants can alter soil chemistry, nutrient cycling, and decomposition rates (Weidenhamer & Callaway 2010). This can leave the environment unsuitable for native plant species (Nalepa & Schloesser 1992, Wilson et al. 2007, Coetzee & Hill 2012, Coetzee et al. 2014). Invasive plants also alter fire regimes in environments where fire is an important ecological process. Changes in the fuel load of ecosystems caused by invasive plants can alter the frequency, seasonality, and intensity of fires (Brooks et al. 2004). Invasive plants can also introduce new diseases/pests that may threaten survival of native species (Moser et al. 2009).

One sector that is heavily impacted by invasive plants is agriculture, which in turn affects many communities and the local economy (Bradshaw et al. 2016). The loss of grazing and productivity in pastures caused by invasive plants can seriously reduce turnover and has a knock-on effect on the economy (Van Wilgen & De Lotto 2011). Invasive plants have also encroached into areas of agricultural crops and can severely reduce overall yield, which causes farmers to lose money (Eschen et al. 2021). Agriculture is essential for livelihoods, provides food security, reduces poverty, and can boost the economy, so it is a fundamentally important sector to protect (Gollin et al. 2002). Apart from agriculture, invasive species also interfere with many other human activities. For example, invasive species have also become a problem on railroads (Benedetti & Morelli 2017), highways (Kalwij et al. 2008, Sharma et al. 2010), and can damage telephone wires (Culliney 2005), thus interfering with normal day-to-day activities. Invasive species also hamper tourism (van Wilgen et al. 2017), recreational activities such as fishing (Richardson & van Wilgen 2004), reduce stream flow in dams (Havel et al. 2015), as well as reducing the price of land and property (Zimmermann et al. 2004, Santo 2017). One of the biggest issues caused by invasive plant species is the reduction of the water table and water quality (Le Maitre et al. 1996, Coetzee & Hill 2012, Havel et al. 2015, Hill & Coetzee 2017). This is a serious issue, particularly in areas where water is scarce and needed for development (Le Maitre et al. 1996).

Invasive plants have a huge economic impact in many countries (Pejchar & Mooney 2009). The economic costs of invasive plant species (including removal and damages) are extreme, with estimated costs of around $3 billion per annum in Australia (Sinden et al. 2004), $34 billion in the U.S.A (Pimentel et al. 2000), $1.4 billion in the U.K (Pimentel et al. 2000) and around $12 billion in South Africa (van Wilgen et al. 2001). It is for these reasons that invasive plant species must be controlled so that communities and countries do not suffer.

1.2 Biological control

Biological control (biocontrol) is a form of invasive species management whereby biological organisms/ natural enemies are used to control or regulate an invasive species population (Stenberg et al. 2021). The practice of biocontrol usually involves releasing either a predator, herbivore, parasite, parasitoid, or pathogen in order to reduce an invasive species population (Hoffmann 1991, Charudattan and Dinoor 2000). The Enemy Release Hypothesis is the theoretical basis of biocontrol (Keane & Crawley 2002). The Enemy Release Hypothesis states that a species outside its indigenous distribution is allowed to proliferate because it is no longer exposed to its natural enemies (Keane & Crawley 2002). The hypothesis predicts that natural enemies are essential in regulating populations, and therefore, biocontrol utilizes this hypothesis by reuniting the target weed with its natural enemy so that populations of the target weed can be reduced (McFadyen 1998). The practice of biocontrol has demonstrated that using natural enemies that are effective biocontrol agents can lower the target weed populations below what is termed the "damage threshold" (Letourneau 1987, Keane & Crawley 2002, Staab & Schuldt 2020) (Fig. 1.1). Once an agent has been released, established, and has reduced the invasive species population, both the agent and weed populations will oscillate but the agent will maintain the weed population at a lower level, hopefully below the damage threshold of the weed. This will allow native species to recover after the invasion (Briese 2000). Over the years, the practice of biocontrol has been successful against numerous invasive plants in many different countries (Wilson et al. 2007, Paterson et al. 2011, Coetzee & Hill 2012, Martin et al. 2018).

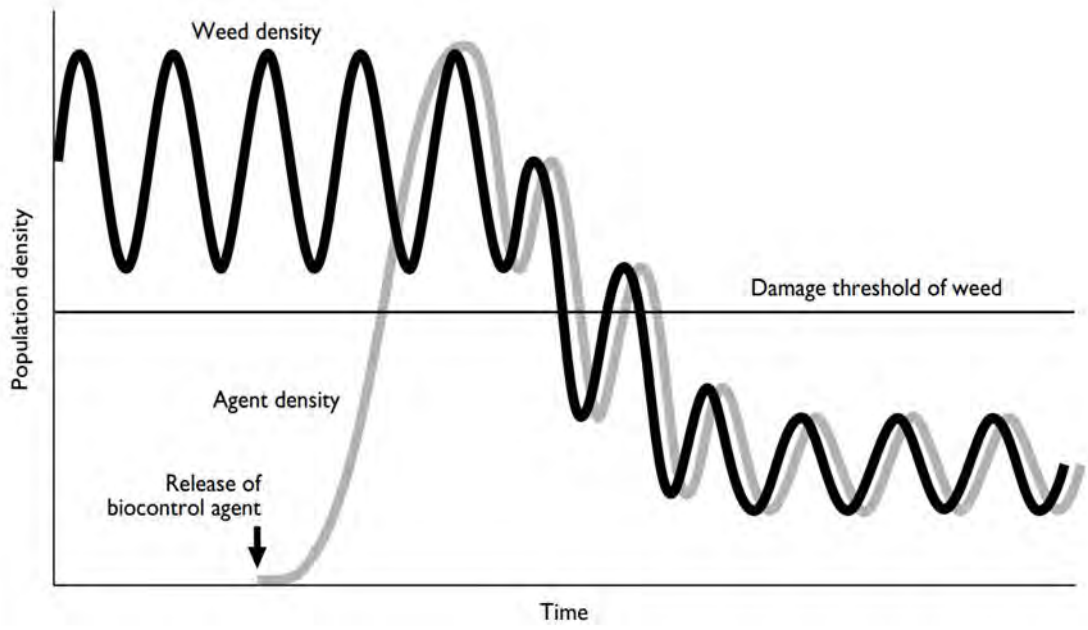

Figure 1.1: The changes in weed density before and after the release of a biocontrol agent (Briese 2000).

The selection and prioritization of potential agents is the first step in the biocontrol process (Goolsby et al. 2006a). In order to find potential agents, surveys are conducted in the invasive species' native habitat for natural enemies associated with the target weed (Goolsby et al. 2006b). Ideally, agents that are prioritized should be the most likely to be damaging enough to result in control of invasive populations of the target weed and be the most likely to be suitably host-specific for release in the intended region of introduction (McFadyen 1998, Paterson et al. 2009, 2014). There are often a very large number of natural enemies associated with a particular target weed, so prioritization is essential as testing all the natural enemies would be impractical and exorbitantly expensive (McFadyen 1998, Paterson et al. 2014). Prioritization is usually based on whether the natural enemies have an efficient mode of damage (such as attacking vulnerable life stages or plant parts) and whether it feeds on closely related plant species. Once a suitable agent is prioritized, it is imported into a quarantine facility where it undergoes pre-release assessments to ensure that it will be safe and effective (McEvoy 1996).

One major criteria for the selection of agents, is host-specificity. It is vitally important that the agent is suitably host specific to the target weed and that it will not damage non-target plants (McEvoy

1996, Marohasy 1998, Paynter et al. 2020). The release of agents that are not suitably host-specific could have serious negative impacts to the receiving environment (Simberloff & Stiling 1996). There is clear evidence that modern host specificity techniques can accurately predict the host range of an agent prior to release and these techniques are still improving, further reducing the chances of non-target impacts and making the practice of biocontrol safer (Hinz et al. 2019, Paynter et al. 2020). Host specificity testing usually involves no-choice and choice testing (McEvoy 1996, Heard 2002).

Both no-choice and choice testing are normally conducted in a laboratory or quarantine facility. No-choice testing entails giving a potential agent one plant species to utilize for development and/or feeding, whilst multi-choice testing entails giving the potential agent multiple species and assessing the preferred 'choice' of host plant (McEvoy 1996, Van Klinken & Heard 2000). No-choice tests can result in false positives. False positives are where an agent utilises a test plant species during host specificity testing, but would never do so under field conditions if it were released (Marohasy 1998). This makes this type of testing very conservative and should be interpreted carefully so that an agent is not rejected, which would be safe if released. False positives are also possible in choice tests. For example, plants exude chemicals that are used as cues by insects to select host plants, however, because multiple plants are often in close proximity when tested, these chemicals build up in the experimental setup and may result in the insect utilizing the wrong plant (Sutton et al. 2017). False negatives occur when an agent does not feed on a plant that it would feed on when released (Marohasy 1998). False negatives are less likely to occur than false positives, but the consequences of false negatives are severe as they result in non-target impacts in the field. False negatives are very unlikely in no-choice testing, and this is why most release applications rely more heavily on no-choice tests, using choice tests only for test plant species that are utilized in no-choice tests. Given the possibility of false negatives and false positives in choice and no-choice testing, data must be interpreted carefully (Murray et al. 2010).

Apart from host specificity testing, pre-release efficacy testing is an important component in the testing of a biocontrol agent (McFadyen 1998). It is important that the agent is suitably damaging and can reduce populations of the target weed once released (McClay & Balciunas 2005). Releasing an

ineffective agent on a target weed takes time and resources but provides no benefits, and could also negatively influence people's perception of biocontrol, and therefore should be avoided wherever possible (McClay & Balciunas 2005). Pre-release efficacy assessments often entail looking into the fitness of a particular agent, usually, its ability to survive and reproduce (Balciunas & Smith 2006). This is because if an agent can survive and reproduce past multiple generations, it increases the likelihood of establishment and impacting the target weed (McFadyen 1998). Despite the importance of predicting the efficacy of biocontrol agents prior to release, pre-release efficacy assessments are thought to lack predictive power and are, therefore, often not included in pre-release assessments (Balciunas & Smith 2006, Paynter et al. 2018). Issues such as climate, predation, and other abiotic factors could impact the efficacy of an agent once released, so predicting the impact an agent is likely to have based on studies in a laboratory setting must be done with caution (Paynter et al. 2018). However, there is evidence that pre-release efficacy testing can be a valuable way to screen agents and allow researchers to select the most effective agent from a suite of prioritized agents with the use of pre-release impact assessments (Jones et al. 2015, 2016) and can at least ensure that agents that are released can damage the target weed under certain conditions.

1.2.1 Success, benefits, and safety of biocontrol

There has been some scrutiny in recent years as to just how safe weed biocontrol is, as there are many concerns that releasing biocontrol agents can have non-target impacts (Crone et al. 2009, Louda & O'Brien 2002). In the early years of biocontrol (1960's), a few non-target impacts were recorded, however, since then, the percentage of non-target impacts of biocontrol agents has reduced overtime from 18.2% (1960's) to 9.9% (1991-2008), as well as the percentage of agents that caused consistent attack to non-targets being reduced from 12% to 1% (Hinz et al. 2019). Importantly, the vast majority of non-target impacts on native species have not led to impacts at the population level, with less than 1% of intentional releases resulting in population-level impacts on native species (Hinz et al. 2019). It is, however, important to note that population-level impacts are not always predicted, and studies are

not always carried out to assess this, so this 1% may be an underestimate. These non-target impacts were also predicated prior to release, however, these risks were considered less important at the time of release than they are today (Downey & Paterson 2016). Consideration also needs to be shown to instances where releasing an agent that may have some risks of non-target impacts might be worthwhile, especially if the risk of not releasing an agent at all is high (Downey & Paterson 2016). Many early examples of non-target impacts would have been avoided with the robust modern host-specificity testing that is required today. With strict regulations, as well as stronger host-range prediction methods now in place, non-target impacts are more accurately predicted, strengthening the safety and perception of biocontrol (Downey & Paterson 2016, Hinz et al. 2019, Paynter et al. 2020).

Biocontrol is now a widely practiced and successful method of control, with over 130 countries participating and over 550 biocontrol agents being released worldwide (Winston et al. 2014, Zachariades et al. 2017). Part of the success and benefit of biocontrol is that in comparison with other methods of control (such as chemical and mechanical control), it is cost-effective and does not rely on constant follow-up for chemical applications or the continuous removal of plants that regrow (Zachariades et al. 2017). Around 70.9% of the agents released globally successfully established themselves on the target weed, and around 55% of the agents caused significant damage to the target plants (Schwarzländer et al. 2018). In South Africa, 136 biocontrol agents have been released, with 92 successfully established on their target plants, whilst 27 have failed to establish (Zachariades 2021). Around 35% of all the agents released in South Africa proved to be highly effective and inflict extensive damage on their target weeds, with a further 38% causing moderate to considerable damage to their target weeds (Zachariades 2021).

Biocontrol has also had a significant benefit on local economies and communities (Wilson et al. 2007). For example, the invasion of Water hyacinth, *Pontederia crassipes* Mart (Pontedariaceae) on Lake Victoria in Uganda, blocked transport links, reduced fishing productivity, and seriously reduced biodiversity (Wilson et al. 2007). With the introduction of the biocontrol agent *Neochetina eichhorniae* (Warner) (Coleoptera: Curculionidae), the infestation declined drastically, resulting in local productivity returning to its normal state, clearing of transport links and marking a return in

biodiversity (Wilson et al. 2007). Without this introduction in a third-world country like Uganda, the long-term economic consequences would have affected thousands of lives. Another example is *Xanthium occidentale* Bertol. (Asteraceae) in Australia which was controlled using two agents, *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff (Erysiphaceae) and *Epiblema strenuana* Walker (Tortricidae) (McFadyen 1998). This biocontrol programme resulted in a return of US$720 000 in Australia, indicating the economic importance of biocontrol (McFadyen 1998). In South Africa, the successful biocontrol of the invasive cactus *Opuntia aurantiaca Lindley* (Cactaceae) resulted in a benefit-cost ratio of 709:1 (van Wilgen et al. 2004, Zachariades et al. 2017). These are just some examples of the benefits of weed biocontrol to biodiversity and society. The benefits from these past programmes are a good indication that the development of new biocontrol agents for invasive alien plants should be considered.

1.3 Cactaceae

The Cactaceae is a family of plants endemic to the America's (Novoa et al. 2015, Guerrero et al. 2019). Only one species, *Rhipsalis baccifera* (J.Müller) Stern, which is also native to the America's but also occurs outside of the America's, particular in parts of Africa and Madagascar (Zimmermann et al. 2009). Most cactus species are succulents, which has allowed them to occur in arid conditions such as deserts, sandy coastal areas, and xeric deciduous forests (Guerrero et al. 2019). Cactus species tend to be slow-growing and have large water storage capabilities (Rebman & Pinkava 2001). Most cactus species can induce crassulacean acid metabolism (CAM) photosynthesis which helps them save water and allows them to cope with intense heat and arid climates. CAM photosynthesis works by closing the stomata during the day and opening them at night, allowing the stomata to reduce the overall water loss that would have been lost during the day (Bräutigam et al. 2017). These adaptations have allowed the Cactaceae to evolve into a great diversity of growth forms and represent over 100 genera encompassing around 1600-1800 species (Figure 1.2).

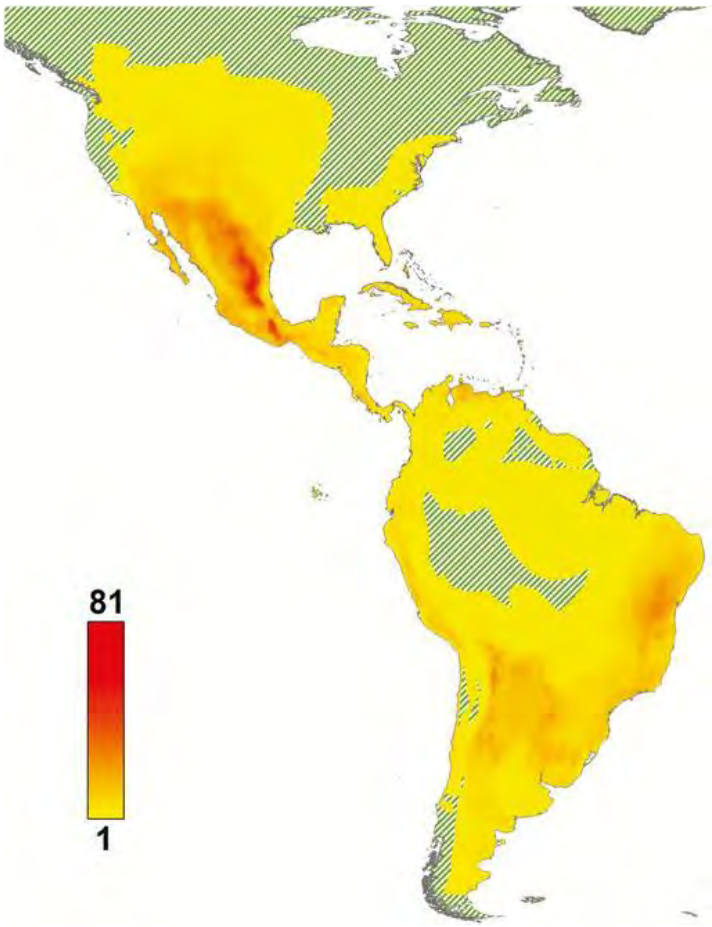

Figure 1.2: Distribution map of the native cactus species richness in North and South America. The scale represents species richness ranging from yellow (n= 1) to red (n= 81). The green stripes represent areas where there are no cactus species present (From Guerrero et al. 2019).

1.3.1 Invasion biology & distribution

The trade of cactus species dates back to as early as the 15th century, when European explorers brought back plants from the America's. Most cactus species were intentionally introduced outside their indigenous distribution for animal fodder, human consumption, and for medicinal and ornamental purposes (Novoa et al. 2015, 2016). However, despite the reasoning behind the introduction of these plants, they started to become overabundant and problematic (Paterson et al. 2011). In South Africa, they created significant issues for agriculture and landowners and have impacted the local environment (Paterson et al. 2011).

One of the earliest introductions of a cactus into South Africa was *Opuntia ficus-indica* Mill, which was introduced in the mid-18th century due to its potential for fodder and fruit production, which is invaluable to farmers in drought conditions (Moran & Zimmermann 1991b, Novoa et al. 2015). Other species were also introduced, and over time, these species ended up escaping from cultivation resulting in the spread of these cactus species in surrounding areas (Novoa et al. 2015). After populations started to establish, other vectors helped the dispersal of cactus species. For example, native animals, such as baboons, small birds, and even elephants, dispersed their seeds by consuming their fruit (Foxcroft 2006). Many invasive cactus species can also reproduce asexually when cladodes (modified stems that function as leaves) break off the main plant and get transported to new areas by sticking to the hair or body of livestock, other wild animals, car tyres, clothes, and shoes of people (Zimmermann et al. 2009, Jones et al. 2014). These cladodes then root into the ground and begin growing in a new area.

Many cactus species have become overabundant in grazing areas, reducing access to water and shade, which are essential for livestock survival in the hotter and drier months (Witt et al. 2017). One of the biggest issues for landowners is that cactus species restrict livestock activity and productivity (Paterson et al. 2021b). Since these grazing areas became so infested with cactus species, livestock were forced into contact with them and became directly harmed by the spines (Witt et al. 2017). Species such as *O. aurantiaca* caused severe damage to the wool of sheep and the hides of cattle, which reduced its economic value (Moran & Zimmermann 1991a). These spiny cladodes are also known to cause severe injuries to livestock, with some injuries even leading to premature culling (Beinart 2011). In Kenya, livestock also consumed the fruit, and because most cactus species have fruit that is covered in glochids (small spines), these accumulate in the gut of the animals, resulting in infections and sometimes death (Witt et al. 2017, Beinart 2011). There have even been circumstances where land was abandoned due to cactus infestations (Witt et al. 2017). Apart from the detrimental impacts to agriculture, it became evident that cactus infestations also had an impact on native biodiversity (Robertson et al. 2011). The spiny cladodes proved to be extremely harmful to native animals, with small birds, reptiles, and mammals often becoming impaled by the large spines (Paterson et al. 2021b). Species such as *Opuntia stricta* (Haw.) Haw. invaded important conservation areas like the Kruger National Park and caused a

reduction in native biodiversity and ecosystem functioning (Robertson et al. 2011). In other countries like Madagascar and Kenya, cactus infestations have also resulted in a decrease in native biodiversity (Witt et al. 2017, Larsson 2004).

Today 35 species of cactus are classified as invasive in South Africa (Department of Environmental Affairs, 2016) (Table 1.1). Without control methods in place, these cacti can result in ecological and economic damage with particular reference to agriculture and wildlife (Paterson et al. 2021b). The cost to remove them mechanically (using machinery or manual labour) has proven to be extremely expensive and not very effective (Zimmermann et al. 2004). Chemical control has proved to be successful for smaller infestations but is not useful for larger infestations (Zimmermann et al. 2004). Chemical control, like mechanical control, is very expensive, and plants can also develop resistance to these chemicals over time, thus making the research and production of new chemicals expensive and time-consuming (DiTomaso 2000). Chemical control procedures also require consistent follow-up removals, which makes it even more expensive in the long run. Biological control is a sustainable method of control that does not required regular follow-up applications and is relatively inexpensive to implement if effective agents are available (Paterson et al. 2021b).

Table 1.1: List of invasive cactus species currently present in South Africa with their respective categories NEM:BA. Categories 1a and b are required by the government to be controlled, removed, or destroyed. Category 2 plants need to have a valid permit and cannot be grown outside permitted areas (Department of Environmental Affairs, 2016).

Species	Common name	Category
Austrocylindropuntia cylindrica (Juss. ex Lam.) Backeberg.	Cane cactus	1a
Austrocylindropuntia subulata (Muehlenpf.) Backeb. subsp. exaltata (A. Berger) D. R. Hunt	Long spine cactus	1b
Cereus hexagonus (L.) Mill.	Queen of the night	1b
Cereus hildmannianus K. Schum.	Queen of the night	1b
Cerues jamacaru D. C.	Queen of the night	1b
Cylindropuntia fulgida (Engelm.) F. M. Knuth var. *fulgida*	Chain-fruit cholla	1b
Cylindropuntia fulgida (Engelm.) F. M. Knuth var. *mamillata* (Schott ex Engelm.)	Boxing glove cactus	1b
Cylindropuntia imbricata (Haw.) F. M. Knuth	Imbricate cactus	1b
Cylindropuntia leptocaulis (D. C.) F. M. Knuth	Pencil cactus	1b
Cylindropuntia pallida (Rose) F. M. Knuth	Pink-flowered sheathed cholla	1a
Cylindropuntia spinosior (Englem.) F. M. Knuth	Cane cholla	1a
Harrisia balansae (K. Schum.) N. P. Taylor & Zappi	Strangler prickly apple	1a
Harrisia martinii (Labour.) Britton	Moon cactus	1b
Harrisia pomanensis (F. A. C. Weber) Britton & Rose	Midnight lady	1a
Harrisia tortuosa (J. Forbes ex Otto & A. Dietr.) Britton & Rose	Spiny snake cactus	1b
Hylocereus undatus (Haw.) Britton & Rose	Night-blooming cereus	2
Myrtillocactus geometrizans (Mart.) Console	Bilberry cactus	1a
Opuntia aurantiaca Lindl.	Jointed cactus	1b
Opuntia elata Link & Otto ex Salm-Dyck	Orange tuna	1b
Opuntia engelmannii Salm-Dyck ex Engelm.	Small round-leaved prickly pear	1b
Opuntia ficus-indica (L.) Mill.	Sweet prickly pear	1b
Opuntia humifusa (Raf.) Raf	Creeping prickly pear	1b
Opuntia leucotricha D. C.	Aaron's-beard prickly pear	1b
Opuntia microdasys (Lehm.) Pfeiff	Yellow bunny-ears	1b
Opuntia monacantha Haw	Drooping prickly pear	1b
Opuntia pubescens J. C. Wendl. ex Pfeiff.	Velvet bur cactus	1a
Opuntia robusta H. L. Wendl. ex Pfeiff	Blue-leaf cactus	1a
Opuntia salmiana J. Parm. ex Pfeiff	Bur cactus	1a
Opuntia spinulifera Salm-Dyck	Saucepan cactus	1b
Opuntia stricta (Haw.) Haw. var. *stricta* and var. *dillenii* (Ker Gawl.) L. D. Benson	Pest pear of Australia	1b
Opuntia tomentosa Salm-Dyck	Velvet opuntia	1b
Peniocereus serpentinus (Lag. & Rodr.) N. P. Taylor	Serpent cactus	1b
Pereskia aculeata Mill.	Barbados gooseberry	1b
Tephrocactus articulatus (Pfeiff.) Backeb.	Pine cone cactus	1a

1.3.2 Biological control of Cactaceae

In 1913, the first biocontrol agent in South Africa was released on the cactus species, *Opuntia monacantha* Haw. (Hill et al. 2020). This agent was a cochineal insect called *Dactylopius ceylonicus* Green (Hemiptera: Dactylopiidae), which, when released, was extremely successful in reducing infestations around South Africa (Lounsbury 1915). Later, in 1934, in efforts to reduce *O. ficus-indica* populations, the release of a moth, *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) was approved for release, and in 1938 the release of a cochineal insect, *Dactylopius opuntiae* Cockerell was also approved and released, with the latter being much more successful as a biocontrol agent than the moth (Annecke & Moran 1978). Since then, biocontrol of *O. ficus-indica* has been successful countrywide with there being very few areas of concern around South Africa (Paterson et al. 2021b) (Fig. 1.3). In 1935, *Dactylopius austrinus* De Lotto, was released against *O. aurantiaca,* commonly known as the "jointed cactus" (Moran & Zimmermann 1991a). The use of *D. austrinus* as a biocontrol agent has helped reduce the cactus to minimal numbers with *O. aurantiaca* now mainly restricted to areas with high rainfall where *D. austrinus* is less effective (Mnqeta & Paterson 2024, Moran & Zimmermann 1991a). Given the success of cochineal biocontrol on invasive Cactaceae, biocontrol was regularly implemented on other cactus species such as *O. stricta* and *Opuntia stricta* var. *dillenii* (Ker Gawl.) LD Benson (Dodd 1940, Mann 1970). Given the successful control of many *Opuntia* species, biocontrol of *Cylindropuntia* species, such as *Cylindropuntia imbricata* (Haw.) F.M. Knuth and *Cylindropuntia fulgida* (Engelm.) F.M.Knuth var. *fulgida* was found to be just as successful using a species of cochineal called *Dactylopius tomentosus* Lamarck (Paterson et al. 2021b, Klein et al. 2020).

Figure 1.3: A large *Opuntia ficus-indica* invasion before (1939) and after (1979) the release of *Dactylopius opuntiae* 'ficus-indica' in Graaff-Reinet, Eastern Cape.

Cochineal insects are successful biocontrol agents because they are host specific to the Cactaceae, spread quickly, and are very damaging (Moran & Zimmermann 1991b). Cochineal are sap-sucking insects that have a complex life cycle (Fig. 1.4). Adult females can lay hundreds of eggs that emerge into small nymphs, also known as 'crawlers', which go through two moults before reaching adulthood (Volchansky et al. 1999). Male crawlers develop into cocoons and emerge as winged adults (Volchansky et al. 1999). Female crawlers differ from male crawlers as once they develop into an adult, they insert their mouthparts into the cladode and become sessile. Once adult females start feeding on a cladode, their mouthparts become immovable and will feed and mate in one spot until death (Volchansky et al. 1999). Adult males, once they emerge from their cocoon, find mature adult females and mate and only live for about 3-5 days. When cochineal feed on a host plant, the cladodes turn yellow and die because their saliva is thought to be toxic to cactus (Torres & Giorgi 2018). Over time, the smaller and younger cladodes are the first to die and fall off the plant, and once a cactus plant is fully infested with cochineal, most of the plant will have fallen to the ground, and the

only remains would be that of the woody stems (Paterson et al. 2011). There is a small difference in cochineal developmental time across the *Dactylopius* species, but generally, cochineal will go through 3-6 generations per year, depending on the particular cochineal species (Guerra & Kosztarab 1992). Factors such as rainfall could limit the spread of crawlers as it can remove the waxy layers of the cactus, exposing them to predators, and in intense rain, can even wash them off the cactus (Moran et al. 1987). Since cochineal are the primary option for biocontrol against invasive cacti species there are several species that are mass-reared and redistributed around the country (Hill et al. 2021).

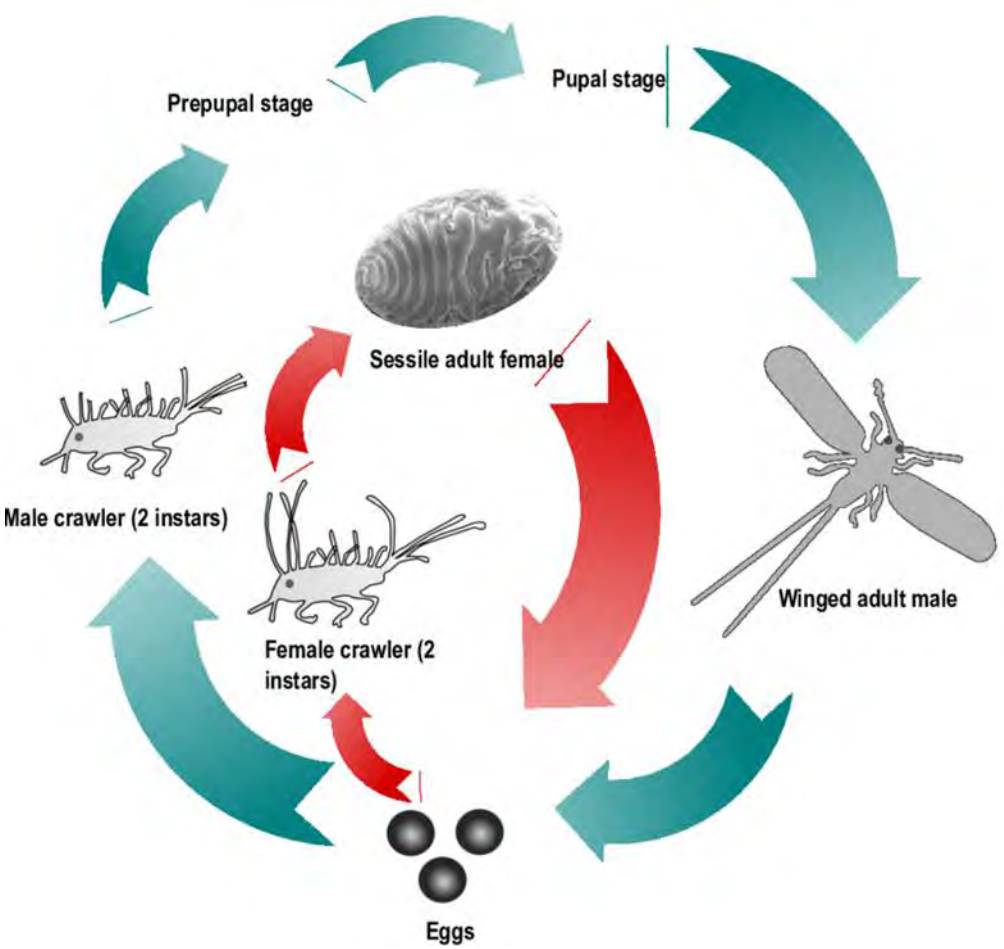

Figure 1.4: Life stages of cochineal insects (From Van Steenderen 2020).

Biocontrol of cactus is generally very successful with most of the agents that have been released on invasive cactus species in South Africa resulting in substantial or complete control (Moran et al. 2021). Today, 15 cochineal biocontrol agents, which include different varieties and lineages, have been released against 15 different cactus species, with 13 species being classified as being under substantial or complete control (Moran et al. 2021, Klein 2011, Paterson et al. 2021b). Since more cactus species are still being introduced into South Africa, it is likely that more cactus species will become naturalized and eventually become problematic (Novoa et al. 2015). Given the successful use of cochineal as biocontrol agents in South Africa, it is likely that using cochineal will be the best option for biocontrol for new emerging cactus invaders (Paterson et al. 2021b).

1.4 *Cylindropuntia pallida* (Rose) F.M Knuth (Cactaceae)

Cylindropuntia pallida [Synonyms: *Cylindropuntia rosea*, *Cylindropuntia rosea* var. *atrorosea* Backeb. *Opuntia rosea* DC. *Grusonia rosea* (DC.) G.D. Rowley *Opuntia pallida* Rose] is also commonly known as the "Douglas pest" or the "Thistle cholla". *Cylindropuntia pallida* is a member of the Bigelovii tribe in the *Cylindropuntia* genus which occurs in its native distribution of Hidalgo, Mexico (Verloove & Guiggi 2020, Bárcenas 2016). The *Cylindropuntia* genus contains approximately 40 species, which have the unique characteristic of having smooth spines covered by a loose papery sheath (Mayer & Rebman 2021). Some research has suggested that *C. pallida* is a hybrid of *C. imbricata* and *Cylindropuntia tunicata* (Lehm.) F.M. Knuth, however, this is yet to be clarified (Laguna et al. 2013). Initially, in South Africa, it was misidentified as *C. fulgida* var. *fulgida* due to its striking similarity in morphological appearance, but it is now known to be *C. pallida* (Henderson & Zimmermann 2003). It has also been misidentified as *C. tunicata*, given its similar morphological appearance, but is identified by the colour of its flower and growth form (Appendix). *Cylindropuntia pallida* has a bush growth form that can reach around 70-80 cm in height and 250 cm in diameter. Like the other species in this genus, the pale green cladodes are cylindrical in shape and are around 42cm in length. The spines are, on average, between 3-5cm in length, and around 10-14 spines can be found per

cladode. The spines are covered by loose sheaths that slide off when pulled. The fruit is around 3-4 cm in length and is covered by spines. They occur singly and are pale green to yellow in colour. The fruit contains many seeds that are pale brown, oval-shaped, and, on average, are around 1mm in length. The feature of *C. pallida* that helps distinguish it from other *Cylindropuntia* species is the salmon-pink colour of the flowers (Fig. 1.5).

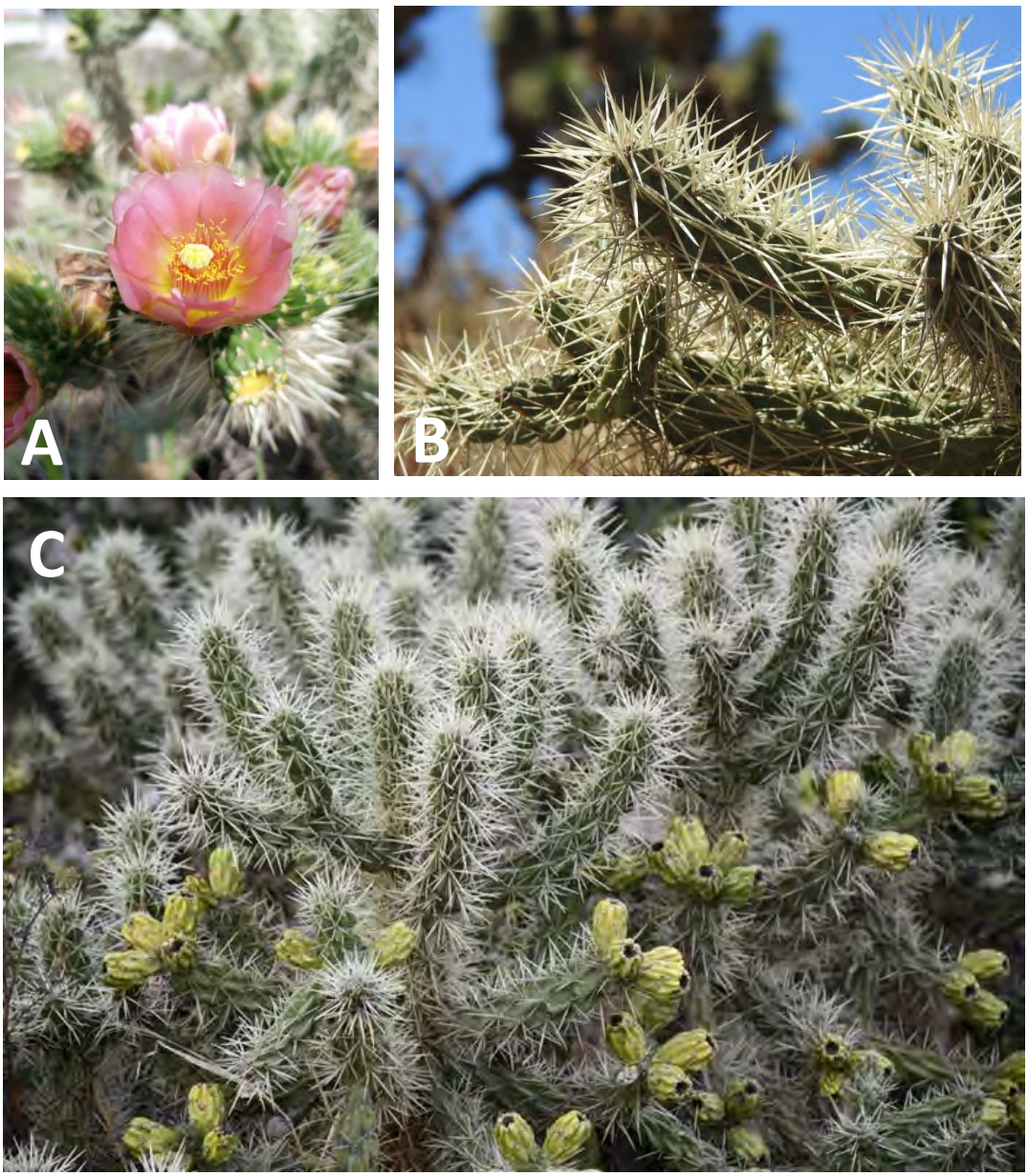

Figure 1.5: A.) A typical *Cylindropuntia pallida* flower with its salmon-pick coloration. B.) Long, green cylindrically shaped cladodes with "pallid" white spines. C.) A large *C. pallida* plant with green/yellow fruit.

1.4.1 Invasive distribution and biology

Cylindropuntia pallida has become a problematic invasive weed in parts of South Africa, Australia, Saudi Arabia, Spain, and Namibia (Deltoro et al. 2013, Jones et al. 2015, Al-Robai et al. 2018, Paterson et al. 2019, Paterson et al. 2021b, Sáez 2016). The cladodes of *C. pallida* can easily fall off the main plant and be carried away by vectors, such as vehicle tyres and animal fur, allowing their populations to spread. Like other invasive cactus species, the plant becomes overabundant and has large spines that are harmful to livestock, and are contaminants of wool, which reduces yields of wool and mohair (Paterson et al. 2021b). The spiny thickets that form in areas with *C. pallida* infestations restrict human and animal movement, reducing access to grazing, shade, and water sources (Fig. 1.6) (Paterson et al. 2021b). Not only does it restrict animal movement, but it can also be dangerous as the spines can impale animals (Deltoro et al. 2013). Further to these issues, *C. pallida* can outcompete native species and become a threat to indigenous biodiversity and ecosystem functioning. In South Africa, *C. pallida* has invaded many areas of agricultural land (Fig. 1.7) in the Eastern Cape and was initially controlled using chemical control (Mokotjomela et al. 2024). This was effective initially as it reduced numbers at the majority of sites where it had been recorded. However, this proved to be ineffective in the long term as complete eradication from sites was rare, with regrowth occurring even after multiple follow-up treatments (Paterson et al. 2021b).

A study by Canavan et al. (2021) compiled a list of top-priority invasive plant species that are prioritized for biocontrol in South Africa. The purpose of the paper was to ensure that investments were directed to the most appropriate weed targets. The Biocontrol Target Selection System (BCTS) was used by assessing 19 different attributes (including success elsewhere of biocontrol programs on the target plant, conflicts of interest, etc.) of invasive species and was scored and ranked on 3 key categories: 1.) Impact of target weed, 2.) Likelihood of achieving success, and 3.) Investment required (Paterson et al. 2021a). This list compiled all major invasive plant species in South Africa, and *C. pallida* was ranked 8th on the list of prioritizations for biocontrol. This further shows why *C. pallida* is an excellent target for biological control.

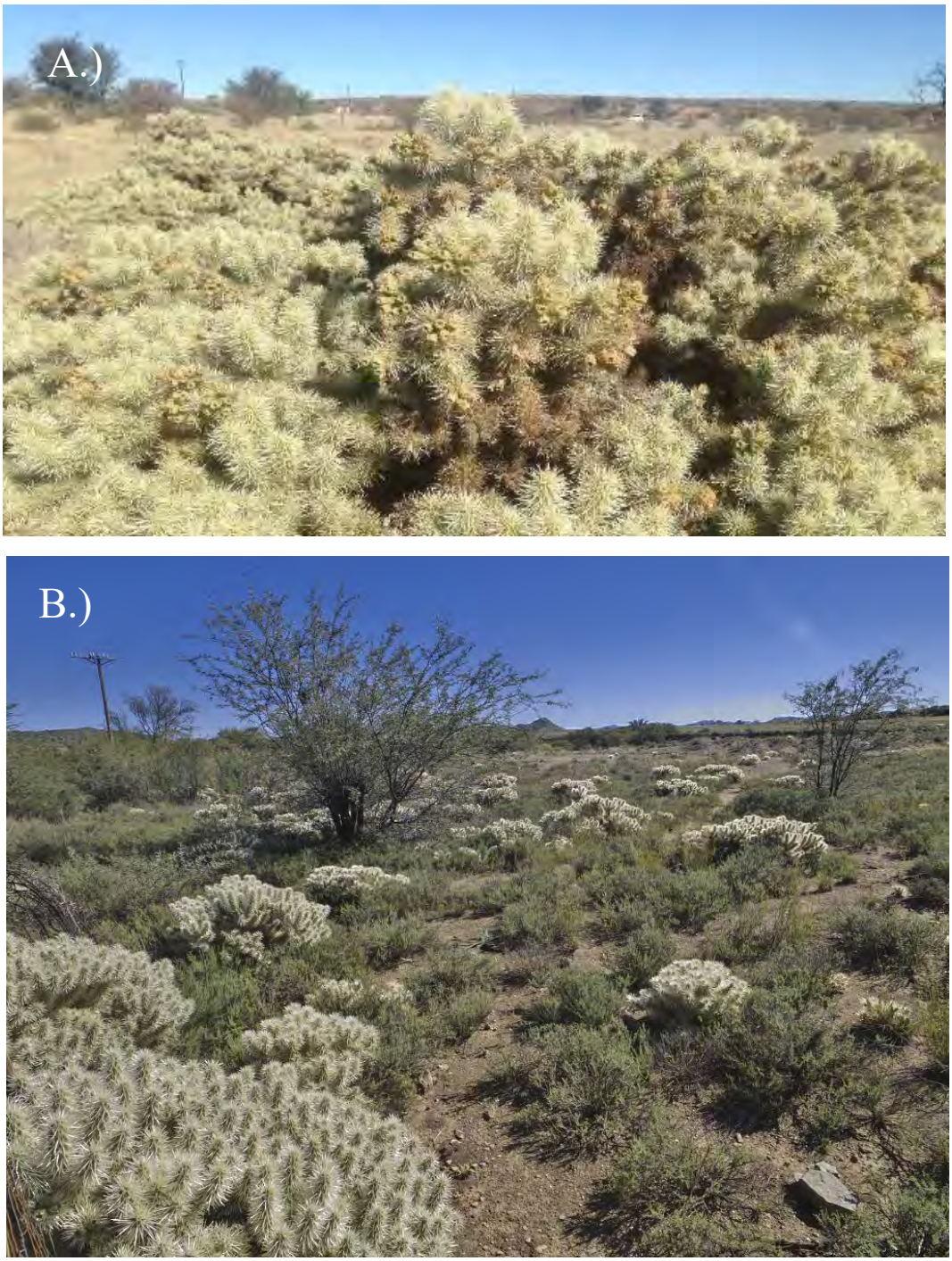

Figure 1.6: A large *Cylindropuntia pallida* infestation near A.) Hotazel, Northern Cape (27°21'54"S, 22°53'16"E) and near B.) Gariep Dam, Free State (30°38'28"S, 25°28'08"E).

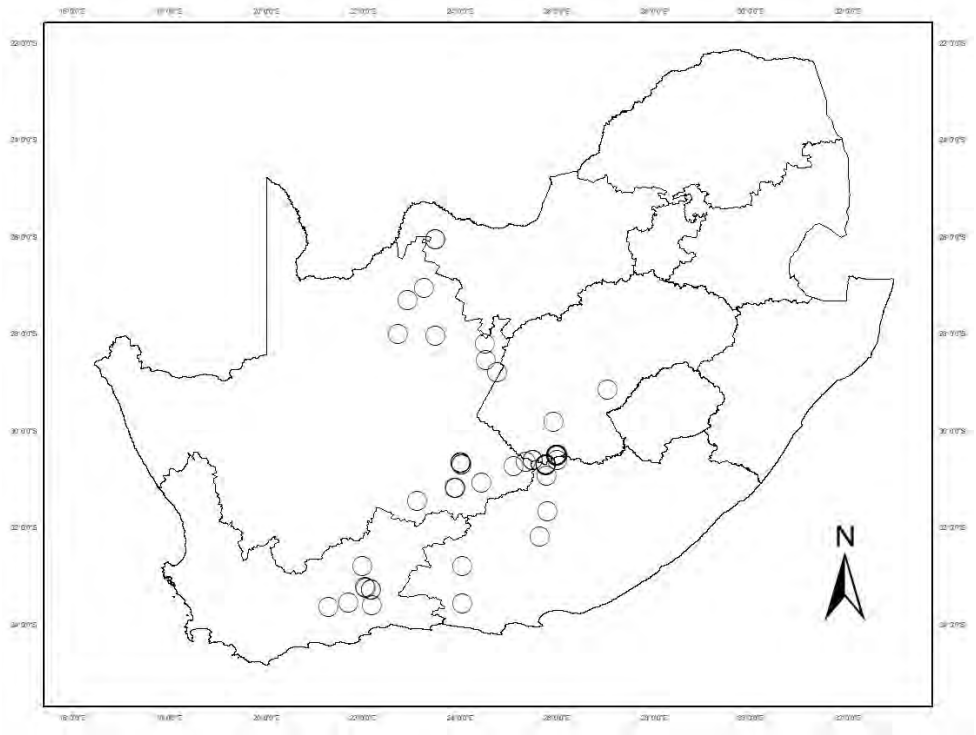

Figure 1.7: Invasive distribution of *Cylindropuntia pallida* in South Africa.

1.5 Aims & rationale

Given that *C. pallida* is a high-priority category 1b plant in South Africa, control methods must be implemented to help alleviate the ecological and economic damage (Department of Environmental Affairs, 2016). The aim of this thesis was to find a suitable biocontrol agent for *C. pallida* in South Africa and to investigate how to select effective cochineal lineages for *Cylindropuntia* weeds.

In Chapter 2, two lineages of *D. tomentosus* were imported into quarantine in South Africa to assess whether they were suitably damaging to *C. pallida* to warrant release. This included the 'californica var. parkeri' lineage, which is used as an agent for *C. pallida* in Australia, and the 'pallida' lineage collected off *C. pallida* in its native distribution in Hidalgo, Mexico. A bioassay was conducted to evaluate the fitness of each cochineal lineage on the target weed. The lineage with the best

performance will be prioritized as a biocontrol agent and undergo host-specificity testing to determine if it is suitably host specific for release in South Africa.

The 'californica var. parkeri' lineage represents a 'new association' and the 'pallida' lineage' represents an 'old association'. A 'new association' is defined as a biocontrol agent utilizing a host which it has not co-evolved with ('californica var. parkeri' on *C. pallida*). 'Old associations', are described as a biocontrol agent utilizing a host that it has a long-standing evolutionary relationship in the indigenous distribution ('pallida' on *C. pallida*). There is currently no agreement as to how to select the most damaging cochineal lineage. In most cases, various lineages are imported, and then they are all tested, and the most damaging are released (Jones et al. 2015, 2016). Knowing how to select damaging lineages in the field would be useful and therefore, in Chapter 3, an evaluation of whether 'new' or 'old' associations are better for Cactaceae biocontrol agent selection was conducted. This was carried out by testing the fitness of three *D. tomenstosus* lineages ('pallida', 'imbricata', and 'cholla') against seven invasive *Cylindropuntia* species present in South Africa to discern whether fitness decreases with phylogenetic distance from the original target weed from which the cochineal was collected off.

The overall aim of this study is to select an effective biocontrol agent for the control of *C. pallida* in South Africa and to improve how we select candidate agents for the control of invasive alien *Cylindropuntia* species in the future. This will not only contribute towards the control of *C. pallida* but also contribute towards the control of other *Cylindropuntia* species, which are becoming increasingly problematic in South Africa and several other arid areas around the world.

Chapter 2

Biocontrol of thistle cholla cactus, *Cylindropuntia pallida*: a successful agent in Australia is incompatible with invasive populations in South Africa.

2.1 Introduction

Some species of cochineal are host-specific to a cactus genus or a single cactus species (Paterson et al. 2021b). For example, *D. opuntiae* is restricted to the cactus genus *Opuntia* whilst *D. tomentosus* (Fig. 2.1) is a species of cochineal with host plants exclusively found within the *Cylindropuntia* genus (Mathenge et al. 2009a, Jones et al. 2016). The life history of *D. tomentosus* is similar to other *Dactylopius* species, except that the female will lay eggs in a mesh-shaped ball that is attached to the end of the female's posterior abdomen rather than laying them singly in a chain. The female incubates the egg mesh for around 17 days (Mathenge et al. 2009a). Once hatched, the crawlers disperse similarly to other *Dactylopius* species and begin to moult into the second instar stage in around 16-18 days. On average, from egg to an adult female capable of oviposition takes around 63.3 days (Mathenge et al. 2009a). Isolation of *D. tomentosus* on *Cylindropuntia* species, and their poor long-distance dispersal has allowed host-adapted lineages to evolve within the species in the native distribution of Southern USA and Mexico (Jones et al. 2016, van Steenderen et al. 2021). These lineages of cochineal exhibit no clear morphological differences, can interbreed with other lineages within the species, and can only be identified by their host range and/or genetic sequencing (Mallet 2005, Mathenge et al. 2015, Jones et al. 2015, 2016, van Steenderen et al. 2021). These cochineal lineages are usually named after the plant species they were originally collected from.

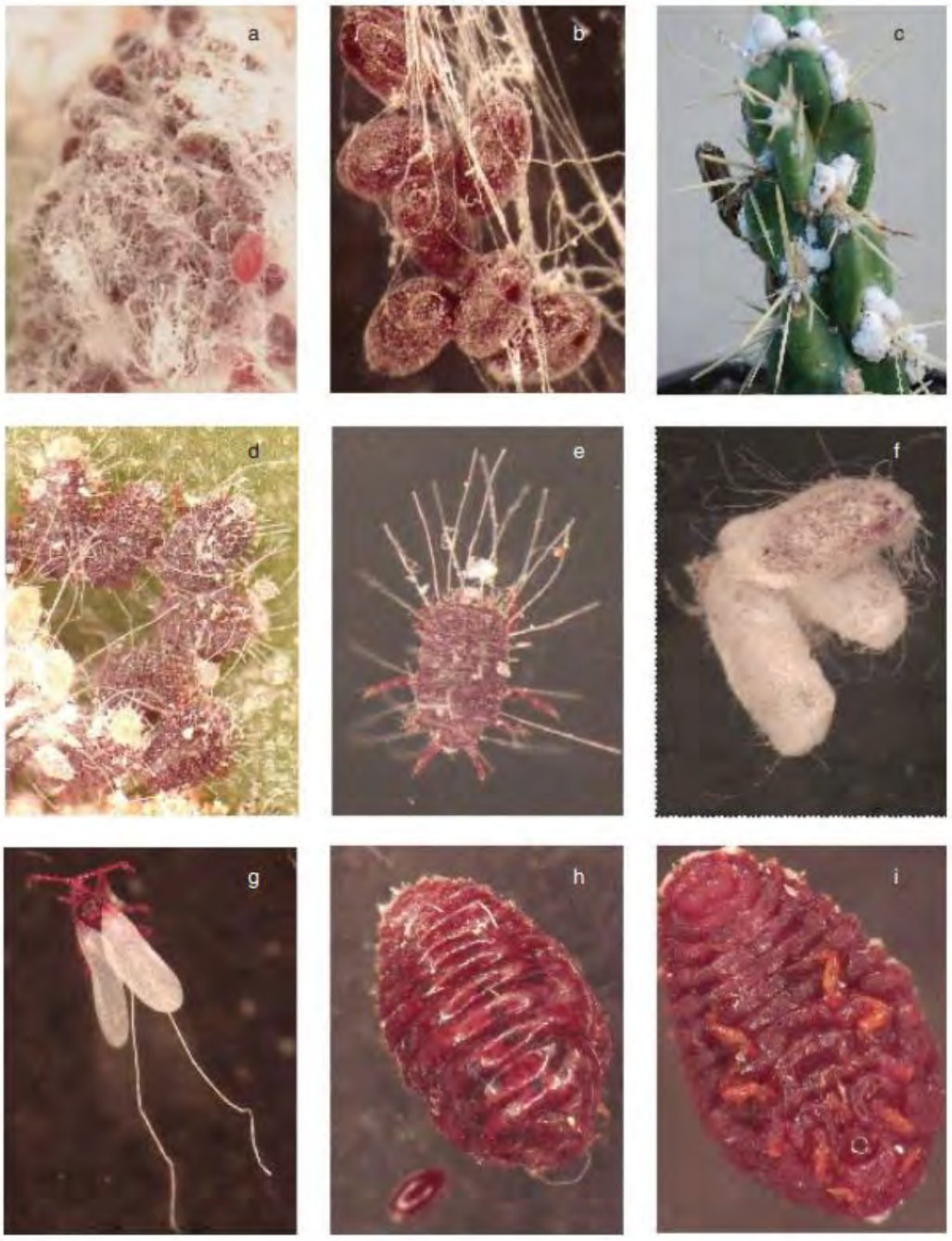

Figure 2.1: The respective life stages of *Dactylopius tomentosus.* a.) eggs covered in wax, b.) close-up of eggs, c.) *D. tomentosus* colonies, d.) crawlers settling at a feeding site, e.) close up of crawler, f.) male cocoon, g.) adult male once emerged from cocoon, h.) dorsal view of adult female, i) ventral view of adult female (Mathenge et al. 2009a).

Some lineages are damaging to particular cactus species but are less effective on others, so it is essential that the correct lineage is released on the correct cactus species to achieve effective control (Mathenge et al. 2009b). In South Africa, the use of the *D. opuntiae* 'ficus' and 'stricta' lineages on *O. ficus-indica* and *O. stricta* respectively, has been extremely successful in reducing the overall infestations and biomass of these species, but using the incorrect lineage (ie., the 'ficus' lineage on *O. stricta*, or the 'stricta' lineage on *O. ficus-indica*) resulted in negligible damage (Volchansky et al. 1999, Githure et al. 1999). In 2008, the use of the *D. tomentosus* 'cholla' lineage resulted in the control of *C. fulgida* var. *fulgida* in South Africa (Klein et al. 2020). This lineage has continued to be an effective agent on *C. fulgida* var. *fulgida* in South Africa as well as *Cylindropuntia fulgida* var. *mammillata* (Schott ex Engelm.) in Australia and South Africa (Jones et al. 2023). Further to this, *C. imbricata* and *Cylindropuntia leptocaulis* (DC.) F.M. Knuth are both under efficient control by the *D. tomentosus* 'imbricata' lineage (Paterson et al. 2021b). Given the success of *D. tomentosus* lineages in the past on *Cylindropuntia* species, biocontrol is considered the best option for the management of *C. pallida* in South Africa (Paterson et al. 2021b).

A lineage of cochineal called *Dactylopius tomentosus* 'californica var. parkeri' was collected from Baja California, Mexico, from the cactus species *Cylindropuntia bernardina* Engelm. ex Parish (=*Cylindropuntia californica* var. parkeri (J.M. Coult.) Pinkava) to control invasive populations of *C. pallida* in Australia (Paterson et al. 2021b). Since cochineal lineages are by convention referred to by the host plant they are collected off, the cochineal released in Australia is referred to as the 'californica var. parkeri' lineage as that was the name of the plant species when it was collected. Testing conducted under quarantine conditions in Australia showed that it was a suitably host-specific and damaging agent for *C. pallida* in Australia (Paterson et al. 2021b). This resulted in the agent's release, and initial results show that it has successfully reduced *C. pallida* infestations in Australia (Unpublished data. A.J. McConnachie). The success of the agent in Australia, and the success of sharing agents between the countries in the past, suggested that *D. tomentosus* 'californica var. parkeri' would be a suitable agent for consideration in South Africa (Paterson et al. 2021b). *Dactylopius tomentosus* 'californica var. parkeri' was imported into quarantine in South Africa to

assess its compatibility with South African *C. pallida* plants. Early signs of poor survival of the 'californica var. parkeri' lineage on South African *C. pallida* during rearing, indicated that it might not be suitable as an agent. Therefore, a second cochineal lineage collected off *C. pallida* in its native range of Hidalgo, Mexico, was brought into quarantine in South Africa, and referred to as *D. tomentosus* 'pallida'.

Insects with limited dispersal ability are likely to develop adaptations to a particular population of a plant species. This is thought to be the case since insects often go through much faster generation times than their hosts (Karban 1982). This can eventually lead the insects to locally adapt to a specific host plant genotype (Edmunds & Alstad 1978). Due to these local adaptations, the insects may not be able to utilize a different population of the same species that is geographically isolated from the populations it has adapted to. This can also be because different plant populations have locally adapted to different conditions in their respective distribution and could have possibly developed different defences that can defend itself better against these insects (Karban 1982). These local adaptations can lead to agent-plant mismatches. This describes a situation where an agent performs poorly on a particular population of the target weed, leading to reduced efficacy and having to find other methods of control or other biocontrol agents for that particular population (Manrique et al 2008).

The aim of this study was to compare the compatibility of the two lineages of cochineal with South African *C. pallida*. This included the successful agent used in Australia, *D. tomentosus* 'californica var, parkeri', and the new potential agent collected off the same species as the target weed in the indigenous distribution, *D. tomentosus* 'pallida'. If either are compatible with South African *C. pallida*, they will be subjected to host-specificity testing and considered for release on *C. pallida* in South Africa.

2.2 Methods & Materials

2.2.1 Collection of cochineal and cladodes

Dactylopius tomentosus 'californica var. parkeri' was collected at a mass-rearing facility in Lightning Ridge in Australia (29°26'12"S, 147°58'54"E), where the agent is mass-reared on *C. pallida*. *Dactylopius tomentosus* 'pallida' was collected off *C. pallida* plants in its native distribution: Hidalgo, Mexico (19°52'23"N, 98°48'31"W). The two cochineal lineages were then imported into the Centre for Biological Control Quarantine Facility, at Rhodes University, Eastern Cape, South Africa ('pallida' = Permit number: P0116839, 'californica var. parkeri' = Permit number: P0109765). All predators found in the cultures were removed. Cochineal-free cladodes of *C. pallida* were collected from the invasive distribution in Cumborah, Australia (29°38'6"S, 147°40'40" E) as a positive control for the 'californica var. parkeri' lineage. The same was done for the 'pallida' lineage collected in Hidalgo, Mexico, at the same site where the cochineal was sourced (19°52'23"N, 98°48'31"W). South African *C. pallida* cladodes were collected in Cradock, Eastern Cape (32°09'51"S, 25°37'09" E).

2.2.2 Experimental set-up

For each cochineal lineage ('californica var. parkeri' and 'pallida'), ten newly emerged crawlers were inoculated onto a single South African *C. pallida* cladode and ten crawlers were placed onto the respective control cladodes (either Australian or Mexican cladodes). Crawlers were removed from their respective cultures using a fine paintbrush and placed in a petri dish, counted under a microscope, and then transferred carefully onto each cladode. The cladodes were separated in closed containers with a mesh covered hole in the lid for ventilation while ensuring that the crawlers could not escape. This was replicated ten times for each cochineal lineage.

Two separate experiments were conducted, one comparing the 'californica var. parkeri' cochineal on South African and Australian cladodes, and the other comparing the 'pallida' cochineal on South African and Mexican cladodes. Having two cochineal lineages within the same facility at the same time was not possible due to the potential for cross contamination through interbreeding. The two

experiments were therefore undertaken at different times and the data are analysed separately for this reason. The quarantine facility was maintained at 25°C with a 14:10 day: night cycle.

2.2.3 Measurements

Identifying damaging cochineal lineages that are compatible with the target weed is an important step in many cactus biocontrol projects (Paterson et al. 2021b). Fitness measurements, such as survival, female weight, days to emergence of offspring, and number of progeny, are good indicators of a cochineal biocontrol agent's compatibility with a host plant (Mathenge et al. 2009b, Czypionka & Hill 2007, Grevstad 1999). These measurements can give a better understanding of whether the potential agent will be an effective agent against the host plant if it were released.

2.2.3.1 Settlement

The proportion of crawlers that settled on each replicate was recorded as an indicator of insect survival. Measurements were taken after two weeks because this is the period when crawlers have either settled on the plant or died (Mathenge et al. 2009a). Settlement was recorded by counting the number of crawlers that had started producing wax (Mathenge et al. 2009a). If a crawler did not produce wax two weeks after inoculation it was presumed to be dead.

2.2.3.2 Female fecundity

Females that produced egg-masses and/or crawlers before 90 days were considered to have reproduced (Mathenge et al. 2009a). Females that had not produced offspring by this time either failed to develop to sexual maturity or were not mated. Only females that had reproduced were included in this fitness metric. For the females that did reproduce, the egg mass and wax associated with each female were removed as soon as eggs/crawlers were seen and placed in separate containers. Females were removed by gently rolling the side of a pin against the female's body under the wax, so that the

female was detached from the cladode. Using a small pin was necessary because the female's mouthparts were embedded into the cladode and a pin gave just enough force to gently remove them from the cladode. Once removed from the cladode, each female was dewaxed gently with a pin, and the female and egg mass were placed in separate containers. After 30 days, the number of progeny produced per female was counted, which was the sum of both the number of eggs and crawlers per reproducing female. This time period was chosen to give the females time to produce all the eggs possible (Mathenge et al. 2009a).

2.2.3.3 Female mass

All surviving females were weighed, including those that did not reproduce, using a Disney fine-balance scale to the nearest (0.01 mg). Adult females were identified by their distinct second moult, as well as their size, since gravid females are larger than non-gravid females (Mathenge et al. 2009b). For females that did reproduce, female body mass (mg), once the wax was removed, was measured using a Disney fine-balance scale to the nearest (0.01 mg). Female mass did not include the egg mass, which was removed and placed in a separate container. After 90 days (since the beginning of the experiment), females that had not reproduced were dewaxed and weighed. These females may have failed to reach sexual maturity or may have not been mated. The mass (mg) of females for each lineage was compared between those that developed on the South African cladodes and the respective controls. A correlation between female mass and number of progeny was calculated for the 'californica var. parkeri' lineage. This only included females that reproduced. For the 'pallida' lineage, the correlation between female mass and number of progeny was not determined. Males were only present in two replicates of the 'pallida' experiment, so the females in the other eight replicates were not mated with and, therefore, did not reproduce. There were, therefore, insufficient numbers of progeny to produce a correlation. The lack of males in most replicates was likely due to chance, as the sex of the crawlers was unknown at the time of inoculation.

2.2.3.4 Days to emergence of offspring

Days to development of offspring was measured as the time crawlers were first seen emerging from the wax of an individual female (Mathenge et al. 2009a, 2015). Therefore, days to development of offspring were only recorded from mated females and not non-mated females. All females that did not reproduce were removed after 90 days.

2.3 Statistical analyses

Insect performance metrics were analysed using a series of general linear models (GLM) and generalised linear mixed models (GLMM), whereby the GLMMs were used to account for non-independence of data points, where necessary (Bolker et al. 2009). For both lineages of cochineal, the proportion of crawlers that settled after two weeks was modelled as a function of the origin of the cladodes that were tested (Australia/Mexico vs South Africa), which was specified as a categorical predictor variable. The model was specified assuming a binomial error distribution with a logit link function, and accounting for unequal variance between females from the same replicate using a random intercept term (Bolker et al. 2009). Female fecundity was modelled as a function of the origin of the cladodes tested, which was specified as a categorical predictor variable. For both the 'californica var. parkeri' and 'pallida' lineages, the GLM was specified assuming a negative binomial error distribution with a log link function, and accounting for non-independence between females from the same replicate using a random intercept term (Bolker et al. 2009). Female body mass (mg) was modelled as a function of the origin of the cladodes that were tested (Australia/Mexico vs South Africa). We assessed whether female body mass differed between the two cochineal lineages ('pallida' and 'californica var. parkeri') and which country each lineage was sourced from (Australia/Mexico vs South Africa), using a gaussian error distribution and an identity link function. For the 'californica var. parkeri' lineage, we relaxed the assumption of equal variance between countries to account for unequal variance. To assess whether there was a correlation between female

body mass (mg) and the number of progeny produced, a negative binomial GLM was specified, with female body mass specified as a continuous predictor. Originally, this model was specified as a GLMM with a random intercept term to account for repeated measurements taken from the same replicate (Bolker et al. 2009). However, the final model was simplified to a GLM due to singularity issues with the GLMM, whereby the within-replicate variation was not sufficient to warrant inclusion in the final model specification (Barr et al. 2013). Female mass was log-transformed prior to model fitting to stabilise its variance. Days to development of offspring was modelled as a function of the origin of the cladodes that were tested. For both the 'californica var. parkeri' and 'pallida' lineages, the GLM was specified assuming a Poisson error distribution with a log link function, and accounting for non-independence between females from the same replicate using a random intercept term (Bolker et al. 2009). Hypothesis testing was performed for each measurement using a likelihood ratio test. For the survival and female mass GLM, a Wald's test, with robust standard errors was used to account for unequal variances between females ($P < 0.05$) (Hothorn & Zeileis 2015). All statistical analyses were done in R version 4.3.3 (R Core Team, 2023). All values presented in the manuscript are mean ± 1 standard error, unless stated otherwise.

2.4 Results

2.4.1 Settlement

For the 'californica var. parkeri' lineage, there was evidence for a statistically significant difference in crawler settlement on the South African cladodes compared with the Australian cladodes ($x^2 = 4.82$, df = 1, P = 0.028) (Fig. 2.2a). Crawler survival was approximately 28 % higher when reared on Australian cladodes (42 ± 9 %), compared with the South African cladodes (14 ± 6 %). For the 'pallida' lineage, there was no evidence of a statistically significant difference in crawler settlement on the South African cladodes compared with the Mexican cladodes ($x^2 = 0.62$; df = 1; P = 0.433). The median crawler settlement on the Mexican cladodes was 48 (± 4) % compared with 40 (± 8) % on the South African cladodes (Fig. 2.2b).

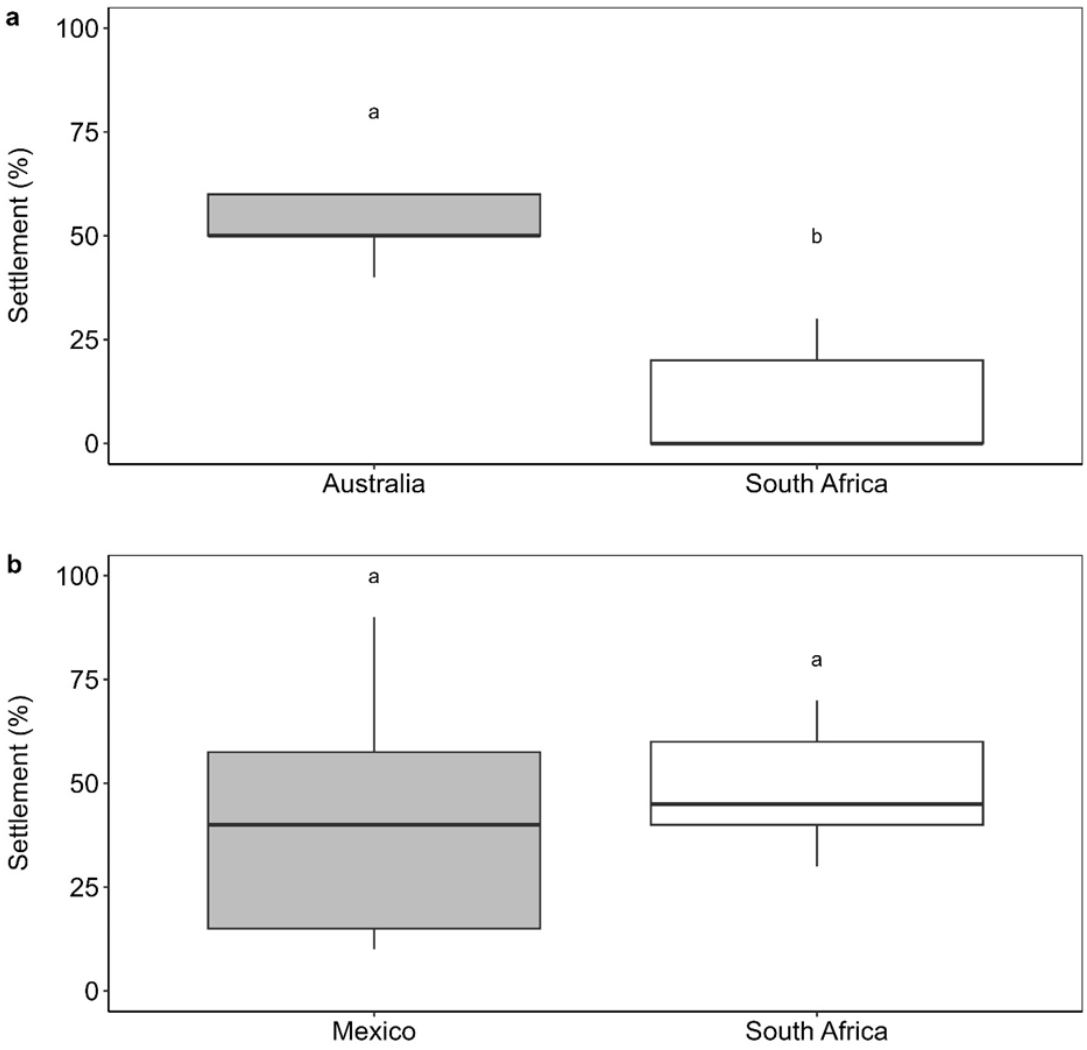

Figure 2.2a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges a.) settlement percentage of crawlers of *Dactylopius tomentosus* 'californica var. parkeri' on Australian and South African *Cylindropuntia pallida* and (b.) *D. tomentosus* 'pallida' lineage on Mexican and South African *C. pallida*. Different small case letters indicate significant differences between groups ($p < 0.05$).

2.4.2 Female mass

For the 'californica var. parkeri' lineage, there was evidence for a statistically significant difference in female mass between adult females on the South African cladodes compared with the Australian cladodes ($x^2 = 3.67$, df = 1, P = 0.055). The average female mass on the Australian cladodes was 3.39 (± 0.57) mg compared with 0.60 (± 0.32) mg on the South African cladodes (Fig. 2.3a). There was evidence for a statistically significant correlation between the number of progeny and female mass across both lineages of cochineal for both the South African cladodes and their respective control cladodes ($x^2 = 73.6$, df = 1, P < 0.001) (Fig. 2.4). The beta coefficient was 0.45 which tells us that as the log of female weight increases by 1 unit, the number of progeny produced increases by 56.8 % on average. For the 'pallida' lineage, there was no evidence for a statistically significant difference in female mass between adult females on the South African cladodes when compared with the Mexican cladodes ($x^2 = 0.23$ df = 1, P = 0.63). The average female mass on the Mexican cladodes was 3.94 (± 0.49) mg compared with 3.64 (± 0.41) mg for the South African cladodes (Fig. 2.3b).

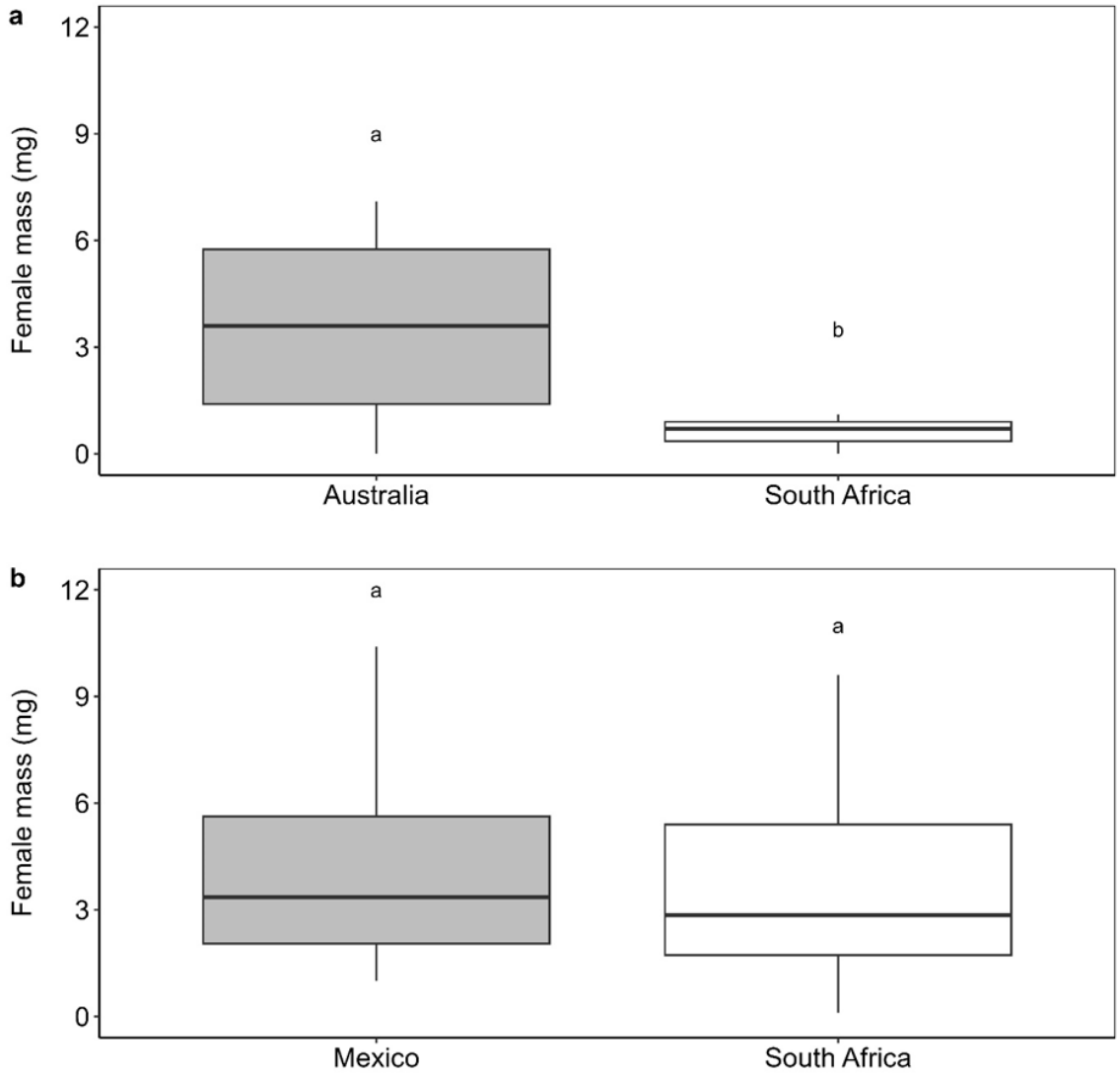

Figure 2.3a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of female mass (mg) of *Dactylopius tomentosus* 'californica var. parkeri' on Australian and South African *Cylindropuntia pallida* and (b.) *D. tomentosus* 'pallida' on Mexican and South African *C. pallida*. Different small case letters indicate significant differences between groups ($p < 0.05$).

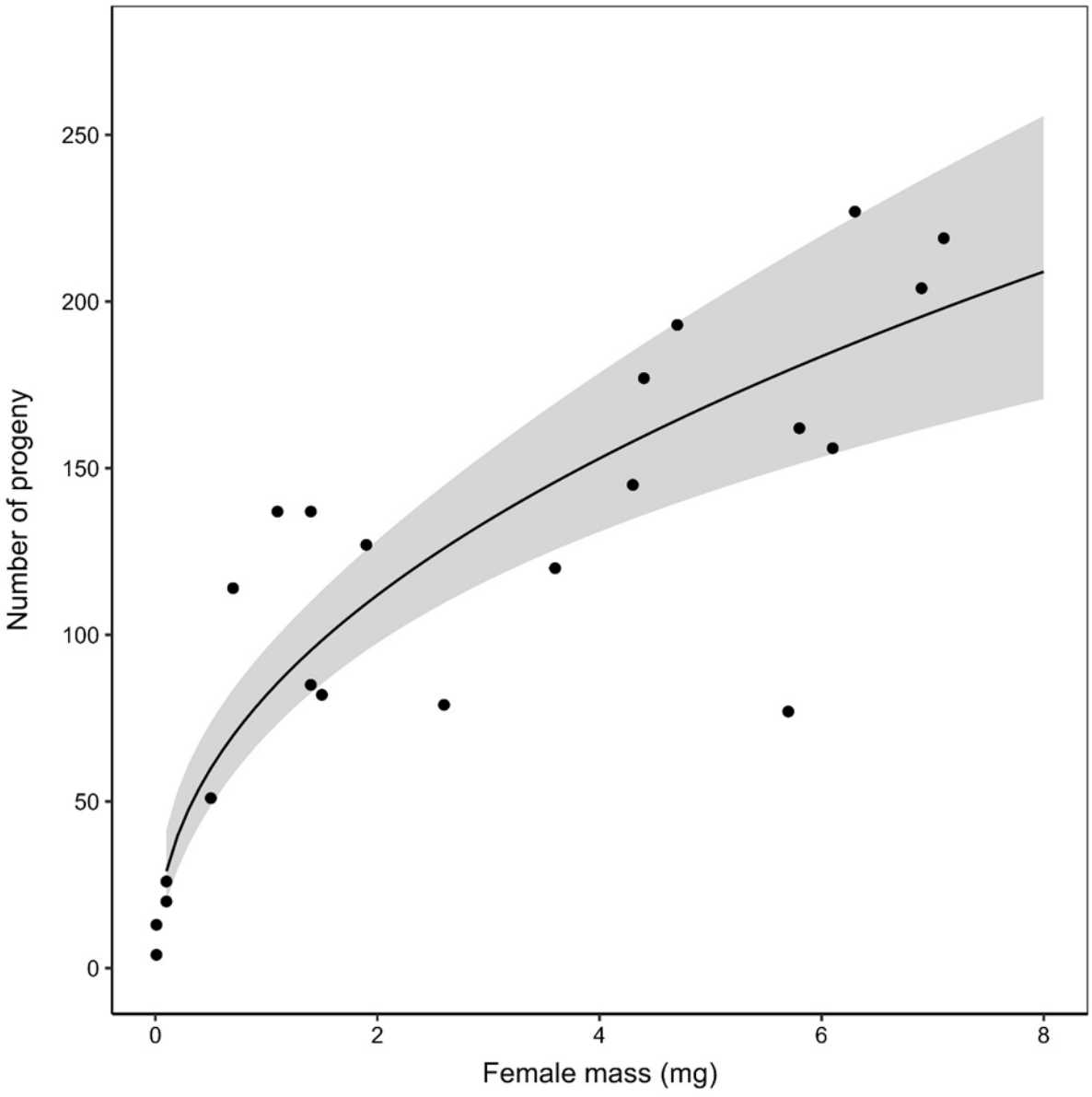

Figure 2.4: Correlation between number of progeny produced per female and the female mass (mg) for the 'californica var. parkeri' lineage. Black line represents the mean expected number of progeny per female and the shaded area represents the 95% confidence interval of the mean.

2.4.3 Female fecundity

For the 'californica var. parkeri' lineage, there was no statistically significant difference between the number of progeny produced per female on the South African cladodes compared with the Australian cladodes ($x^2 = 0.46$, df = 1, P = 0.497). The average progeny produced per female on the Australian cladodes (n = 19) was 121 (± 16) compared with 88 (± 38) on the South African cladodes (n = 3) (Fig. 2.5a). For the 'pallida' lineage, there was no statistically significant difference in the number of progeny produced per female on the South African cladodes compared with the Mexican cladodes ($x^2 = 1.49$, df = 1, P = 0.222). The average progeny produced per female on the Mexican cladodes (n = 2) was 65 (± 17) compared with 96 (± 16) on the South African cladodes (n = 8) (Fig. 2.5b).

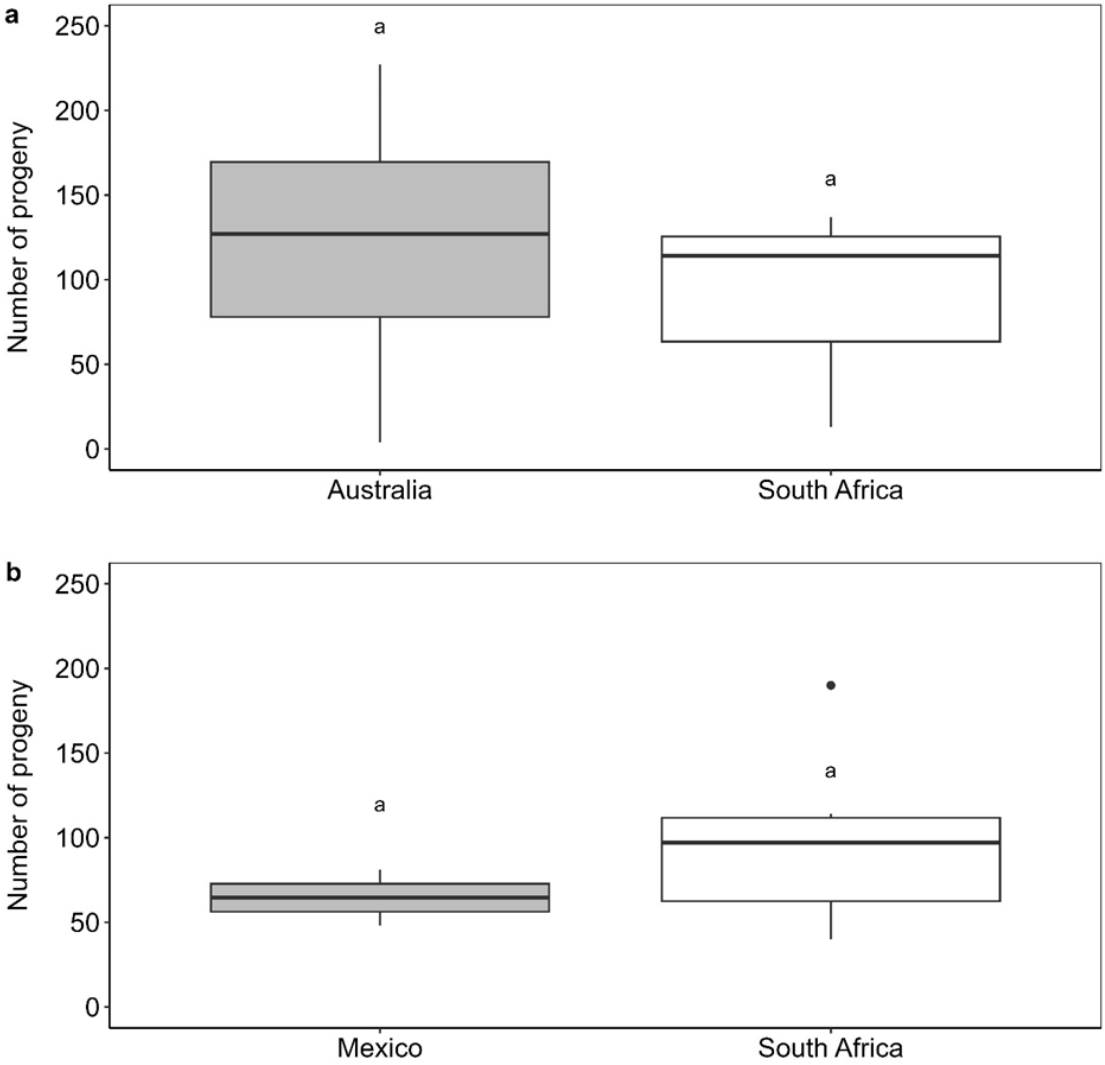

Figure 2.5a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of progeny produced by *Dactylopius tomentosus* 'californica var. parkeri' females on Australian and South African *Cylindropuntia pallida* and (b.) *D. tomentosus* 'pallida' females on Mexican and South African *C. pallida*.

2.4.4 Duration to emergence of offspring

For the 'californica var. parkeri' lineage, there was no statistically significant difference between the days to emergence of offspring per female on the South African cladodes compared with the Australian cladodes ($x^2 = 0.05$, df = 1, P = 0.818). The average days to emergence of offspring per female on the Australian cladodes (n = 19) was 59.9 (± 0.06) compared with 61 (± 0) on the South African cladodes (n = 3). For the 'pallida' lineage, there was no statistically significant difference between the days to emergence of offspring per female on the South African cladodes versus the Mexican cladodes ($x^2 = 0.44$, df = 1, P = 0.509). The average days to emergence of offspring per female on the Mexican cladodes (n = 2) was 87.5 (± 3.5) compared with 92.5 (± 2.6) on the South African cladodes (n = 8).

2.5 Discussion

Despite being an effective agent against *C. pallida* in Australia, *D. tomentosus* 'californica var. parkeri' is unlikely to be successful for biocontrol of *C. pallida* in South Africa. The 'californica var. parkeri' lineage had significantly higher settlement, female weight, and fecundity on the Australian *C. pallida* cladodes than on the South African cladodes, under the same conditions. The 'californica var. parkeri' lineage had low values of settlement, female mass, and low fecundity, which suggests that it is unlikely that populations of this cochineal would even persist on the South African plants in the field. The 'pallida' lineage had similar fitness results to that of the Mexican control plants and showed a much higher fitness than that of the 'californica var. parkeri' lineage on South African plants. These results indicate that the 'pallida' lineage should be prioritized for host-specificity testing in order to be released as a biocontrol agent against *C. pallida* in South Africa.

The differences in host suitability recorded in this study may have resulted from local adaptations of cochineal lineages to certain host plant genotypes in the indigenous distribution (Kawecki & Ebert 2004). Local adaptations in the field of biocontrol can lead to an agent-plant mismatch (Manrique et al. 2008). An example of an agent-plant mismatch is the release of the

biocontrol agent *Hydrellia pakistanae* Deonier (Diptera: Ephydridae), a shore fly used for the biocontrol of *Hydrilla verticulata* (L. f.) Royle (Hydrocharitaceae), a submerged aquatic weed, in the USA (Center et al. 1997). The agent was only able to establish populations in southern USA and was not effective and had poor establishment in the northern areas of the USA (Center et al. 1997). Studies showed that this was because a dioecious genotype occurred in the southern areas, and a monoecious genotype occurred in the northern areas, and the agent was not able to effectively attack the monoecious plants (Center et al. 1997). A similar issue was seen with the release of *Floracarus perrepae* Knihinicki & Boczek (Acariformes: Eriophyidae), a leaf-galling mite on *Lygodium microphyllum* (Cav.) (Lygodiaceae), an invasive climbing fern in Florida, USA (Boughton & Pemberton 2011). Distinct haplotypes of the plant and mite occurred in certain areas of the indigenous distribution and mites collected from the plant genotype matching the weed population were more damaging to the target weed populations (Goolsby et al. 2006a). The results of this study suggest that the population of *C. pallida* in Australia originated from a different source in the indigenous distribution, with local adaptations that make it susceptible to different cochineal lineages than the South African population. It is also possible that the californica var. parkeri' lineage developed adaptations to the Australian *C. pallida* post-release, but that these adaptations make it unsuitable on South African plants.

The taxonomic origin of *C. pallida* is still unknown, and more work is required to understand its taxonomic relationship within the genus (Laguna et al. 2013). However, it is thought that it might be a hybrid of *C. imbricata* and *C. tunicata* (Johnson et al. 2009). Some studies have shown that hybridization can complicate the search for a biocontrol agent because populations in the invasive range often have very different genotypes that are not present in the native range (Fritz et al. 1999; Ellstrand & Schierenbeck 2000). One example of hybridization complicating effective biocontrol is *Lantana camara* L. (Verbaceae) in South Africa (Baars & Neser 1999, Urban et al. 2011, Vardien et al. 2012). Biocontrol research on *L. camara* in South Africa dates back to as early as 1961, and since then 26 biocontrol agents have been released, but success has been variable because the invasive populations in South Africa are composed of multiple hybrids and ornamental varieties (Urban et al.

2011, Zachariades, 2021, Zalucki et al. 2007). The impacts of biocontrol agents differ on each of the hybrids and varieties, resulting in overall reduced levels of control (Urban et al. 2011). Given that *C. pallida* is thought to be a hybrid in its native range, it is possible that invasive populations in other countries are very different from each other. Hybrids are known to have increased genetic variability within their native habitat, and introduced populations are likely not to have been sourced from the same population in the indigenous distribution (Harms et al. 2020). This increases the likelihood that the populations in South Africa and Australia are different from each other.

Another example of hybrid populations affecting the use of agents in biocontrol is that of the native *Myriophyllum sibiricum* Kom (Haloragaceae) and the invasive *Myriophyllum spicatum* L. in the USA (Borrowman et al. 2014). Both native and introduced populations have produced hybrid offspring throughout the areas they co-occur (Borrowman et al. 2014). This has led to several difficulties in predicting the efficacy of the biocontrol agent *Euhrychiopsis lecontei* (Dietz) (Coleoptera: Curculionidae), a native weevil on these plants. The weevil showed a great deal of variability in its performance across the lineages of the *Myriophyllum* species (Borrowman et al. 2014). Therefore, given that hybrids create very different genotypes in populations, it can impact the efficacy of biocontrol agents on hybrid species and can be difficult to predict.

For other invasive cactus species, agents have been shared between Australia and South Africa with both countries having success in reducing cactus invasions (Paterson et al. 2011). Species such as *C. imbricata* are controlled in both countries using the *D. tomentosus* 'imbricata' lineage as well as *C. fulgida* var. *fulgida* and *C. fulgida* var. *mamillata* both being controlled using the *D. tomentosus* 'cholla' lineage (Paterson et al. 2019). Several *Opuntia* species such as *O. stricta* and *O. ficus-indica* are controlled respectively with the *D. opuntiae* 'stricta' and 'ficus' lineages in both countries (Paterson et al. 2021b). Sharing agents between countries has been extremely valuable and cost effective and should still be continued in the future given the success in the past. However, the results of our study indicate that factors such as local adaptation and genetic mismatches between the agent and the target weed genotype need to be taken into consideration, even if the target weeds are thought

to be the same species and are morphologically identical. An agent in one country might not always be suitable for another, as is the case for *C. pallida*.

The 'californica var. parkeri' lineage is unsuitable for *C. pallida* in South Africa, but the 'pallida' lineage could be a promising biocontrol agent. The 'pallida' lineage performed just as well on the South African plants as the 'californica var. parkeri' lineage did on the Australian plants. Therefore, if there are similarities in these fitness parameters, we could expect the 'pallida' lineage to be similarly damaging on *C. pallida* in South Africa as the 'californica var. parkeri' lineage is on *C. pallida* in Australia. Host-specificity testing of the 'pallida' lineage has commenced, and if it proves to be suitably host-specific, an application for release will be submitted to the relevant authorities in South Africa. As the species *D. tomentosus* is known to be restricted to host plants in the *Cylindropuntia* genus, it is very likely that the lineage will be suitably host specific for release.

Chapter 3

In with the old and out with the new: Are old associations or new associations better for the biological control of Cactaceae?

3.1 Introduction

Co-evolution between herbivorous insects and their host plants has resulted in host plant specialization in the majority of phytophagous insect species. Host specialization in insects is thought to have evolved because of the vast diversity of plants which possess a wide range of characteristics, such as nutritional quality and secondary metabolites (Jaenike 1990). Insects have adapted to particular characteristics of a host plant over time, which has resulted in an increase in the insect's fitness and survival on that particular host plant (Via 1990). In the same way the insects adapt to a host plant, the host plant also adapts characteristics to combat or resist the insect herbivore. This results in a decrease in the insect's fitness on other plant species, which leads to a specialized plant-insect relationship (Jaenike 1990). Isolated populations of the same insect species can also lead to host-adapted lineages (like those discussed in *D. opuntiae* and *D. tomentosus* on cactus species) to a particular plant species (Mathenge et al. 2015, Paterson et al. 2021b). This is essentially a precursor to speciation, but on a finer scale within a particular species, and the lineages that have developed can still interbreed with one another.

The practice of biocontrol takes advantage of these specialized host-adapted relationships, and it is generally thought that the closer the relationship between the agent and host plant, the more effective the agent will be, given the evolutionary history with one another (Goolsby et al. 2006b). This is why agents are usually sourced from the origin population of the target weed within the native distribution (Goolsby et al 2006a). These specialized insect-plant relationships that have co-evolved over a long

period of time are described as an ‘old association’. However, due to intentional (biocontrol agents) and unintentional releases, many insect species have moved to new areas and have formed new relationships with plant species that they did not co-evolve with, which are described as ‘new associations’ (Hokkanen & Pimentel 1984).

Problematic ‘new associations’ can come about between insects and plants when insect species enter a new environment and utilize hosts that they have not come into contact with before (Garnas et al. 2016). These species can cause damage to native plant species and agricultural plants when they enter a new environment (Goldson et al. 2005). Since the plants have never previously been associated with the insect, they fall under the category of ‘new associations’. One example is that of the Polyphagous shot-hole borer beetle *Euwallacea fornicatus* (Eichhoff) (Coleoptera: Curculionidae) and its associated fungal symbiont, *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki et O’Donnell (Hypocreales: Nectriaceae) in South Africa (de Wit et al. 2022). The beetle has now become invasive in almost all provinces in South Africa and has established on over 100 plant species, both native species and important agricultural crops. Although there are no comprehensive studies showing that *E. fornicatus* is less damaging to plant species in its indigenous distribution, theory suggests that the native plant species in South Africa are more susceptible due to the fact that they have not co-evolved with *E. fornicatus* and, therefore, have not developed suitable defences (Hokkanen & Pimentel 1984). Other examples include the Asian longhorned beetle *Anoplophora glabripennis* Motchulsky (Coleoptera: Cerambycidae), introduced from Asia into North America which killed millions of native trees (Meng et al. 2015). Many of these invasive insects, however, are generalists and have a much wider host range than that of the more specialized insects typically used in biological control (Rafter & Walter 2020). However, these generalist insects will have evolved certain adaptations to feed and survive on certain plants, and therefore, if invasive insects feed on new hosts, these hosts usually have a phylogenetic relationship with its original host species (Roques et al. 2006). ‘New associations’ are also created in the release of certain biocontrol agents, with some evidence suggesting that agents with ‘new associations’ can be just as effective, or even more effective, than agents with ‘old associations’ with the target weed (Jones et al. 2016, Hokkanen & Pimentel 1984). For example, the use of the biocontrol

agents *D. tomentosus* 'cholla' on *C. fulgid*a in both South Africa and Australia and the 'bigelovii' lineage on *Cylindropuntia spinosior* (Englem.) F.M. Knuth in Australia both represent 'new associations' (Mathenge et al. 2009b, Sheehan & Potter 2017).

There is evidence to support that 'new associations' make for effective biocontrol agents (Hokkanen & Pimentel 1984, 1989), but there is also evidence to suggest that 'old' associations' in biocontrol are more effective (Goeden & Kok 1986). Some evidence has suggested that agents which represent a 'new association' would be more beneficial as they lack the inherent homeostasis that could prevent successful biocontrol (Pimentel 1963). Essentially, 'newer associations' may be a more effective agent as the target weed may not have developed suitable defenses against a 'newer' enemy as it has not evolved alongside it and, therefore, may be more susceptible to damage (Hokkanen & Pimentel 1984). The use of 'old associations' as effective agents is justified by the evidence of a long-standing co-evolutionary relationship with a target host so that the insect has adapted to combat the plant's defenses and survive for multiple generations (Goeden & Kok 1986). The idea of 'new associations' has caused some controversy in the biocontrol community because it could potentially be a dangerous idea in the practice of biocontrol, as importing exotic pests that are less host-specific could have negative impacts on the environment (Goeden & Kok, 1986). This is because most 'new associations' insects are oligophagous, meaning that although the insect's diet breadth is limited to some extent, it can still feed on other plant species besides the target weed, making them less host-specific (Cates 1981). Ideally, monophagous insects, meaning only one host species can be utilized, are the safest options for biocontrol (Cates 1981). Monophagous agents that have 'old associations' in biocontrol have proved to be successful and are thought to be safer for releases as it is highly unlikely these agents would feed on anything other than its target host plant (Goeden & Kok 1986).

In cactus biocontrol, particularly biocontrol targeting the *Cylindropuntia* species, lineages of *D. tomentosus* are host-specific to the *Cylindropuntia* genus but have different efficacies on target species within the genus (Jones et al. 2015, 2016). Cochineal lineages are, by convention, named after the host plant they were collected from; for example, the 'pallida' lineage was named because it was collected off *C. pallida*. Investigations into the efficacies of several different lineages of *D. tomentosus* on

invasive *Cylindropuntia* species present in Australia showed variability in efficacy and host specificity amongst species in the *Cylindropuntia* genus (Jones et al. 2015, 2016). For example, the 'acanthocarpa x echinocarpa' lineage of *D. tomentosus* which was collected off a suspected hybrid between *Cylindropuntia acanthocarpa* (Engelm. & J. M. Bigelow) F. M. Knuth and *Cylindropuntia echinocarpa* (Engelm. & J. M. Bigelow) F. M. Knuth had a wide host range and was shown to be effective on several target species including *C. tunicata*, *C. imbricata*, *Cylindropuntia kleiniae* (DC.) F.M. Knuth, and *C. fulgida* (Jones et al. 2016). This indicates that 'new associations' were effective in the control of these weeds. However, the 'imbricata' and 'cholla' lineages had a much narrower host range and a preference for *C. imbricata* and *C. fulgida* respectively. Another lineage, 'cylindropuntia spp', which was collected off an unidentified *Cylindropuntia* species, also showed a wide host range and proved to be an effective agent, with efficacy trials showing plants dying between 12 and 30 weeks on *C. imbricata*, *C. kleiniae*, and *C. leptocaulis* (Jones et al. 2016). The average development time from egg to oviposition of *D tomentosus* is between 6-8 weeks, meaning that in these efficacy trials, between 2-4 generations were enough to kill these plants, making them highly effective agents (Mathenge et al. 2009b). This resulted in its release as a biocontrol agent on *C. kleiniae* and *C. leptocaulis* in Australia (Sheehan & Potter 2017, Jones et al. 2016).

This variability in efficacies raises interest into the 'new' vs 'old' association debate as most of the agents used in Australia on invasive *Cylindropuntia* species represent 'new associations' (Sheehan & Potter 2017). However, the release of the 'cylindropuntia spp' lineage shows that there is no clear indication as to what plant species to collect agents from. This is because if cochineal was collected off an unidentified species, there is clearly no concrete method or understanding as to which plant species to collect cochineal off (Jones et al. 2016). Evidence suggests that selecting an agent from the origin of the target weed can also make for a good biocontrol agent (Chapter 2). Releasing the most effective lineage is very important, and understanding how to select the most effective agent can help with the agent prioritization stage and further improving the practice of biocontrol, saving both time and resources. Due to their host-specificity and the existence of known lineages, *D. tomentosus* makes for

a good study system to investigate whether ‘old’ or ‘new’ associations are more or less damaging to *Cylindropuntia* species.

In this study the aim is to investigate whether ‘new’ or ‘old’ associations of *D. tomentosus* lineages are more effective biocontrol agents. This was done by investigating the fitness of various cochineal lineages on their primary host plants and target weeds, as well as other *Cylindropuntia* species of varying phylogenetic distance from the target weed. To do this, an experiment was designed to investigate whether there is a correlation between the fitness of cochineal lineages and phylogenetic distance from the host plant using the *Cylindropuntia* genus as an example. This study will hopefully give insight as to whether ‘new’ or ‘old’ associations are better for biocontrol on *Cylindropuntia* species and will hopefully give an indication into whether we can better select agents off the target weed itself (old associations) or from close relatives of the target weed (new associations).

3.2 Methods & Materials

3.2.1 Collection of cochineal and cladodes

Three lineages of *D. tomentosus* were tested, these being the ‘pallida’ ‘imbricata’ and ‘cholla’ lineages. The ‘pallida’ lineage was imported from Hidalgo, Mexico (19°52'23"N, 98°48'31"W) (Chapter 2). The ‘imbricata’ lineage was collected at a mass-rearing station managed by the Centre for Biological Control in Kariega, South Africa (33°43'17"S, 25°25'54"E). The ‘cholla’ lineage was collected on a population of *C. fulgida* var. *mammillata* in Prince Albert, South Africa (33°13'24"S, 22°1'47"E). Each of the *Cylindropuntia* species used in the experiment were collected at various localities around South Africa (Table 3.1).

Table 3.1: The localities of the *Cylindropuntia* species collected in their invasive range in South Africa and the current *Dactylopius tomentosus* lineage used or considered for biocontrol. The names for each species are abbreviated in brackets. These abbreviations are used in the results section.

Cactus species	**Agent used or considered in South Africa**	**Locality**
Cylindropuntia pallida (Rose) F. M. Knuth (*C. pa*)	'pallida'	Denmark farm, Cradock, Eastern Cape (32°09'51"S, 25°37'09" E)
Cylindropuntia imbricata (Haw.) F.M. Knuth (*C. i*)	'imbricata'	Dagaboer farm, Cradock, Eastern Cape (32°30'03"S, 25°50'17" E)
Cylindropuntia spinosior (Englem.) F.M. Knuth (*C. s*)	None	Beaufort West, Western Cape (32°20'20"S, 22°34'42" E)
Cylindropuntia prolifera (Englem.) F.M. Knuth (*C. pr*)	None	Beaufort West, Western Cape (32°19'49"S, 22°36'26" E)
Cylindropuntia kleiniae (DC.) F.M. Knuth (*C. k*)	None	Oudtshoorn, Western Cape (33°34'33"S, 22°12'52" E)
Cylindropuntia fulgida (Engelm.) F.M. Knuth var. *fulgida* (*C. fulvf*)	'cholla'	Prince Albert, Western Cape (33°16'02"S, 22°09'03" E)
Cylindropuntia fulgida (Engelm.) F.M. Knuth var. *mamillata* (Schott ex Engelm.) Backeberg (*C. fulvm*)		Rhodes University, Eastern Cape (33°18'38"S, 26°31'04" E)







Top left: *C. imbricata*

Middle left: *C. prolifera*

Bottom left: *C. pallida*

Bottom middle: *C. fulgida* var. *fulgida*

Top right: *C. spinosior*

Middle right: *C. kleiniae*

Bottom right *C. fulgida* var. *mamillata*

3.2.2 DNA extraction and analyses

The sequences of all species except *Cylindropuntia pallida* used in this study were taken from Mayer & Rebman (2021). Since there were no representatives for *C. pallida* available on GenBank, additional sequences for the species were obtained using the PCR protocol described by Mayer & Rebman (2021). Sequences of *C. imbricata*, *C. prolifera*, *C. spinosior*, *C. kleiniae*, *C. fulgida* var. *fulgida*, and *C. fulgida* var. *mamillata* were available on Genbank and therefore were downloaded for use in this study.

Mayer and Rebman (2021) sequenced two intergenic spacer regions, namely using the *trnH*$^{(GUG)}$*-psbA* and *trnQ*$^{(UUG)}$*-5′rpS16* primers. Both primer pairs were tested in the present work, but the *trnQ*$^{(UUG)}$*-5′rpS16* region consistently produced large sections of noisy chromatogram peaks with low-quality scores. Only the *trnH*$^{(GUG)}$*-psbA* region was therefore retained for further analysis.

3.2.2.1 Extraction and PCR protocol

DNA was extracted from *C. pallida* using the CTAB extraction protocol (Doyle & Doyle 1987). For both primer pairs, a 25 µL PCR reaction mix, composed of 9 µL of PCR grade water, 1 µL of forward primer, 1 µL of reverse primer, 12 µL Taq, 0.5 µL of BSA, and 2 µL of DNA template (approximately 60 ng/ml) was used. For both primer pairs, amplification of the target regions was conducted according to the following PCR protocol: 80°C, 5 min; 35 cycles (95°C, 1 min; 50°C, 1 min with a ramp of 0.3°C/s; 65°C, 4 min); 65°C, 5 min. Products were viewed on a 1% agarose gel, and successfully-amplified samples were sent to Macrogen Europe (https://order.macrogen-europe.com/) for sequencing in the forward direction.

3.2.2.2 Sequence analysis

Sequences were manually inspected and trimmed where appropriate to ensure high-quality output and aligned in the online MAFFT v7 (https://mafft.cbrc.jp/alignment/server/) server using the default parameters (Katoh et al. 2019).

3.2.3 Phylogenetic distance

The similarity of pairwise distances (p-distances) between DNA sequences of species has helped with understanding which species are more genetically similar than others (Van de Peer & Salemi 2009). Therefore, utilizing p-distance metrics with up-to-date DNA sequences of a particular set of species could be a useful tool in measuring fitness across closely related species and identifying whether there is a correlation between fitness measurements and phylogenetic distance. Phylogenetic distance was calculated as the p-distance between the 'host plant' of each cochineal species tested and the other plant species tested. For example, for the 'pallida' cochineal, *C. pallida* plants were defined as the host plant, and the pairwise genetic distances were calculated between *C. pallida* and the remaining plants for each cochineal species. The analysis was run in MEGA-11, where 1000 bootstrap repeats were applied with default settings (K2P parameter model with partial deletion) (Tamura et al. 2007).

3.2.4 Experimental set up

Each lineage ('cholla', 'pallida' and 'imbricata') was tested on seven different *Cylindropuntia* species, including two varieties of *C. fulgida* collected from various localities in South Africa (Table 3.1). For each *Cylindropuntia* species, a single cladode was placed in a container and inoculated with ten crawlers of a single *D. tomentosus* lineage. Crawlers were removed from their respective cultures and inoculated in the same way as described in Chapter 2. This was replicated ten times for each plant species and each cochineal lineage (3 cochineal lineages X 7 cactus species = 21 treatments). Measurements were taken of the crawler's development and survival every 3-4 days. Once males began to pupate, all cladodes of the same species were placed in a closed insect cage so that adult males would have a chance to mate with more females. This was done because, if by chance, no males emerged in a container, then no reproduction would take place, and this would affect the fitness calculations.

Having multiple cochineal lineages within the same facility at the same time was not possible due to the possibility of cross-contamination through interbreeding. The 'pallida' lineage is still undergoing host-specificity testing and, therefore, was conducted in a quarantine facility at 25°C with a 14:10 day:

night cycle. The ‘cholla’ and ‘imbricata’ lineages were conducted at the same time but in two separate labs to avoid cross-contamination under similar conditions to the quarantine facility.

3.2.5 Measurements

3.2.5.1 Survivorship

The proportion of crawlers reaching various developmental life stages, including 1st instar, 2nd instar, adult, and those that reproduced were counted. Survival to the 1st instar was counted once crawlers started producing wax. Survival to the 2nd instar was recorded when crawlers had moulted, and the discarded exuviate was present. Adult females were identified and counted when the experiment was concluded, and females were weighed. The total number of individuals involved in reproduction was counted for each replicate once the experiment was concluded. This included all females that had reproduced and any adult males that emerged. For each of the three cochineal lineages tested (‘imbricata’, ‘pallida’, and ‘cholla’), the correlation between the proportion of crawlers that survived to each life stage (1st instar, 2nd instar, adult, and reproduction) and phylogenetic distance between plant species tested was modelled as a function of the phylogenetic distance, which was specified as a continuous predictor variable. The model was specified assuming a binomial error distribution with a logit link function, and accounting for potentially autocorrelated measurements taken from the same plant species across cochineal lineages using a random intercept term (Bolker et al. 2009). A cochineal by phylogenetic distance interaction term was included in the saturated model to test the hypothesis that the phylogenetic distance correlation with insect fitness parameters was cochineal-species specific. Hypothesis testing was performed using a series of likelihood ratio tests comparing the saturated model with a null model without the interaction term, and another null model without the interaction term and the phylogenetic distance term.

3.2.5.2 Female Fecundity

Female fecundity was measured in the same way as described in Chapter 2. The only difference is that females that did not reproduce are included here for the statistical analyses. For each of the three cochineal lineages tested, the correlation between the number of progeny produced per female and phylogenetic distance between plant species tested was modelled as a function of the phylogenetic distance, which was specified as a continuous predictor variable. We included a cochineal by phylogenetic distance interaction term in the saturated model to test the hypothesis that the phylogenetic distance correlation with insect fitness parameters was cochineal-species specific. The model was specified assuming a negative binomial error distribution with a log link function, and accounting for potentially autocorrelated measurements taken from the same plant species and same replicate across cochineal lineages using a random intercept term (Bolker et al. 2009). Since many females did not reproduce, we tested for zero-inflated values and found that the data was not zero-inflated. Hypothesis testing was performed using a series of likelihood ratio tests comparing the saturated model with a null model without the interaction term, and another null model without the interaction term and the phylogenetic distance term.

3.2.5.3 Female mass

Female mass (mg) was measured in the same way as described in Chapter 2. For each of the three cochineal lineages tested, the correlation between female mass and phylogenetic distance between plant species tested and individual replicates was modelled as a function of the phylogenetic distance, which was specified as a continuous predictor variable. The model was specified assuming a gamma error distribution with a log link function, and accounting for potentially autocorrelated measurements taken from the same plant species across cochineal lineages using a random intercept term (Bolker et al. 2009). A gamma error distribution was used as it is impossible for a female to weigh less than zero and needed to account for confidence values being greater than zero. A cochineal by phylogenetic distance interaction term was included in the saturated model to test the hypothesis that the phylogenetic distance

correlation with insect fitness parameters was cochineal-species specific. Hypothesis testing was performed using a series of likelihood ratio tests comparing the saturated model with a null model without the interaction term, and another null model without the interaction term and the phylogenetic distance term.

3.2.5.4 Fitness index

In order to calculate the fitness of the three lineages across all of the *Cylindropuntia* species, a fitness index was calculated for each species (see equation below). For each of the three cochineal lineages tested, the correlation between the fitness index and phylogenetic distance between plant species tested was modelled as a function of the phylogenetic distance, which was specified as a continuous predictor variable. The model was specified assuming a zero-inflated (Zi) gamma error distribution with a log link function, and accounting for potentially autocorrelated measurements taken from the same plant species across cochineal lineages using a random intercept term (Bolker et al. 2009). A cochineal by phylogenetic distance interaction term was included in the saturated model to test the hypothesis that the phylogenetic distance correlation with insect fitness parameters was cochineal-species specific. Hypothesis testing was performed using a series of likelihood ratio tests comparing the saturated model with a null model without the interaction term, and another null model without the interaction term and the phylogenetic distance term.

$$\text{Fitness} = \frac{\text{Settlement (\% of crawlers surviving to 1}^{st}\text{ instar) X Fecundity (Average No. Progeny)}}{\text{Days to Emergence (First generation crawlers when removed with eggs)}}$$

All statistical analyses were performed in R version 4.3.3 (R Core Team, 2023). All values presented in the manuscript are mean ± 1 standard error, unless stated otherwise.

3.4 Results

Abbreviations of the *Cylindropuntia* species are given in Table 3.1.

3.4.1 Survivorship

1st instar survival

a.) Performance

For 1st instar survival there was a statistically significant interaction term that explained settlement rates between cochineal lineages and *Cylindropuntia* species ($x^2 = 86.05$, df = 12, $P < 0.01$) (Fig. 3.1a). This indicates that there is a difference in 1st instar survival between plant species, however, this difference depends on which cochineal treatment is applied. For the 'cholla' lineage, we saw that the average 1st instar survival was highest for *C. fulvf* (5± 0.37), *C. fulvm* (6 ± 0.45) *and C. i* (6 ± 0.34). In comparison, it was significantly lower on *C. pr* (2 ± 0.26). For the 'imbricata' lineage, 1st instar survival was highest on *C. i* (7 ± 0.73). In contrast, it was significantly lower on *C. pr* (3 ± 0.37) and *C. pa* (3 ± 0.49). The 'pallida' lineage was highest on *C. pa* (5 ± 1.64) but similar to *C.s* (4 ± 0.63). In comparison, it was significantly lower on *C. k* (1 ± 0.36).

b.) Phylogenetic correlation

For 1st instar survival, there was no statistical evidence for a cochineal-specific correlation between phylogenetic distance and 1st instar survival ($x^2 = 0.60$, df = 2, $P = 0.74$). However, there is statistical evidence for a significant correlation between phylogenetic distance and 1st instar survival, averaged across the three cochineal species ($x^2 = 60.52$, df = 1, $P < 0.001$). This correlation was negative, with 1st instar survival decreasing by approximately 2% per 0.01 unit increase in phylogenetic distance, averaged across the three cochineal species tested (Fig. 3.1b).

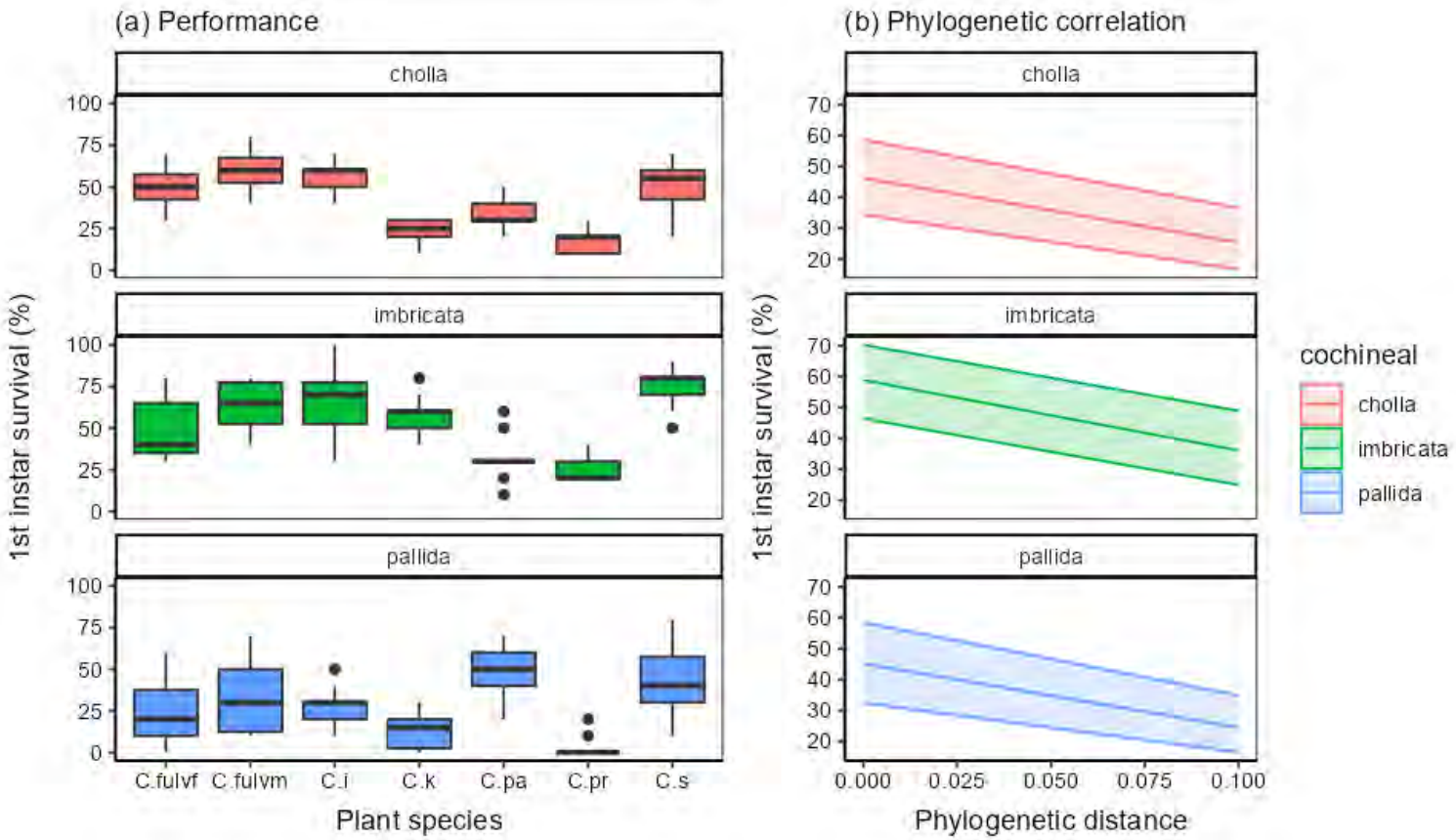

Figure 3.1a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of a.) 1st instar survival rates (%) of each cochineal lineage across the various *Cylindropuntia* plant species and (b.) correlation between phylogenetic distance and 1st instar survival rates for each cochineal lineage. The coloured line represents the mean expected 1st instar survival per replicate, and the shaded area represents the 95% confidence interval of the mean.

2nd instar survival

a.) Performance

For 2nd instar survival, there was a statistically significant interaction term that explained settlement rates between cochineal lineages and *Cylindropuntia* species ($x^2 = 136.98$, df = 12, $P < 0.01$) (Fig. 3.2a). This indicates that there is a difference in 2nd instar survival between plant species, however, this difference is dependent on which cochineal lineage is being looked at. For the 'cholla' lineage, we saw that the 2nd instar survival was highest for *C. fulvf* (4 ± 0.35) and *C. fulvm* (5 ± 0.45) respectively. In comparison, it was significantly lower on *C. k* (1 ± 0.3). For the 'imbricata' lineage, 2nd instar survival was highest on *C. s* (7 ± 0.4), *C. i* (5 ± 0.79), and *C. fulvm* (5 ± 0.54) respectively. In contrast, it was significantly lower on *C. pr* (1 ± 0.34). No crawlers survived to 2nd instar on *C.k*. The 'pallida' lineage was highest on *C. pa* (4 ± 0.47). In comparison, it was significantly lower on *C. s* (1 ± 0.29), *C. fulvm* (1 ± 0.48), and *C. fulvf* (1 ± 0.40). No crawlers survived to 2nd instar on *C. pr* and *C. k*.

b.) Phylogenetic correlation

For 2nd instar survival there was no statistical evidence for a cochineal-specific correlation between phylogenetic distance and 2nd instar survival ($x^2 = 4.22$, df = 2, $P = 0.12$). However, there is statistical evidence for a significant correlation between phylogenetic distance and 2nd instar survival, averaged across the three cochineal species ($x^2 = 92.66$, df = 1, $P < 0.001$). This correlation was negative, with 2nd instar survival decreasing by approximately 2% per 0.01 unit increase in phylogenetic distance, averaged across the three cochineal species tested (Fig. 3.2b).

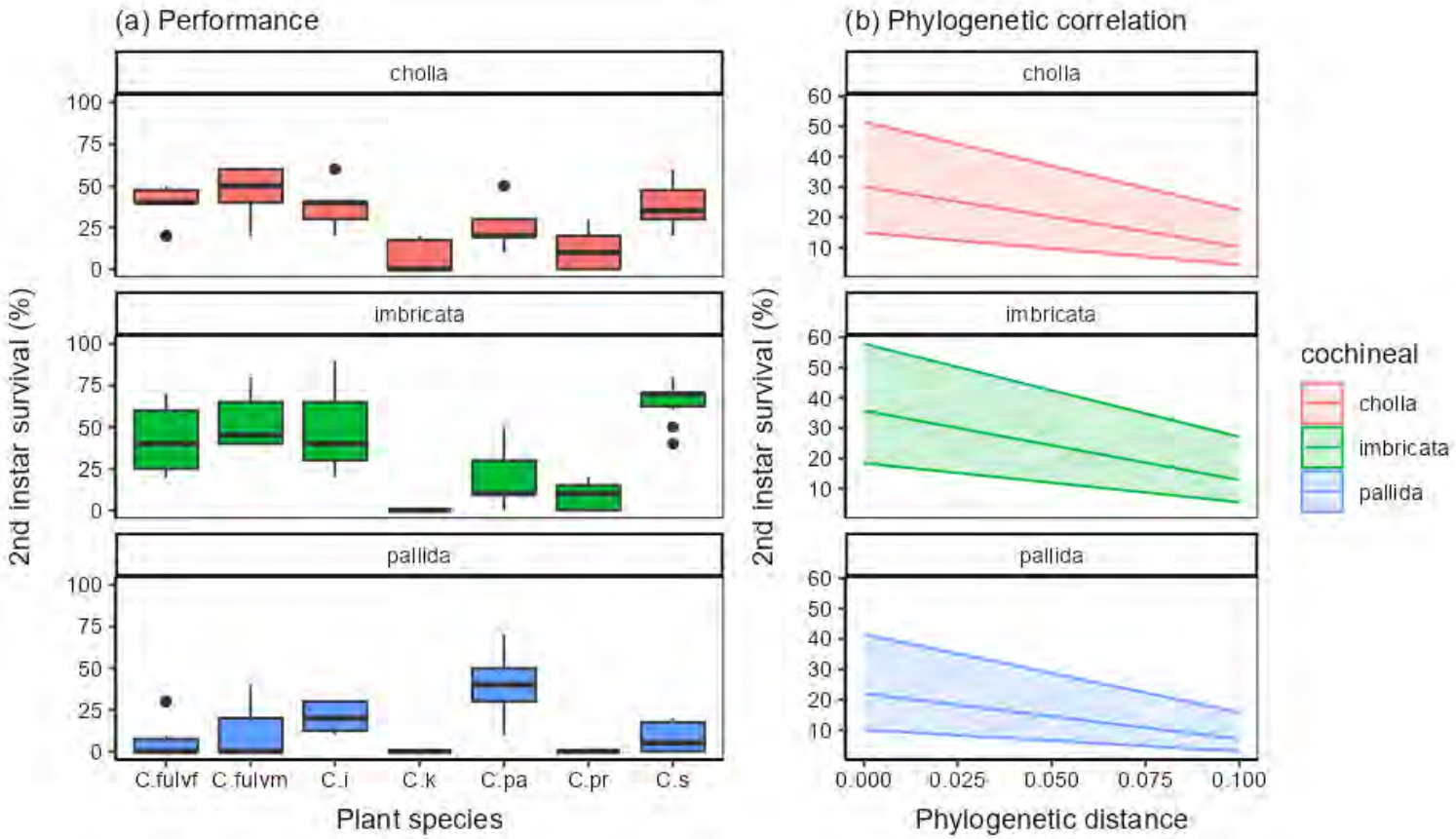

Figure 3.2a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of 2nd instar survival rates (%) of each cochineal lineage across the various *Cylindropuntia* plant species, and (b.) correlation between phylogenetic distance and 2nd instar survival rates for each cochineal lineage. The coloured line represents the mean expected 2nd instar survival per replicate, and the shaded area represents the 95% confidence interval of the mean.

Adult survival

a.) Performance

For adult survival, there was no statistically significant interaction term that explained adult survival between cochineal lineages and *Cylindropuntia* species ($x^2 = 1$, df = 1, P =0.31) (Fig. 3.3a). This shows that there is no difference in adult survival rates between plant species, however, this depends on which cochineal lineage is being applied. For the 'cholla' lineage, we saw that adult survival was highest for *C. fulvf* (4 ± 0.45) and *C. fulvm* (4 ± 0.64), respectively. In comparison, it was significantly lower on *C. pr* (1 ± 0.41). For the 'imbricata' lineage, adult survival was highest on *C. s* (4 ± 0.4), *C. i* (4 ± 0.63), and *C. fulvm* (5 ± 0.54) respectively. In contrast, it was significantly lower on *C. pr* (1 ± 0.34). No crawlers survived to adult for *C. k*. The 'pallida' lineage was highest on *C. pa* (3 ± 0.39) and *C. i* (2 ± 0.47). In comparison, it was less than zero and significantly lower on *C. fulvm* (0.4 ± 0.27), *C. fulvf* (0.3 ± 0.30) and *C. s* (0.2 ± 0.13). No crawlers survived to adult on *C. pr* and *C. k*.

b.) Phylogenetic correlation

For adult survival, there was no statistical evidence for a cochineal-specific correlation between phylogenetic distance and adult survival ($x^2 = 1.88$, df = 2, P = 0.39). There is statistical evidence for a significant correlation between phylogenetic distance and adult survival, averaged across the three cochineal species ($x^2 = 79.37$, df = 1, $P < 0.001$). This correlation was negative, with adult survival decreasing by approximately 2% per 0.01 unit increase in phylogenetic distance for the 'imbricata' and 'cholla' lineage and 1% per 0.01 unit for the 'pallida' lineage (Fig. 3.3b).

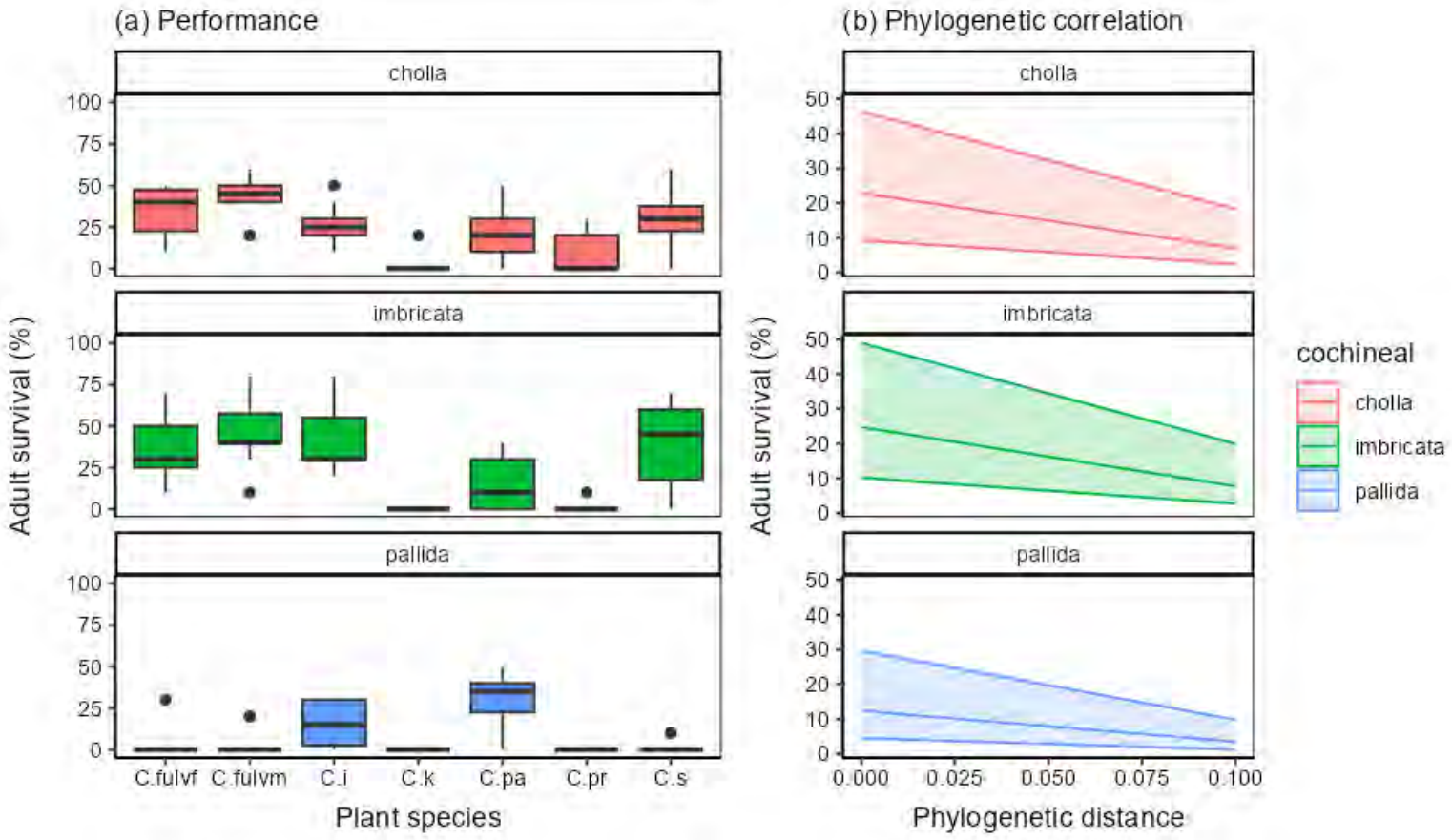

Figure 3.3a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of a) adult survival rates (%) of each cochineal lineage across the various *Cylindropuntia* plant species and (b.) correlation between phylogenetic distance and adult survival rates for each cochineal lineage. The coloured line represents the mean expected adult survival per replicate and the shaded area represents the 95% confidence interval of the mean.

Reproduction rates

a.) Performance

For reproduction rates, there was a statistically significant interaction term that explained settlement rates between cochineal lineages and *Cylindropuntia* species ($x^2 = 249.82$, df = 12, $P < 0.01$) (Fig. 3.4a). This means that there is a difference in reproduction rates between plant species, however, it is cochineal dependent. For the 'cholla' lineage, we saw that the proportion of crawlers that ended up reproducing was highest for *C. fulvf* (2 ± 0.35) and lowest for *C. pr* (0.22 ±0.22). Surprisingly, not as many crawlers managed to reproduce on *C. fulvm* (1 ± 0.27). For the 'imbricata' lineage, reproduction was similar across *C.i* (3 ± 0.84), *C. s* (3 ± 0.76), *C. fulvf* (3 ± 0.71), and *C. fulvm* (3 ± 0.59). No reproduction took place on *C. pr* and very little took place on *C. pa* (0.33 ± 0.27). For the 'pallida' lineage, reproduction only took place on *C. pa* (2 ± 0.47) and *C.i* (1 ± 0.42). No crawlers managed to reproduce on *C.k* for all three lineages.

b.) Phylogenetic correlation

For reproduction rates, there was no statistical evidence for a cochineal-specific correlation between phylogenetic distance and reproduction rates ($x^2 = 5.85$, df = 2, $P = 0.05$). There is statistical evidence for a significant correlation between phylogenetic distance and reproduction rates, averaged across the three cochineal species ($x^2 = 57.39$, df = 1, $P < 0.001$). On average, reproduction rates decreased by approximately 2% per 0.01 unit for an increase in phylogenetic distance for the 'imbricata' lineage and around 1% for the 'pallida' and 'cholla' lineage (Fig. 3.4b).

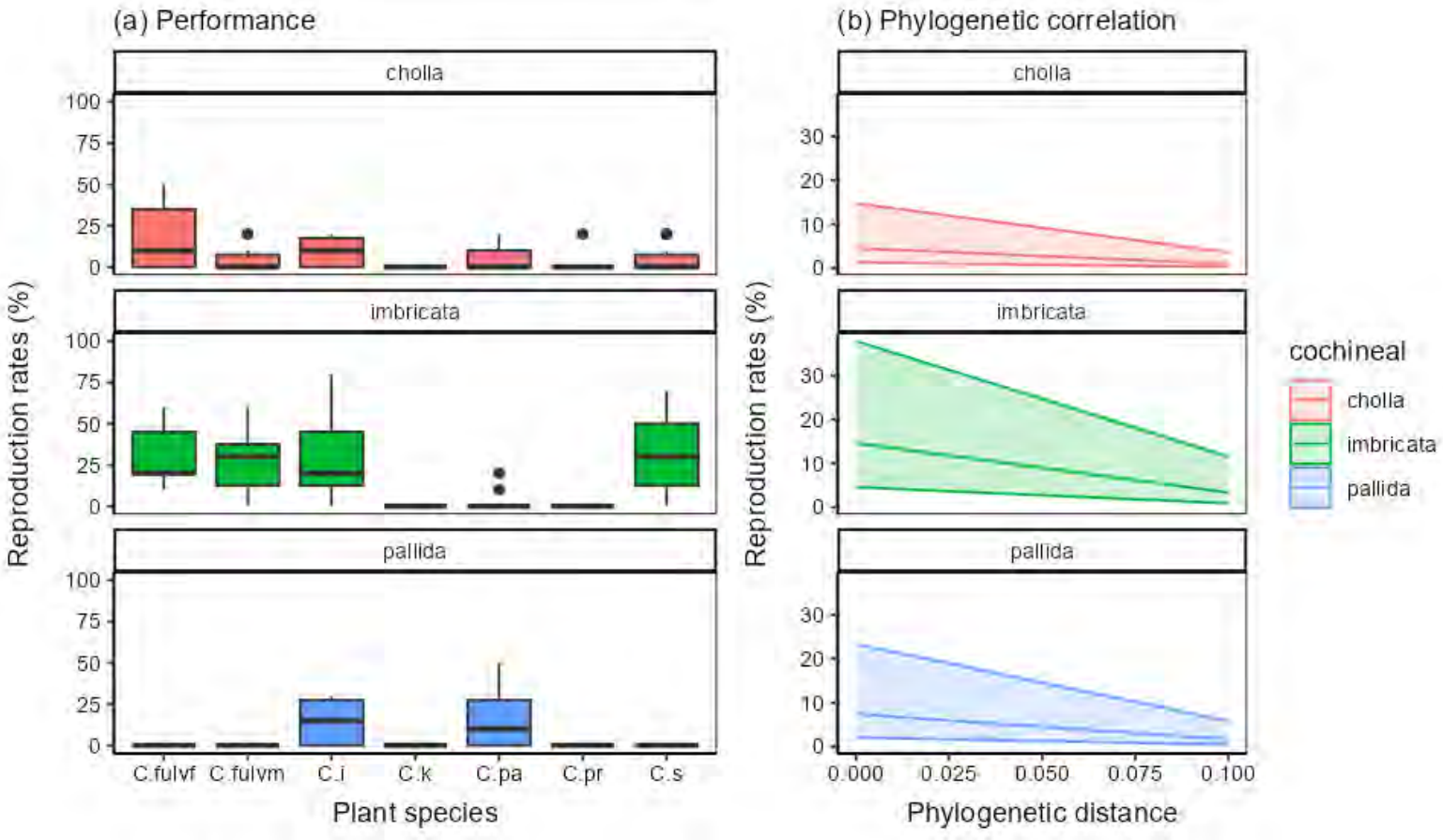

Figure 3.4a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of reproduction rates (%) of each cochineal lineage across the various *Cylindropuntia* plant species and b.) correlation between phylogenetic distance and reproduction rates for each cochineal lineage. The coloured line represents the mean expected reproduction rate per replicate, and the shaded area represents the 95% confidence interval of the mean.

3.4.2 Female Fecundity

a.) Performance

There was a statistically significant interaction term that explained female fecundity between cochineal lineages and *Cylindropuntia* species ($x^2 = 1754.90$, df = 2, $P < 0.01$) (Fig. 3.5a). There is, therefore, a difference in female fecundity between plant species, however, this is dependent on which cochineal treatment is applied. For the 'cholla' lineage, we saw that the number of progeny was highest for *C. fulvf* (54 ± 13). In comparison, the rest of the species were significantly lower, with *C. s* (3 ± 0.15) and *C.pa* (6 ± 2.42) being the lowest. For the 'imbricata' lineage, female fecundity was highest on *C. i* (46 ± 9.30), *C. fulvf* (43 ± 7.60) and *C. s* (36 ± 6.52). In contrast, the lowest was on *C. pa* (3 ± 1.80). No progeny was produced on *C. pr*. For the 'pallida' lineage progeny was only produced on *C. pa* (46 ± 11.7) and *C. i* (113 ± 28.9). No reproduction occurred in any of the three lineages for *C.k*, and therefore, no progeny was produced.

b.) Phylogenetic correlation

For female fecundity, there was no statistical evidence for a cochineal-specific correlation between phylogenetic distance and number of progeny produced per female ($x^2 = 10.41$, df = 5, $P = 0.06$). There is no statistical evidence for a significant correlation between phylogenetic distance and female fecundity, averaged across the three cochineal lineages ($x^2 = 2.35$, df = 1, $P = 0.13$) (Fig. 3.5b).

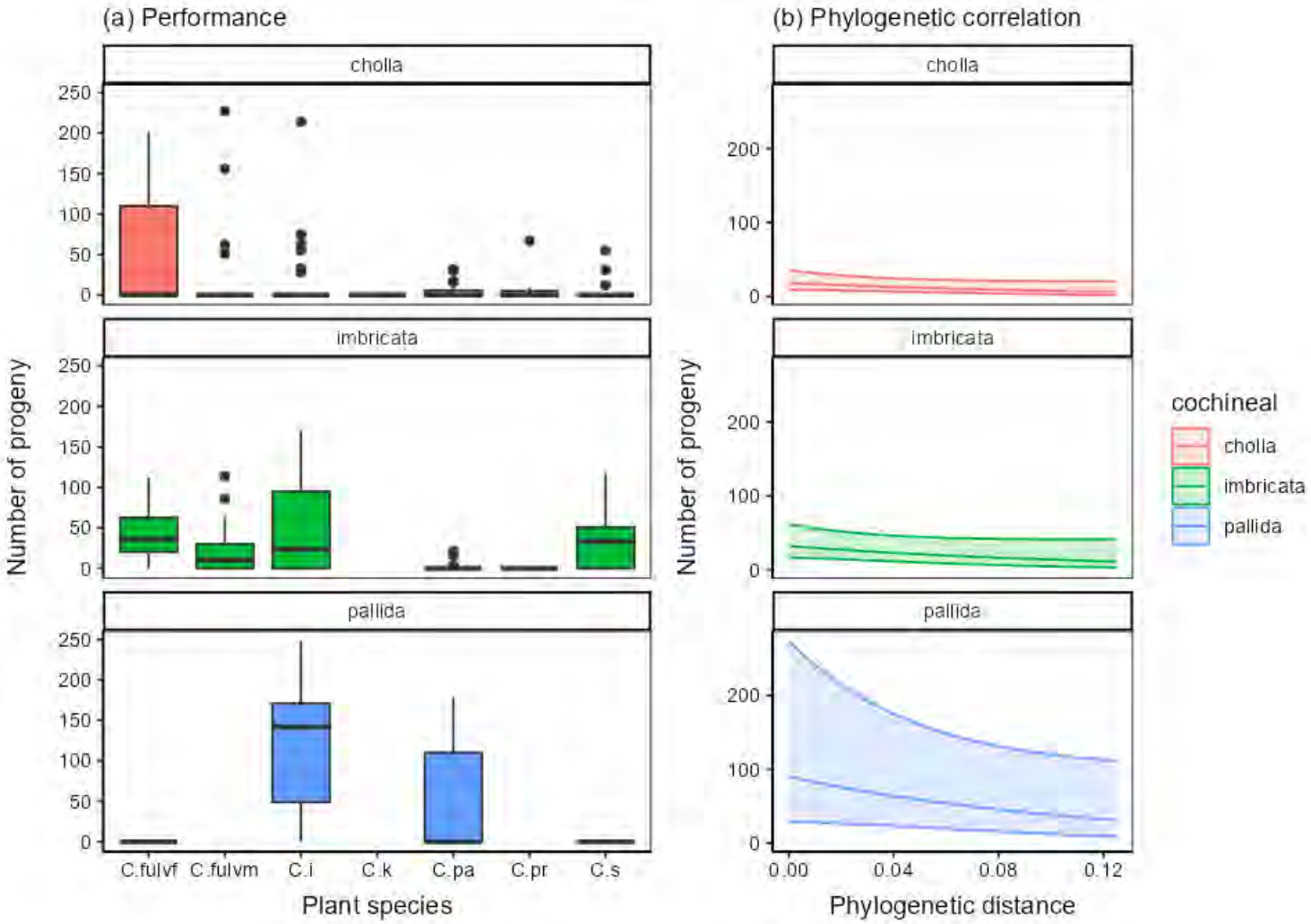

Figure 3.5a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of number of progeny per female of each cochineal lineage across the various *Cylindropuntia* plant species and b.) correlation between phylogenetic distance and number of progeny for each cochineal lineage. The coloured line represents the mean expected number of progeny per female, and the shaded area represents the 95% confidence interval of the mean.

3.4.3 Female mass

a.) Performance

There was a statistically significant interaction term that explained body mass (mg) between cochineal lineages and *Cylindropuntia* species ($x^2 = 52.02$, df = 8, $P < 0.05$) (Fig. 3.6a). This indicates that there is a difference in cochineal body mass between plant species, however, this difference depends on which cochineal treatment is applied. For the 'cholla' lineage, we saw that the body mass (mg) was highest for *C.i* (2.42 ± 0.42) mg and *C. fulvf* (2.49 ± 0.36) mg, respectively. In comparison, it was lower on *C. k* (0.55 ± 0.15) mg and *C. fulvm* (1.62 ± 0.31) mg. For the 'imbricata' lineage, average body mass was highest on *C. i* (3.36 ± 0.43). In contrast, it was lowest on *C. pr* (0.4 ± 0) mg and on *C. fulvf* (2.33 ± 0.35) mg. For the 'pallida' lineage, female body mass (mg) was highest on *C. pa* (2.81 ± 0.36) mg but similar to *C. i* (2.74± 0.77). In comparison, it was significantly lower on *C. s* (0.15 ± 0.05) mg and *C. fulvf* (0.23 ± 0.09) mg.

b.) Phylogenetic correlation

There was no statistical evidence for a cochineal-specific correlation between phylogenetic distance and female mass (mg) ($x^2 = 2.40$, df = 2, $P = 0.30$). There is statistical evidence for a significant correlation between phylogenetic distance and female mass (mg), averaged across the three cochineal lineages ($x^2 = 4.64$, df = 1, $P < 0.05$) (Fig. 3.6b). On average, female mass (mg) decreased by approximately 12 (± 3) % per 0.01 unit increase in phylogenetic distance for each cochineal lineage (Fig. 3.6b).

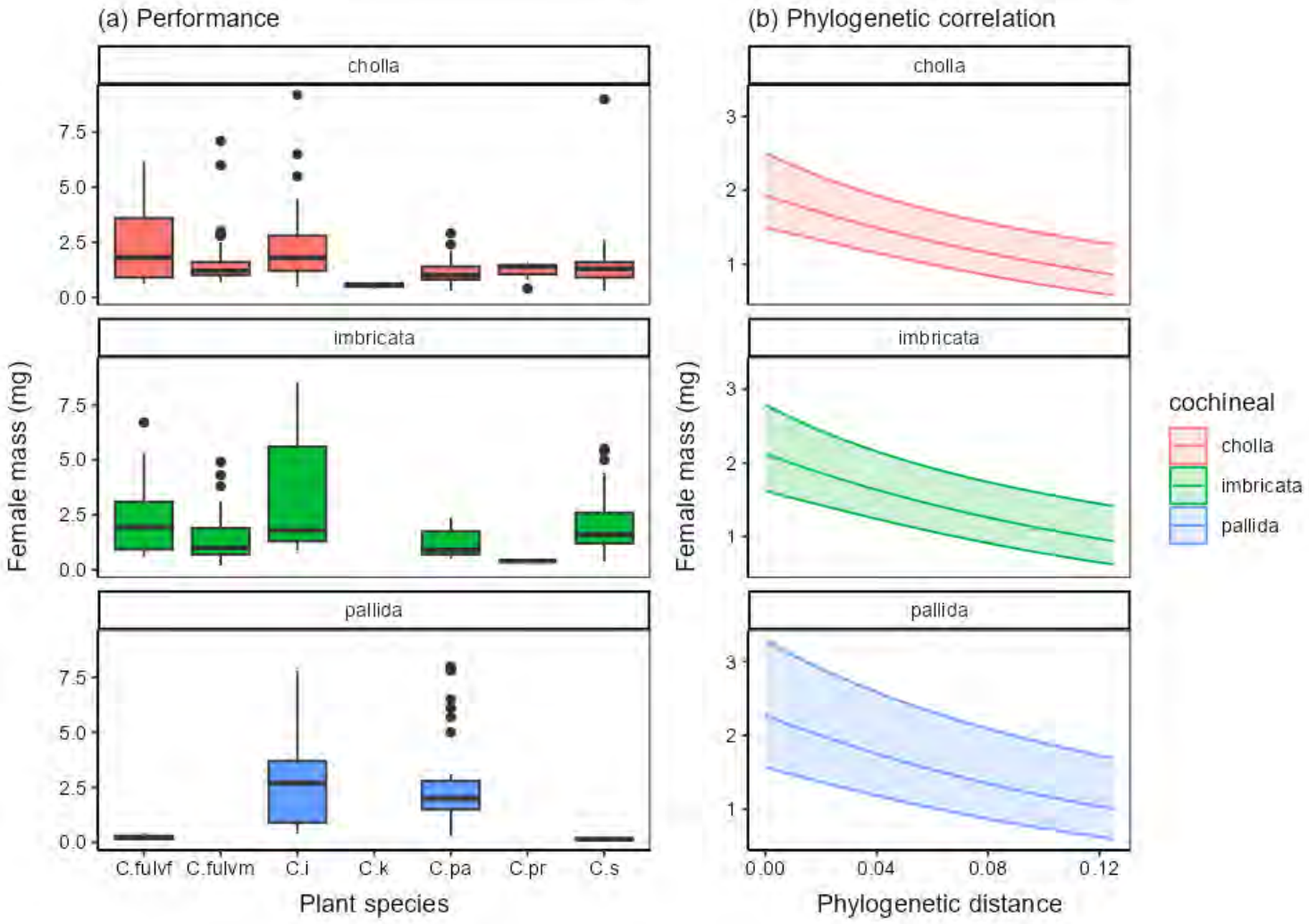

Figure 3.6a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of female mass (mg) per female of each cochineal lineage across the various *Cylindropuntia* plant species and (b.) marginal effects showing the correlation between phylogenetic distance and female mass (mg) for each cochineal lineage. The coloured line represents the mean expected female mass (mg) per female, and the shaded area represents the 95% confidence interval of the mean.

3.4.4 Fitness index

a.) Performance

For the 'cholla' lineage, we saw that the average fitness index was highest for *C.fulvf* (3.91 ± 1.14) (Fig. 3.7a). In comparison, it was lower on *C. pr* (0.14 ± 0.12), *C. s* (0.27 ± 0.25), and *C. pa* (0.25 ± 0.11). For the 'imbricata' lineage, the average fitness index was highest on *C. i* (4.68 ± 1.51) and *C.s* (2.90 ± 0.56) and lowest on *C. pa* (0.11 ± 0.07). No reproduction took place on *C. pr* and, therefore, scored zero on the fitness index. For the 'pallida' lineage, the average fitness index was highest on *C. pa* (5.05 ± 1.50) but similar to *C. i* (3.25 ± 1.33), and due to no reproduction on the remaining species, they scored zero on the fitness index. *C.k* was the only species to have a zero score on the fitness index for all three cochineal lineages.

b.) Phylogenetic correlation

There is no statistical evidence for a cochineal-specific correlation between phylogenetic distance and the fitness index ($x^2 = 5.48$, df = 2, P = 0.06). There is statistical evidence for a significant correlation between phylogenetic distance and the fitness index, averaged across the three cochineal species ($x^2 = 25.04$, df = 1, $P < 0.01$) (Fig. 3.7b).

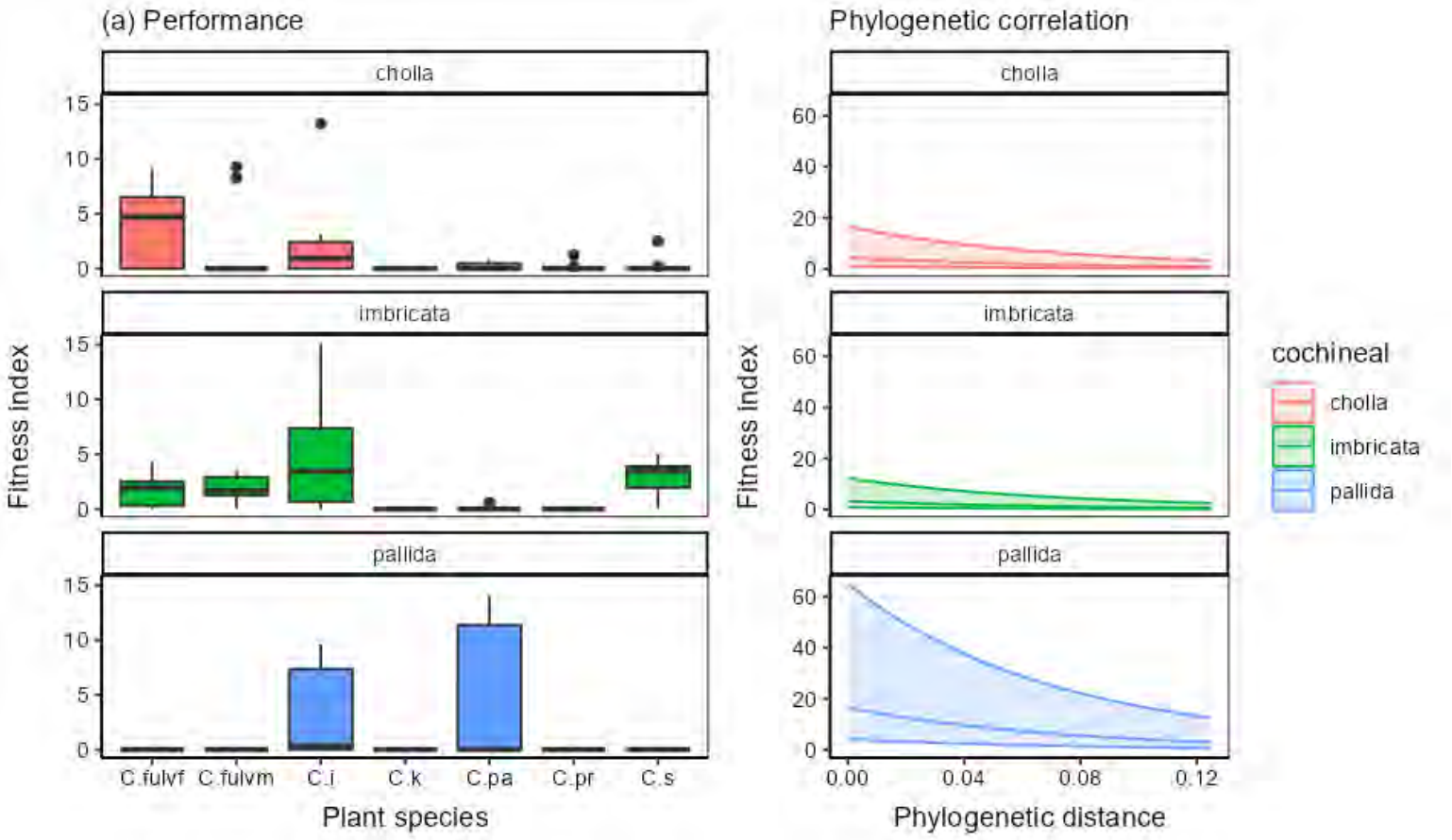

Figure 3.7a: Box and whisker plot representing the interquartile range (box) and whiskers representing the lower and upper ranges of fitness index calculated per replicate of each cochineal lineage across the various *Cylindropuntia* plant species and (b.) correlation between phylogenetic distance and the fitness index for each cochineal lineage. The coloured line represents the mean expected fitness index per replicate, and the shaded area represents the 95% confidence interval of the mean.

3.5 Discussion

The results of this study show that cochineal lineages in the *Cylindropuntia* genus, in general, have higher fitness on their original host plant associated with plant species and its closest relatives and decrease on more phylogenetically distant species. For every fitness measurement assessed in this study, there was a negative correlation between the fitness measurements and phylogenetic distance, indicating that agents sourced on the original target species and very closely related species should be prioritized, rather than sourcing agents off more distantly related species. This evidence supports the idea that 'old associations' are more effective biocontrol agents than 'new associations'.

'New associations' have been shown to be effective agents for some cactus biocontrol programmes (Jones et al. 2015, 2016). However, the main issue with this approach is that it is very difficult to know which plants to search for 'new associations' and what constitutes a 'new association'. Anything that is not found on the target weed itself could be considered a 'new association', but it would be impossible to survey all the relatives of a targeted weed, and one does not know how phylogenetically distant from the target weed one should search. For example, the use of the 'cylindropuntia spp' lineage against *C. kleiniae* and *C. leptocualis* in Australia, clearly shows that there is no clarity in what species to collect off in biocontrol agent selection (Jones et al. 2016). The results of this study provide evidence that since fitness decreases with phylogenetic distance from the target weed, agents should be collected off the origin of the target weed and its very close relatives, therefore aiding future cactus biocontrol agent selection.

In the case of the 'californica var. parkeri' lineage, it was collected off *C. bernardina* (formerly *C. californica* var. *parkeri*) and has been released as a biocontrol agent against *C. pallida* and *C. prolifera* in Australia, where it is an effective agent (Unpublished data, A.J McConnachie). Given that this lineage was not collected off *C. prolifera*, it could technically be classified as a 'new association'. However, both *C. prolifera* and *C. bernardina* occur in sympatry in Baja California and are two of the most common cactus species in this region. If the definition of a 'new association' is a species that has not had an evolutionary association with another species, rather that it forms a new relationship with a species that it has never come into contact with, we can assume that 'new associations' must be collected

off species that are geographically separate from the target weed (Hokkanen & Pimentel 1984, 1989). Even with the poor long-distance dispersal ability of cochineal, we can assume that since the 'californica var. parkeri' lineage was collected off *C. bernardina*, where it occurs in sympatry with *C. prolifera*, it has likely been associated with *C. prolifera* over the course of its evolutionary history, making it an 'old association' and not a 'new association'. This means that it is highly likely that the 'californica var. parkeri' lineage has been previously associated with *C. prolifera*, and, therefore, should not be classified as a 'new association' on that plant. Further surveys could find the same cochineal lineage on *C. prolifera* in its native range. Given that the results of this study indicate 'older associations' should be prioritized, selecting agents off species that occur in sympatry with the target weed could be classified as an 'old association' and, therefore, could also be collected.

There are several successful biocontrol agents that are attributed to 'new associations' (Jones et al. 2015, 2016). However, there are very few circumstances where 'new associations' are compared against the efficacy of 'old associations' on the same target weeds, such as in this study (Table 3.2). For example, the 'cylindropuntia spp' lineage has been released against *C. leptocaulis*, due to its efficacy as a biocontrol agent (Sheehan & Potter 2017). However, the efficacy of a 'leptocaulis' lineage was not tested against *C. leptocaulis*, and therefore, there is no evidence to support whether the 'leptocaulis' lineage would be a better biocontrol option than the 'cylindropuntia spp' lineage (Jones et al. 2016). In the case of initial testing for *C. fulgida* in Australia, the fitness and efficacy of both the 'cholla' and the 'fulgida' lineage were tested on *C. cholla* and *C. fulgida* (Mathenge et al. 2009b). Both *C. cholla* and *C. fulgida* also occur in Baja California and are thought to be very close relatives of one another, but their native ranges do not overlap. In this instance, the fitness and efficacy of the 'cholla' lineage was greater than that of the 'fulgida' lineage on *C. fulgida* and therefore resulted in the release of the 'cholla' lineage, giving evidence that a 'new association' can be more effective than an 'old association' (Mathenge et al. 2009b). However, the fitness of the 'imbricata' lineage represents an 'old association,' since it had higher fitness on *C. imbricata* than any other lineage tested (Paterson et al. 2021b). Therefore, there seems to be evidence both for and against 'new associations', further complicating agent selection.

Table 3.2: Current biocontrol agents used in South Africa against their respective target weeds, whether they represent a 'new' or 'old' association, as well as if they were compared against the use of another association.

Cochineal agent	Target weed	Association	Was it compared against another association?	References
D. ceylonicus	*O. monacantha*	'old association'. Early surveys reveal that in its native range, the agent was associated and discovered on the target weed.	'new association' not tested	Lounsbury 1915, Mann 1969
D. austrinus	*O. aurantiaca*	'new association' The geographic ranges of the agent and weed do not co- occur.	'old association' not tested	Moran & Cabby 1979, Moran & Zimmermann 1991a)
D. tomentosus 'imbricata'	*C. imbricata*	'old association' Collected in 1925 off *C. imbricata* in Texas.	'new associations' have been tested and are shown to be more effective in Australia.	Jones et al. 2016, Paterson et al. 2021b
	C. leptocaulis	'new association'.	'old association' not tested.	Paterson et al. 2021b
D. tomentosus 'cholla'	*C. fulgida*	'new association' Collected off *C. cholla* in Baja California, geographically isolated from *C. fulgida.*	'old association' was tested and found to be less effective.	Mathenge et al. 2009b, Klein et al. 2020
D. opuntiae 'stricta'	*O. stricta* *O. cespitosa* *O. engelmanii* 'Northern cape/ Free State variety'	Unknown, due to no knowledge as to which species the lineage was collected from.		Paterson et al. 2021b, Winston et al. 2014, Dodd 1940
D. opuntiae 'ficus'	*O. ficus-indica* *O. tomentosa*			

The relationships between phytophagous insects and their host plants help reveal evolutionary processes (Bernays & Chapman 1994). Insects can sometimes be used to help resolve taxonomic issues of plant species since insects tend to utilize closely related plant species that share chemical

characteristics that they have adapted to (Mathenge et al. 2010a). For example, a non-target attack of the host-specific agent *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae) on *Azolla filiculoides* Lam. (Azollaceae) in South Africa was reported on the native *Azolla pinnata* R.Br. subsp. *africana* (Desv.) R.M.K.Saunders & K.Fowler, which was a serious issue as host-specificity testing showed that *S. rufinasus* could not feed on this species (Hill 1998, McConnachie et al. 2004, Madeira et al. 2016). Through the use of genetic barcoding, it was shown that these species were actually *Azolla crisata* Kaulf, which is in the same clade as the target weed and expected to be a suitable host (Madeira et al. 2016). This study showed that insects are often better taxonomists than people, and the non-target feeding was, in fact, exactly what the phylogenetic approach to host specificity testing predicted (Madeira et al. 2016). Aphids (Hemiptera: Sternorrhyncha), as well as lineages of cochineal species, like those seen in *D. tomentosus*, can also be used to elucidate host plant taxonomy (Eastop 1979, Mathenge et al. 2010a). It is possible that the performance of cochineal species could help clarify and resolve taxonomic issues of the Cactaceae in future.

Since closely related plants largely share similar characteristics, this could be why some 'new associations', that are collected off closely related species from the target weed were considered effective. The important delineation is that some 'new associations' are associated with an agent that was collected off very close relatives (Like 'cholla' on *C. fulgida*), but whether cochineal collected off the actual target weed would be equally, or even more damaging, is unknown. The results of this study suggest that agents collected off the target weed are likely to be more effective, but can also be effective on its closest relative, which is why the 'cholla' lineage made for an effective agent on *C. fulgida*. Since the 'cholla' lineage performed best on *C. fulgida* and not on *C. cholla*, it could suggest that maybe there the chemical composition is slightly more favourable on *C. fulgida* than on *C. cholla* (Mathenge et al. 2009b). However, agents collected from more distantly related species will likely not share these characteristics as strongly as *C. cholla* and *C. fulgida* and, therefore, may have poor fitness. The taxonomic identification of *Cylindropuntia* species is therefore crucial in agent selection so that agents can be collected from the target weed and its closest relatives.

One concern of 'new associations' is that of safety (Goeden & Kok, 1986). Agents with 'new associations' can be unpredictable since they are generally oligophagous and are able to damage and utilize hosts that they do not have a long-standing evolutionary relationship (Goeden & Kok. 1986). This means that agents with 'new associations' are likely to have more of a propensity to attack other plant species and could have non-target impacts (Goeden & Kok. 1986). In the opposite case, if a biocontrol agent is selected from its original host plant ('old association'), it is unlikely that it will attack any other species when the main host is present. Every newly prioritized agent, being either a 'new' or 'old' association, undergoes rigorous host specificity testing prior to release. With this in mind, decisions will be made as to whether it is safe enough to be released, and therefore, it does not matter whether it is classified as a 'new' or 'old' association (Dennill & Moran 1989). There is no evidence to suggest that 'new associations' in biocontrol have had negative impacts, so one might argue that they are just as safe as 'old associations'. In the case of *D. tomentosus*, the risk of a new association having non-target impacts does not apply, given its adaptations and life history, to survive solely on *Cylindropuntia* hosts (Mathenge et al. 2009a). So even if we do decide to select a lineage of *D. tomentosus* that represents a 'new association' (like 'cholla' on *C. fulgida*), it will not have the same safety issues as those present in other biocontrol programmes where the insects have a wider host range that extends to multiple plant families.

For future *Cylindropuntia* biocontrol programmes, *D. tomentosus* lineages should be collected off the source population of the target weed. If agents cannot be sourced off the main target weed, then agents should be collected off its closest relatives. There may be exceptions where the next closest relative will make for a more effective agent ('cholla' on *C. fulgida*), but this study suggests that generally, we are likely to find more effective agents on the original target weed. The results of this study add to the evidence that, in general, biocontrol agents with 'old associations' should be prioritized over ones with 'new associations'.

Chapter 4

General discussion

This thesis helped select a damaging candidate biocontrol agent for *C. pallida* in South Africa and investigated how to select the most damaging *D. tomentosus* lineages for invasive *Cylindropuntia* species. The work presented in this thesis identified a promising new agent in the 'pallida' lineage for South African *C. pallida.* It had comparable fitness with the 'californica var. parkeri' lineage on Australian *C. pallida* plants, and since this agent is effective against *C. pallida* in Australia, the 'pallida' lineage should be successful against South African *C. pallida* (Chapter 2). The results in this study provide suitable evidence to support the project moving on to the host-specificity testing phase. The surprising result of the 'californica var. parkeri' lineage being ineffective on South African *C. pallida* indicates that there is clearly a difference between the *C. pallida* plants in Australia and South Africa and presents a rare example in cactus biocontrol where agents used in one country is not suitable for another. Furthermore, there is evidence to support that 'old associations' are more effective biocontrol agents, suggesting that 'old' associations should be prioritized in future *Cylindropuntia* biocontrol programmes using *D. tomentosus* (Chapter 3). In this chapter, I will discuss the importance of pre-release efficacy testing in biocontrol programmes (4.1), the use of host-adapted lineages in cactus biocontrol in South Africa (4.2), and the hybridization of cochineal lineages and its implications for biocontrol (4.3).

4.1 Importance of pre-release efficacy testing in biocontrol programmes

Host-specificity is considered the most important testing procedure in the biocontrol practice (McEvoy 1996). As discussed, species of cochineal can be host-specific to cactus genera, and host-adapted lineages are host-specific to a particular cactus species (Chapter 2, Mathenge et al. 2009a). *Dactylopius tomentosus* has been shown to be host-specific to the *Cylindropuntia* genus, and different lineages of this species are usually more effective on one or a few species, making certain lineages highly host-

specific to a select few cactus species (Mathenge et al. 2009a, Paterson et al. 2021b). This means that when prioritizing *D. tomentosus* agents, they are already safe for release, and because of this, the selection of the most damaging and effective cochineal agent becomes relatively more important, especially since Cactaceae species are found almost exclusively in the America's (Zimmermann et al. 2009, Novoa et al. 2015, Guerrero et al. 2019).

Pre-release efficacy testing is an important procedure in biocontrol, ensuring that the most effective agent possible is released on the target weed (Pearson & Callaway 2005). The use of measurements such as survival to each life stage, female weight, female fecundity, and fitness indices help researchers understand whether an agent will survive and reproduce well on the target plant (Venter et al. 2022). Agents that have high rates of survival and reproduction on a particular species indicate the host is suitable for future generations. Also, the higher the fitness of an agent, the quicker the population will grow and, therefore, will achieve faster control, such as in many *Dactylopius* species (Volchansky et al. 1999). By using and comparing these fitness metrics, we can select the agent that is the most damaging option.

It is, however, important to note that although fitness measurements can help us predict whether an agent will establish or not, fitness is not the only factor that will determine an agent's efficacy in the field. Some researchers believe that trying to predict an agent's efficacy in a laboratory facility is a difficult or even impossible task (Balciunas & Smith 2006, Paynter et al. 2018). This is motivated partly because the testing lacks the variability of conditions and factors present in the invasive range, such as temperature, predation, and rainfall (Paynter et al. 2018). However, one can argue that although it is difficult to predict the impact these factors will have on the efficacy of the agent, it still provides good evidence that under suitable conditions, the agent can survive and reproduce, thus increasing the likelihood of agent success and excluding those agents that we are certain will not be suitably damaging. In the context of Cactaceae biocontrol programmes, there is reliable evidence from other cochineal studies (especially with *D. tomentosus*) that shows that high cochineal fitness is correlated with high levels of control and, therefore, continues to be a useful way of predicting agent success in the field (Mathenge et al. 2009b, Moran & Zimmermann 1991b). For example, fitness metrics (number of

progeny, female weight, and settlement) were used to identify the 'cholla' lineage as an effective biocontrol agent for *C. fulgida* var. *fulgida* in South Africa (Mathenge et al. 2009b). Post-release evaluations now show that the agent has been effective in the control of *C. fulgida* var. *fulgida* countrywide (Klein et al. 2020).

Extrapolating results from the lab to the field can be difficult; however, in some instances, relative performance can be compared if we have a suitable comparison (Chapters 2 & 3). For example, the performance (survival, female mass, and fecundity) of the 'californica var. parkeri' on the Australian *C. pallida* plants was similar to the performance of the 'pallida' lineage of South African *C. pallida* plants (Chapter 2). Since we already know that the 'californica var. parkeri' lineage is an effective agent against *C. pallida* in Australia (Unpublished data, A. McConnachie), we can assume that it is possible that the 'pallida' lineage will perform just as well on South African *C. pallida*. Further to this, the 'imbricata' and 'cholla' lineage was highest on *C. imbricata* and the two *C. fulgida* varieties, respectively (Chapter 3). Since we know that both lineages have been effective agents on these species in the field, we can extrapolate these results from the lab to the field (Klein et al. 2020, Paterson et al. 2021b). For future Cactaceae biocontrol programmes, comparing the fitness of a new agent with a successful one (with its target weed) as a control could be a useful approach for pre-release efficacy testing.

Understanding whether an agent will be effective is important as, ideally, the invasive weed populations must be reduced as fast as possible to avoid any indirect non-target impacts (Pearson & Callaway 2005). There have been instances whereby host-specific agents have been released, but do not bring about effective control over their target species. The release of ineffective agents increases the chances of indirect non-target impacts (Pearson & Callaway 2005). One example of indirect non-target impacts is the interference of food web interactions in higher trophic levels whereby other organisms are affected by the release of a biocontrol agent (Pearson et al. 2000, Nuessly & Goeden 1984, McEvoy 1996). This was observed with the release of the fruit-fly, *Uruphora* species as a biocontrol agent against the invasive Knapweeds, *Centaurea diffusa* (Asteraceae) Lam. and *Centaurea maculosa* (Asteraceae) Lam (Pearson et al. 2000). The *Urophora* species have had very little impact on

the two target weeds but continued to produce numerous offspring in the winter season (Pearson et al. 2000). Since the populations continued to grow, but did not kill the invasive weeds, the *Uruphora* flies provided a substantial food source for Deer Mice and has resulted in an increase of populations of deer mice. Deer mice are vectors of a disease called hantavirus, which can be a fatal disease to humans. Since deer mice populations increased, it increased the probability of transmission to humans (Ortega et al. 2004). In the America's, the hantavirus can be fatal, having a fatality rate that ranges from <1% to 12%, which depends on the susceptibility of someone infected by the hantavirus (Bi et al. 2008). This could have serious consequences for human health in these areas and, therefore, is an extreme concern (Ortega et al. 2004). This example had the public very concerned, however, a study showed that there was no significant increase in hantavirus infections and that deer mice populations returned to normal levels (Messing & Brodeur 2018).

Indirect non-target impacts of biocontrol agents are often attributed to an agent's abundance in the field (Holt & Hochberg 2001). If an agent is released and becomes increasingly abundant, without effectively reducing a target weeds population, the higher the likelihood of indirect effects (Pearson & Callaway 2005). Population dynamics play a role here, for example, even if an agent has high initial populations, but is effective in reducing the weed's population, then the populations of both the weed and agent will oscillate, causing the agent's populations to drop when the target weeds population is low and therefore reduces the chances of an overabundant population and therefore lowers the risk of unpredictable indirect effects (Holt & Hochberg 2001, Briese 2000). Therefore, releasing effective agents is vitally important to reduce non-target impacts associated with indirect effects, further increasing the safety and perception of biocontrol. In the case of *D. tomentosus*, the level of control on other invasive *Cylindropuntia* species is generally very good and therefore no indirect non-target impacts have been recorded (Paterson et al. 2021b, Klein et al. 2020).

Although predicting an agent's efficacy based on fitness data can be difficult, it is still a worthwhile procedure in biocontrol programmes and can help screen agent candidates before continuing to host-specificity testing. Given that fitness studies have helped predict cochineal efficacy in other cactus biocontrol programmes, there is evidence to support that the 'pallida' lineage will be an effective

agent against *C. pallida* in South Africa (Chapter 2). Given that it will likely be an effective agent in the field, it, therefore, means that it is unlikely to have indirect non-target impacts. For future *Cylindropuntia* biocontrol programmes, we should use pre-release efficacy testing like those conducted in this thesis to predict an agent's efficacy with a benchmark species that has a known efficacy in the field to predict whether new cochineal lineages are going to be damaging enough to be released.

4.2 The use of host-adapted lineages in cactus biocontrol in South Africa

Cochineal insects have developed adaptations to survive and feed exclusively on plants within the Cactaceae (Moran & Zimmermann 1991b). Factors such as low mobility, their sessile nature (apart from the nymphal stage and adult males), and their poor long-distance dispersal ability have allowed the evolution of host-adapted lineages (Miller & Kosztarab 1979, Mathenge et al. 2010a). Host-adapted lineages can be driven by having multiple generations of insects on the same plant genotype, which results in limited gene flow between populations adapted to different plant genotypes (Karban 1992). The performance of cochineal lineages is directly linked to phylogenetic distance, indicating that the fitness and performance of *D. tomentosus* are greatest on the original host plant of each lineage and decreases on less closely related *Cylindropuntia* species (Chapter 3). These results show that for future cactus biocontrol control programmes, prioritizing agents on the original target host and its closest relatives is preferred.

It is recommended that agents be sourced from the target weed populations in the indigenous distribution rather than close relatives. Only when it is impossible to collect from the target weed should close relatives be considered. Previously, it has been suggested that agents be sourced from different species where the agent represents a 'new association' (Hokkanen & Pimentel 1984). Given that evidence now shows that 'old associations' are more effective biocontrol agents, taxonomy and a clear understating of phylogenetic relationships become important when collecting agents off target and non-target species. Taxonomic confusion has been a big issue in many cactus biocontrol programmes (Paterson et al. 2021b). For example, *Trichocereus spachianus* is a high-priority invasive weed in South

Africa, but taxonomic issues severely hampered early biocontrol efforts (Paterson et al. 2021b). This was because *T. spachianus* has undergone numerous taxonomy changes, previously being identified as *Echinopsis spachiana* (Lem.) H. Friedrich & G.D. Rowley and *Echinopsis schickendantzii* F.A.C. Weber (Schlumpberger & Renner 2012). However, further investigation revealed that the plant species should fall within the *Trichocereus* genus. Recently, it was classified as *Soehrensia spachianus* (Lem.) Schlumpb (Korotkova et al. 2021), but was quickly reclassified under *Trichocereus* (Roberto Kiesling 2023, pers. comms., Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)). Not only has *T. spachianus* faced numerous taxonomic difficulties, but identifying the origin of its native distribution has also proved to be challenging (Albesiano 2015). It is thought that its native distribution is in northwestern Argentina and southern Bolivia. However, localities where the plant has not been planted are not known and it is possible that no natural populations exist. This has hindered the biocontrol of *T. spachianus* since the collection of natural enemies has been difficult (Paterson et al. 2021b). Closely related plant species have been considered as potential sources of agents, but identifying the most appropriate close relatives has been challenging because of the taxonomic confusion surrounding the species.

Another issue related to cactus taxonomy is that many hybrids and varieties are still being discovered and are extremely difficult to identify in the field (Novoa et al. 2019). For example, *C. bigelovii* (a close relative of *C. tunicata* and *C. pallida*) has been shown to have multiple different varieties in its native distribution that are extremely difficult to separate from one another without the use of genetic techniques (Mayer & Rebman 2021). Another species, *Opuntia engelmanii* Salm-Dyck, is an invasive species in South Africa that has many different varieties across the country (Paterson et al. 2021b). In the indigenous distribution, *O. engelmanii* is thought to have the widest range of any *Opuntia* species in North America, resulting in numerous varieties, locally adapting to the variable environments (Weniger 1978). This species belongs to a complex that has formed many interclade hybrids, resulting in numerous different entities, and has caused issues with taxonomic classification amongst these species (Majure & Puente 2014). Around 15 different intraspecific varieties have been described in the past, but it is not clear whether some varieties are distinct species on their own (Weniger

1978). Given this confusion, 3 distinct morphotypes (Northern Cape/ Free State, Limpopo, and Kenyan varieties) were classified in South Africa in order to aid the biocontrol efforts of *O. engelmanii* (Paterson et al. 2021b). The biocontrol of *D. opuntiae* 'stricta' proved to be an effective agent at one site of the Northern Cape/ Free State variety, which was surprising since previous laboratory studies showed that the cochineal was not effective against this particular variety (Paterson et al. 2021b). To further biocontrol efforts, 15 other cochineal lineages (6 *D. opuntiae* and 9 *Dactylopius confusus* Cockerell) were collected in areas of southwestern USA off known varieties of *O. engelmanii* (Paterson et al. 2021b). None of the imported lineages were found to be effective or suitable for the Northern Cape/ Free State variety or the Limpopo variety, but suitable lineages were found for the Kenyan variety (Paterson et al. 2021b). This example highlights the difficulty of selecting multiple agents for different varieties of a single species and given the example of the *D. opuntiae* 'stricta' being a successful agent against one population of the Northern cape/ Free State variety and not on other populations, there is likely to be variation between these different populations, further complicating biocontrol efforts. Ideally, the origin of each variety of *O. engelmanii* should have been identified and agents should have been collected there.

The biocontrol of *C. pallida* is an interesting situation where the 'californica var. parkeri' lineage (which represents a 'new association') is an effective agent in Australia, but ineffective against populations in South Africa (Chapter 2). The 'pallida' lineage (which is an 'old association') makes for a better agent for South African *C. pallida*. One might assume that *C. pallida* in Australia is more closely related to *C. bernardina* whilst *C. pallida* in South Africa is likely closely related to *C. pallida* in Hidalgo, Mexico, given that cochineal performance is directly linked to the phylogenetic distance (Chapter 3). However, given that *C. pallida* is a suspected hybrid between *C. tunicata* and *C. imbricata*, it is possible that the *C. pallida* found in Australia was sourced from a different area and might have a different origin from the *C. pallida* found in South Africa (Laguna et al. 2013). This is because hybrids in their native habitat are known to have increased genetic variability amongst populations and, therefore, introduced populations would have been sourced from two different populations (Harms et al. 2020). Given the necessity of certain chemical characteristics for lineages of *D. tomentosus*, it could

also be that the *C. pallida* in Australia might share similar chemical characteristics to *C. bernardina*, which could also be due to local adaptation in its invasive range (Chapter 2).

It is possible that the populations of *C. pallida* in Australia could have hybridized with another *Cylindropuntia* species that is closely related to *C. bernadina*, hence why the 'californica var. parkeri' lineage was a suitable agent for its control in Australia (Unpublished data, A.J. McConnachie). Hybridization within the Opuntioideae is known to be a common phenomenon, with around 64% of species having polypoidal genes (which is a likely result of hybridization) (Pinkava 2002). Genetic techniques can be used to investigate the parent plants of hybrids, like in the case of *Cylindropuntia* x *fosbergii* (C.B. Wolf) Rebman, M.A. Baker & Pinkava (Mayer et al. 2011). The use of Amplified Fragment Length Polymorphisms (AFLPs, Vos et al. 1995) makes for an efficient way to test whether a species has recently diverged (Koopman 2005). This technique helped show that *C.* x *fosbergii* was indeed a hybrid and provided evidence that one of its parent plants was *C. bigelovii* (Mayer et al. 2011). Although the study does not provide information about the geographic origin of the hybrid, it can confirm whether it is a hybrid or not and can elucidate parent plant species. In the case of *C. pallida*, a similar study could be conducted using AFLP's to help determine the parent plants for Australian and South African *C. pallida*, in order to better understand why the performance of the 'californica var. parkeri' lineage was high on Australian plants and not South African plants. Further genetic analyses could also be done on both *C. pallida* in Australia and South Africa to see how different the plant species are in terms of their phylogenetic relationship.

Sharing biocontrol agents between countries has been successful in the past, and this should still be done wherever possible so that resources are shared (Paterson et al. 2019). If the agent is not useful or effective, like in the case of the 'californica var. parkeri' lineage on South African *C. pallida*, agents should then be prioritized on the original host species and its closest relatives (Chapter 2). This is because there is evidence to suggest that biocontrol agents have better fitness and are best selected on the original target host and closely related species (Chapter 3). Therefore, researchers should continue to utilize 'older' associations where possible, as they would likely be a more effective and safer solution

for Cactaceae biocontrol. This study also provides general support for the use of 'old associations' rather than 'new associations' in weed biocontrol programmes against non-cactus targets.

4.3 Hybridization of cochineal lineages and its implications for biocontrol

If the 'pallida' lineage is approved for release, one potential issue that could hamper its efficacy in the field is hybridization with other lineages of *D. tomentosus* that are already present in the country ('cholla' and 'imbricata'). In some cases, hybridization has resulted in increased fitness of biocontrol agents (Szűcs et al. 2012). Studies have shown that first-generation (f1) hybrid offspring can have higher fitness (heterosis) due to the masking of recessive alleles (Lynch 1991). One example of this is the use of *Longitarsus jacobaeae* (Waterhouse) (Coleoptera: Chrysomelidae) against the weed *Jacobaea vulgaris* Gaertn. (Asterales: Asteraceae) in North America (Szűcs et al. 2012). Certain releases of the agent in some areas were ineffective, and therefore, researchers collected another lineage of *L. jacobeae* from a different area of its native distribution (Szűcs et al. 2012). Researchers then did hybridization testing with the two lineages (Szűcs et al. 2012). Their results showed that the fitness of the second generation (f2) was much higher than the original lineage. In this study, they suggested that hybridizing different lineages could help make for a more successful biocontrol agent and should be investigated in other biocontrol programmes (Szűcs et al. 2012).

Hybridization can also lead to outbreeding depression in a population and, therefore, can reduce the impact of the biocontrol agent (Vorsino et al. 2012). Outbreeding depression is the result of a breakup of genetic complexes caused by hybridization of very different genotypes and can have serious impacts such as hybrid infertility or inviability in future generations (Lynch 1991). There have not been many studies investigating the negative impacts of future generations of hybridizing biocontrol agents, but of the studies that have been done, there have been very few instances where the hybridization of biocontrol agents has resulted in negative impacts (Goldson et al. 2003, Benvenuto et al. 2012).

In the case of cochineal insects, some studies have investigated the hybridization of lineages to assess the potential impact of their use as biocontrol agents. In one study involving *D. opuntiae*,

hybridization between the 'ficus' and 'stricta' lineage resulted in the f1 hybrids being able to attack both *O. ficus-indica* and *O. stricta* (Hoffmann et al. 2002). The f2 crosses and back-crosses resulted in instances where some f2 back-crosses were host-specific and others would feed on the host plants of both parents (Hoffmann et al. 2002). This is problematic because when f2 crosses produce offspring that are host-specific to a different species than what is present in the field, it could result in a population decrease and, therefore, lower levels of control overall. Understanding the potential hybridization of lineages is, therefore, very important as it could risk the biocontrol of other successfully controlled weeds.

Another study was conducted to investigate whether the hybridization of the 'imbricata' and 'cholla' lineages of *D. tomentosus* would result in negative consequences for the respective biocontrol abilities (Mathenge et al. 2010b). This study showed that future generations of a 'cholla x imbricata' lineage had increased fitness that was greater than the original parent lineage and suggested that where *C. imbricata* and either one of the two *C. fulgida* varieties co-occur, there is likely to be no reduction in efficacy, and possibly better levels of control overall (Mathenge et al. 2010b).

It is important to find out if a new biocontrol agent can hybridize with other lineages released in the country, as it could impact the efficacy of the agent and hamper the control of the target species. *Dactylopius tomentosus* lineages are known to hybridize, and if the 'pallida' lineage is shown to be safe and effective, and is approved to be released, hybridization trials should be conducted to investigate the fitness of a 'pallida x cholla' and 'pallida x imbricata' lineage. The 'pallida' lineage was shown to have poor survival and fitness on both *C. fulgida* varieties, whilst it had high fitness on *C. pallida* and *C. imbricata* (Chapter 3). In areas where *C. fulgida* and *C. pallida* co-occur, it is highly unlikely that hybridization would be possible since the 'pallida' lineage could not survive on *C. fulgida* and the 'cholla' lineage could not survive on *C. pallida* (Chapter 3). Since the 'pallida' lineage can survive on *C. imbricata*, there is a hybridization risk where populations of *C. pallida* and *C. imbricata* co-occur, and therefore, it is important to see whether a 'pallida x imbricata' lineage could impact the control of both plant species. Since there is a lot of geographic overlap in the range of these two species, it would be valuable to conduct hybrid testing between the 'pallida' and 'imbricata' lineages (Fig 4.1). This has

been done with other cochineal lineages, so similar methods can be used in order to test whether hybrid offspring will impact the biocontrol of either *C. pallida*, *C. imbricata*, or both.

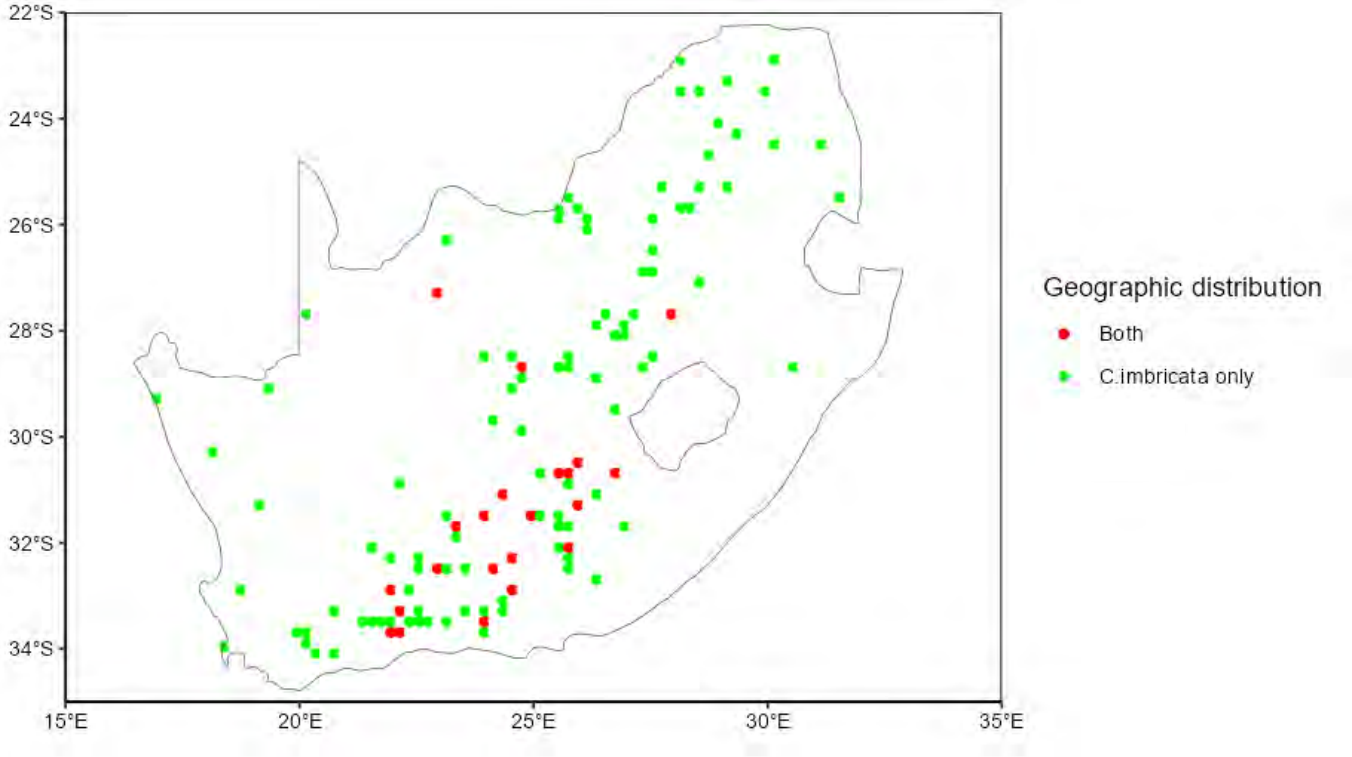

Figure 4.1: Invasive distribution of *Cylindropuntia imbricata* (green) and where *Cylindropuntia pallida* (red) share the same distribution in South Africa. Distributional data was downloaded from GBIF (GBIF.org 2024-2025).

4.4 Conclusion

The research in this thesis helped identify a potential biocontrol for *C. pallida* in South Africa. The 'pallida' lineage represents an 'old association' and is very likely a host-specific agent, but host-specificity testing is being carried out to confirm this. This research also confirmed that 'old associations' are generally more effective biocontrol agents than 'new associations' and should be prioritized for future *Cylindropuntia* biocontrol programmes. More studies investigating 'new' and 'old' associations could be beneficial in the future to understand how to select the most effective agents in other weed biocontrol programmes. This could help streamline biocontrol programmes in the future and make sure that we are accurately prioritizing and selecting the right candidate agents.

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Appendix

Figure A1: Collection of *Dactylopius tomentosus* 'pallida' in Hidalgo, Mexico.

Figure A2: The differences between *Cylindropuntia tunicata* and *Cylindropuntia pallida* in their native ranges. The yellow flower (top-left) is *C. tunicata* and the pink flower is *C. pallida* (top-right). The growth forms differ, with *C. tunicata* growing horizontal and shrub-like (Middle) whilst *C. pallida* grow with tall trunks (Bottom).