Investigations into biological control options for *Lycium ferocissimum* Miers, African Boxthorn (Solanaceae) for Australia

THESIS

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Abstract

Lycium ferocissimum Miers (Solanaceae) (African boxthorn or boxthorn) is a shrub native to South Africa, and has become naturalised and invasive in Australia and New Zealand. The plant is listed on the Noxious Weed List for Australian States and Territories. Although other control methods are available, biological control presents a potentially sustainable intervention for reducing populations of this weed in Australia.

In South Africa, the plant has been recorded from two allopatric populations, one in the Eastern Cape Province, the other in the Western Cape Provinces, however, there taxonomic and morphological uncertainties are reported in the literature. Therefore, before native range surveys for potential biological control agents could be considered, the taxonomic uncertainty needed to be resolved. The two geographically distinct areas, as well as the Australia population were sampled to assess morphological and genetic variation. All samples collected in Australia were confirmed as *L. ferocissimum*, with no evidence of hybridisation with any other *Lycium* species. Nuclear and chloroplast genetic diversity within *L. ferocissimum* across South Africa was high, and Australia was low, with no evidence of genetic seperation. One of the two common chloroplast haplotypes found across Australia was found at only two sites in South Africa, both in the Western Cape, suggesting that the Australian lineage may have originated from this region. Ten samples from South Africa, putatively identified in the field as *L. ferocissimum*, were genetically characterised as different (unidentified) *Lycium* species. The majority of plants sampled were confirmed as *L. ferocissimum*, sharing a common haplotype (haplotype 5) with sampled specimens from Australia.

Morphological analyses across different *Lycium* species in South Africa did not identify any leaf or floral characteristics unique to *L. ferocissimum*, and thus morphological identification in the native range remains problematic.

Surveys for phytophagous insects on *L. ferocissimum* were carried out regularly over a two-year period in the two regions. The number of insect species found in the Eastern Cape Province (55) was higher than in the Western Cape Province (41), but insect diversity based on Shannon indices was highest in the Western Cape Province. Indicator species analysis revealed eight insect herbivore species driving the differences in the herbivore communities between the two provinces. Based on insect distribution, abundance, feeding preference and available literature, three species were prioritised as potential biological control agents. These include the leaf-chewing beetles, *Cassida distinguenda* Spaeth (Chrysomelidae) and *Cleta eckloni* Mulsant (Coccinellidae), and the leaf-mining weevil, *Neoplatygaster serietuberculata* Gyllenhal (Curculionidae).

Native range studies such as this are perhaps the most technically difficult and logistically time-consuming part of the biological control programme. Yet, the entire outcome of a programme depends on the suite of potential agents feeding on the weed. The information gained during this stage significantly contributed to the prioritization of agents for further host-range testing and possible release. Here we showed how molecular and genetic characterisations of the target weed can be used to accurately define the identity and phylogeny of the target species. In addition, the study also highlighted the importance of considering plant morphology and how phenotypic plasticity may influence infield plant identifications while conducting native range surveys. By gaining further information during long-term and wide spread native range surveys we were not just able to provide a list of herbivorous insect fauna and fungi associated with the plant, but were able to prioritise the phytophagous species that held the most potential as biological control agents.

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Authorship contribution statement

The candidate (Mr Evans Vusani Mauda) co-conceived the idea, providing data, undertook the data analysis, writing up the results and reviewed and edited the manuscripts published. The data provided on the maunscripts were hence cited in the thesis to avoid plagiarism.

Chapter 1

1.1 Introduction

1.1.1 Plant-insect interactions

Invasive plant species in the introduced region often lack natural enemies with which they are normally associated in the native region. As such, these species have a high chance of becoming invasive due to this lack of natural enemies associated with them. Often plants in the native region have co-evolved with insect enemies that force the plant to invest some of its resources in defence against such predation (Stotz *et al.*, 1999; Messing & Wright, 2006). In the invaded region, plants that have become naturalised and invasive lack enemies that will prompt them to allocate resources to defence mechanisms, shifting most of their resources to reproduction (Mello & Silva-Filho, 2002). If introduced in an invaded region, natural enemies are self-sustaining and little to no augmented releases are required once these insects have established themselves and reunited with their native host plant (Hill, 1998; Messing & Wright, 2006; Hill & Coetzee, 2017). One example of insect-plant reunion is the invasive aquatic weed, *Salvinia molesta* DS Mitchell (Salviniaceae) and the small weevil, *Cyrtobagous salviniae* Calder & Sands (Erirhinidea) in Australian and South African waterways. The weevil as the natural enemy of the weed caused substantial control in the two countries, clearing up the thick mats of the plants (Messing & Wright, 2006; Martin *et al.*, 2018b).

1.1.2 Biological invasion

Biological invasion of alien invasive species has been recorded as the second biggest threat to biodiversity of native flora and fauna, with habitat destruction being the biggest (Keane & Crawley, 2002; Nuñez *et al.*, 2005; Simberloff, 2005; Simberloff *et al.*, 2013). Hence, there is a need to control such species once they have naturalised and established themselves in the introduced areas. Invasive species have been found to support pest species in the invading region and can be detrimental to the economy of a country if not managed (Parsons & Cuthbertson, 2004; Noble & Adair, 2016; Gagic *et al.*, 2018; Ireland *et al.*, 2019b). Invasion by alien species in most instances would be responsible for habitat fragmentation as they create islands of populations in the ecosystem by possibly driving out some of the native species that cannot compete with them for resources, leaving them disjointed from other species with which they previously co-existed (see also Wilson *et al.*, 2016).

1.1.3 Enemy Release Hypothesis (ERH)

Biological control of alien invasive species is often characterised by the perception of risks and restrictive regulatory procedures (e.g. USA) (Schwarzländer *et al.*, 2018). Applications of biological control of weeds based on ecological, evolutionary and experimental science is a highly successful discipline dating back centuries. Alien invasive species are often managed using the classical biological way of re-uniting them with their natural enemies from their respectively native region (Hinz *et al.*, 2019). The Enemy Release Hypothesis (ERH) assumptions are that specialist herbivores specific to the invading weed are not present in the invaded region and the weed's population is not regulated (Keane & Crawley, 2002). The hypothesis further assumes that native plant species have more pressure from both specialist and generalist herbivores than has the invasive plant population (Cripps *et al.*, 2006; Canavan *et al.*, 2019). Furthermore, Orians and Ward (2009) indicate that there are various selective pressures in a plant's native region where there are specialist and generalist herbivores that can shape its populations. Orians and Ward (2009) also point out that native herbivorous insects would not switch to new hosts, thereby leaving the invasive species to out-compete the native species for resources. Canavan *et al.* (2019) suggest that since the ERH forms a key assumption in biological control of weeds, it is important to test some of these assumptions and understand the role of enemy release before any biological control programme is initiated. The ERH is based on several logical arguments that state that populations are regulated by natural enemies; that natural enemies will regulate native species rather than introduced exotic species (Gagic *et al.*, 2018), and finally that invasive populations will capitalise on the absence of natural enemies, increasing their population (Keane & Crawley, 2002). However, there are risks and high costs of releasing an exotic species notwithstanding the fact that a good biological control agent inflicts substantial damage to the target plant and has minimal non-target impacts (Raghu *et al.*, 2006).

1.2 Lycium ferocissimum *as a model for the Enemy Release Hypothesis*

Lycium ferocissimum Miers (Solanaceae) is a shrub commonly known as African boxthorn. The plant is native to South Africa, where it occurs primarily in the Western and Eastern Cape provinces, but some botanical records show occassional occurence outside these areas (Figure 1.2; Haegi, 1976; Venter, 2000). The shrub has become naturalised and invasive in a number of countries around the world (Noble & Rose, 2013). In Australia, it is listed on the Noxious Weed List in some Australian States and is a weed of National Significance (Adair, 2013; Noble & Rose, 2013; Noble *et al.*, 2014). Australian landowners and government have tried to manage the weed with integrated mechanical and chemical control, but these methods have proven costly and unsustainable, which has led the Australian government to initiate a biological control programme against the shrub (Noble & Rose, 2013). However, little is known about the species in the native range, including the associated insect herbivores. Additionally, in the native range there is taxonomic and phylogenetic confusion and the plant is able to hybridise with a number of its closely related and morphologically similar species, all of which will need to be investigated for a successful biological control programme (Venter, 2000; Adair, 2013).

1.3 Lycium ferocissimum *Miers (Solanaceae)*

Lycieae is a tribe of flowering plants in the subfamily Solanoideae of the family Solanaceae. The tribe Lycieae, a large cosmopolitan genus, is one of the largest tribes within Solanaceae and consists of 92 species, (Levin *et al.*, 2007, 2011). Within the Lycieae tribe, the *Lycium* genus is the largest genus with 88 species (Haegi, 1976; Levin *et al.*, 2007, 2011). *Lycium,* according to Haegi (1976), is a genus comprising 80 to 90 species distributed in extra-tropical America and southern Africa, but Venter (2000) suggested that worldwide *Lycium* has approximately 75 species in arid to desert environments of the new world (see also Fukuda *et al.*, 2001). *Lycium spp.* are mostly woody deciduous or evergreen and ending in a spine (Noble & Adair, 2016). *Lycium ferocissimum* is native to South Africa where it is is found in arid to semi-arid environments (Coates Palgrave, 2005; Levin *et al.*, 2007).

1.3.1 Morphological description

Several authors have described the morphological features of *L. ferocissimum,* but these are inconclusive and contradictory, making field identifications difficult as it shares similar morphological attributes with a number of closely related species, causing complications and misidentifications. According to Venter (2000) and Coates-Palgrave (2005), *L. ferocissimum* is a rigid thorny branched shrub growing up to 2 – 3 m in height. Adair (2013) describes it as a dense glabrous shrub growing up to 6 m in height and 3 m wide. Haegi (1976) indicates that sometimes the shrub grows from 2 - 8 m in height, forming impenetrable thickets depending on the habitat and climate.

Lycium ferocissimum is described by both Adair (2013) and Noble & Adair (2016) as a rigid shrub with lateral branches ending in spines up to 15 cm long. However, Coates Palgrave (2005) suggests that the branchlets form spines only 5 – 7 cm in length (see also Haegi, 1976). Flowers are funnel-shaped with white or pale purplish petals with a deeper purple throat, present most of the year, but mainly produced in summer (Figure 1. 1; Venter, 2000; Adair, 2013; see also Haegi, 1976 for a description).

According to Venter (2000) leaves are clustered on stems with 3 - 6 leaves per fascicle, sometimes solitary on young stems. Adair (2013) describes the leaves as fleshy and often in clusters of 5 - 12 leaves per fascicle (Figure 1. 1; see also, Haegi, 1976). Noble & Adair (2016) described the plant stem as silver-grey when young, turning light brown to grey as it matures and becomes fissured with age (see also Noble & Rose, 2013), whereas Venter (2000) described the stem as rigid, with the young stems dull green and older stems pale grey to pale pinkish brown.

Figure 1. 1. *Lycium ferocissimum* Miers (Solanaceae) leaves on fascicles and deep throat flower in the Eastern and Western Cape provinces, South Africa (Adapted from Noble & Adair, 2016).

1.4 Distribution

1.4.1 Distribution in the indigenous range

In South Africa, the shrub is spread across three biomes: fynbos, grassland and savanna, and a number of different environments including coastal, inland, valleys, riverside and plains (Figure 1. 2; Mucina & Rutherford, 2006). *Lycium ferocissimum* occurs on sand dunes and sand flats along coastal areas, while inland it is found on the sandy soils of arid grassy plains and in the karoo (Haegi, 1976; Venter, 2000; Rutherford *et al.*, 2012; Adair, 2013).

There are a few records of the plant in Namibia and Lesotho (http://newposa.sanbi.org/sanbi). Its distribution overlaps with several morphologically similar and closely related species (such as *Lycium afrum* L. and *Lycium horridum* Thunb.) with which it can hybridize (Venter, 2000). *Lycium ferocissimum* is not widely distributed around Africa, but there are a few accounts of its occurrence in Tunisia and Morocco, although it is doubtful if the shrub is indigenous there (Venter, 2000; Adair, 2013).

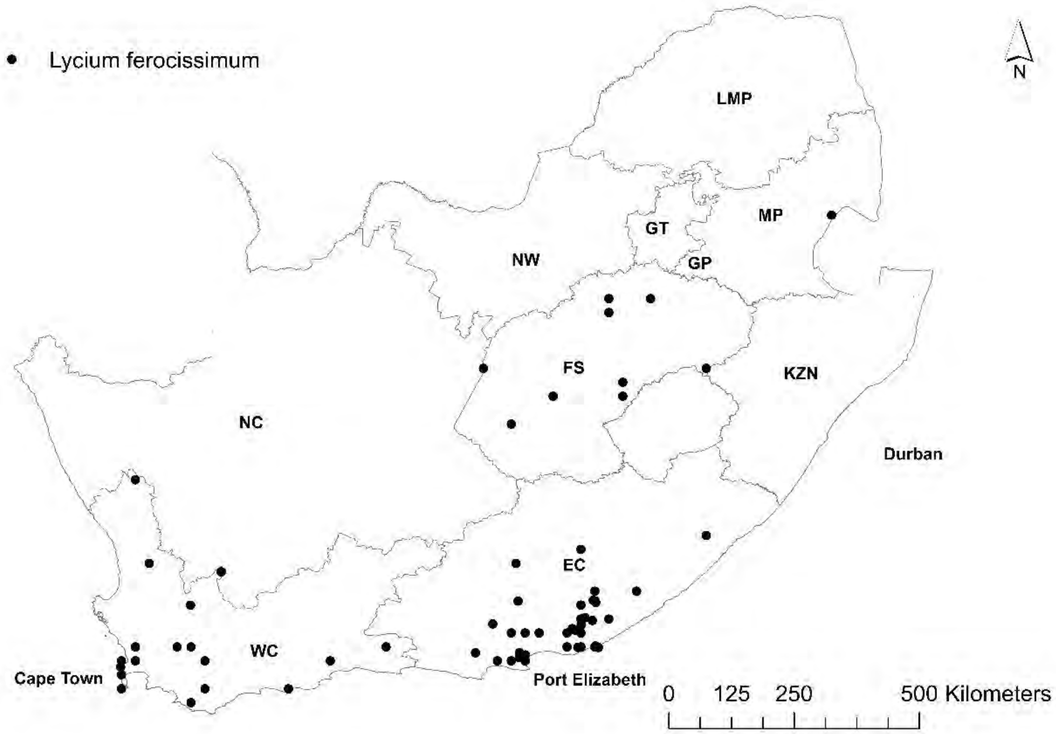

Figure 1. 2. Distribution of *Lycium ferocissimum* Miers (Solanaceae) in South Africa (SANBI, 2016; Venter, 2000).

1.4.2 Distribution in invaded range

Apart from South Africa, Morocco, and Namibia the shrub is also recorded in other parts of the world, e.g. California, (USA) (Haegi, 1976), where it is naturalised but rare, as well as Australia and New Zealand where it has been declared a major weed (Haegi, 1976; Adair, 2013; Noble & Adair, 2016). Parsons & Cuthbertson (2004) showed that *L. ferocissimum* occurs in similar climatic conditions in its native and invaded ranges. Rutherford *et al.* (2012) found that the shrub thrives and performs well in disturbed soils with low nitrogen levels and high phosphorous, potassium and magnesium. In New Zealand, the shrub is restricted to coastal areas in South and North Island (Adair, 2013). In Australia, *L. ferocissimum* grows on all soils and is not restricted by saline soils (Noble & Adair, 2016).

The density of the shrub increases in semi-arid, temperate areas close to seasonal or permanent water sources (Noble & Adair, 2016).

1.4.3 Distribution of Lycium ferocissimum *in Australia*

In the mid-twentieth century *L. ferocissimum* was introduced into Australia as a hedge plant due to barbed wire shortages caused by World War II (Adair, 2013; see also Haegi, 1976). The plant escaped cultivation and is now naturalised and abundant in South Australia, Victoria and New South Wales (Haegi, 1976; Noble & Rose, 2013). The plant has become a common weed in agriculture, rangelands, and in disturbed areas around towns and cities. In the southern part of Australia *L. ferocissimum* is found in almost all the coastal and inland areas, but is less common in southern Queensland and southern Western Australia and Tasmania (Figure 1. 3). *Lycium ferocissimum* in Australia does not occur at altitudes above 1000 m (Noble & Adair, 2016), and also occurs on Lord Howe and Norfolk islands (Figure 1. 3). The spread of the shrub is facilitated by invasive bird species, for example starlings (Haegi, 1976; Noble *et al.*, 2014). Other animals, such as the Tasmanian pademelon, feed on the fruits, thus facilitating the spread of seeds and increasing the shrub's distribution (Haegi, 1976; Adair, 2013; Noble *et al.*, 2014).

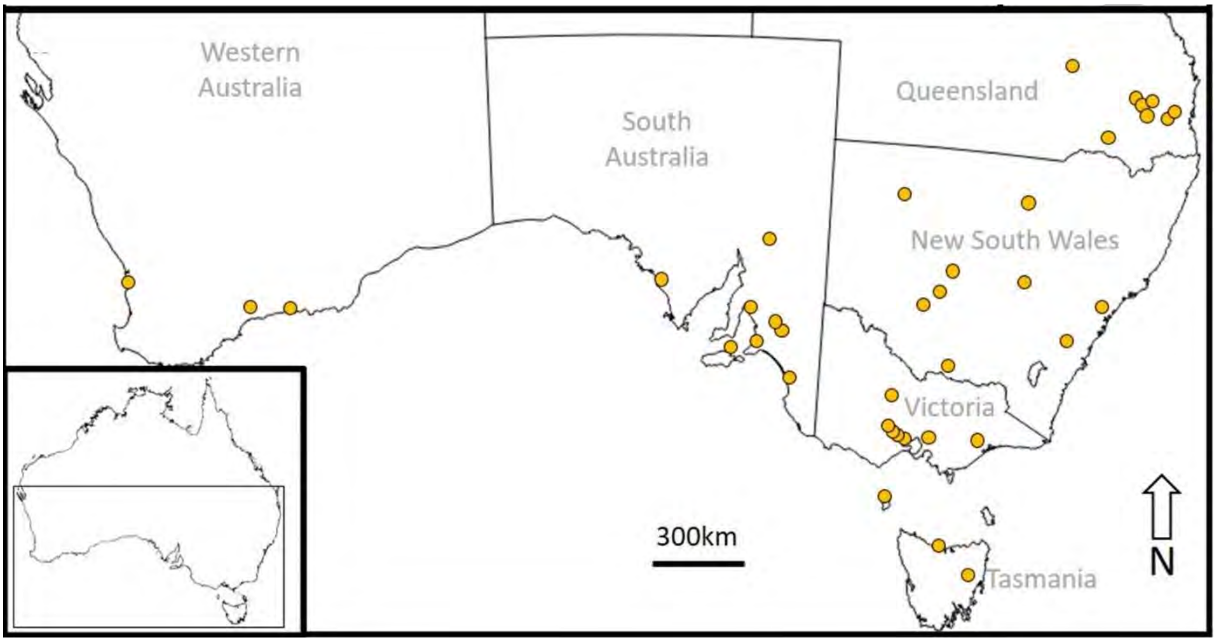

Figure 1. 3. Distribution of *Lycium ferocissimum* Miers (Solanaceae) in Australia were data was collected indicated in yellow (Adapted from McCulloch *et al.,* 2020).

1.5 Lycium ferocissimum *in Australia*

1.5.1 Impacts on Biodiversity

In Australia a broad range of environments are negatively impacted by *L. ferocissimum* (Adair, 2013). The shrub degrades faunal habitats, thus directly impacting native biodiversity (Adair, 2013; Noble & Adair, 2016). Haegi (1976) showed that the plant has significant impacts on a range of vegetation species and communities, as 18 species and nine vegetation communities are negatively impacted by *L. ferocissimum*. Several Ramsar wetlands have been affected by the invasion of *L. ferocissimum* and management of these sites is becoming difficult (Adair, 2013). Coutts-Smith & Downey (2006) found that *L. ferocissimum* has threatened biodiversity in thirteen catchment management areas in New South Wales and there is evidence of a decline in biodiversity.

1.5.2 Impacts on Agriculture

Venter (2000) and Adair (2013) showed that the shrub contains substances making it unpalatable to livestock. *Lycium ferocissimum* has also been shown to host a range of pathogens and agricultural pests (such as the tomato/potato psyllid) that are of concern to Australia (Adair, 2013; Noble *et al.*, 2014). Some studies have found that *L. ferocissimum* would act as a host for the psyllid in winter (Adair, 2013; Noble *et al.*, 2014). *Lycium ferocissimum* has been found to be susceptible to, and hence a carrier of a wide range of viruses that attack solanaceous crops such as tomatoes, egg plants and potatoes (Adair, 2013; Noble *et al.*, 2014; Noble & Adair, 2016).

1.5.3 Benefits

The shrub can withstand herbivory because of its hardiness, thorns and limited palatability, thus it can reduce overgrazing in degraded areas (Rutherford *et al.*, 2012). In Australia, *L. ferocissimum* provides protection to a variety of native fauna, where native vegetation or habitat have been degraded and lost through disturbance (Noble & Adair, 2016).

Lycium ferocissimum provides a wide variety of benefits such as providing fruits and leaves for the pademelon, a Tasmanian marsupial. Two native endangered and vulnerable species (*Isoodon obesulus* and *Peramele gunnii*) have been recorded eating fruits of *L. ferocissimum* (Adair, 2013; Noble & Adair, 2016). Bee keepers benefit from *L. ferocissimum* during drought when nectar and pollen for brood rearing becomes scarce (Noble & Rose, 2013). The shrub provides protection for the Australian greater stick-nest rat against its primary predator, the barn owl (Noble & Adair, 2016). Noble and Rose (2013) showed that in some coastal places, little penguins (*Eudyptula minor*) utilize the shelter of *L. ferocissimum* shrubs for protection against predators. There is evidence of the shrub being poisonous to chickens, but poultry farmers use the plant for protection against hawks, dogs and cats (Adair, 2013; Noble & Rose, 2013; Noble & Adair, 2016).

1.6 Legislation on Lycium ferocissimum *in Australia*

Lycium ferocissimum has been declared a weed in seven States in Australia, under the Biosecurity and Agriculture Management Act 2007 (Table 1. 1; Haegi, 1976; Noble & Adair, 2016). The shrub was designated a Weed of National Significance by the Australian Government in 2012 (Adair, 2013). The Western Australia's South Coast Natural Resource Management (NRM) have completed a management programme for the shrub (Noble and Rose 2013) while, Western Australia State Department of Parks and Wildlife have also undertaken management stratergies for *L. ferocissimum* on the Beagle Islands south of Geraldton.

Table 1. 1. Australian declaration status of *Lycium ferocissimum* Miers (Solanaceae) in different states across Australia (Adapted from Noble & Adair, 2016).

States	Legislature	Declaration status	Description
Australian Capital Territory	*Pest Plants & Animals Act 2005*	Declared weed	Pest plant that must be suppressed (C2), and propagation & supply prohibited (C4).
New South Wales (NSW)	*Noxious Weeds Act 1993*	Declared weed	Not to be sold, propagated or knowingly distributed throughout NSW.
Northern Territory (NT)	*Weeds management Act 2001*	Declared weed	To be eradicated if found & not to be introduced in NT.
Queensland	*Land Protection Act 2002*	Declared weed	Illegal to sell the shrub or its seeds
South Australia	*Natural Resources Management Act 2004*	Declared weed	Management actions prescribed on regional basis.
Tasmania	*Weed Management Act 1999*	Declared weed	Containment across most of the Tasmanian land area.
Victoria	*Catchment & Land Protection*	Declared weed	Regionally controlled
Western Australia	*Biosecurity & Agriculture Management Act 2007*	Not declared weed	Entry of the shrub permitted.

1.7 Management of Lycium ferocissimum *in Australia*

Birds and other fauna contribute to the spread and introduction of *L. ferocissimum* to new areas. Control of *L. ferocissimum* in Australia is challenging due to its broad distribution across a range of environments, and its resilience to both chemical and mechanical management techniques (Parsons & Cuthbertson, 2004). Noble and Rose (2013) therefore suggested that one of the prior strategies for management of the plant would be to start managing the populations from the list of infested areas towards sites which are heavily infested with the plant (see also Brougham *et al.*, 2006). They also suggested that among the first considerations for weed management, must be to know the pathways of spread. *Lycium ferocissimum,* due to its fast capacity of regeneration from root stock, stem and seed, requires integrated management strategy to control its aggressiveness (Adair, 2013; Noble & Rose, 2013; Noble & Adair, 2016; see also Zimmermann & Olckers, 2003). Biological control is considered a potential option to complement conventional control methods for *L. ferocissimum* in Australia (Julien, 2006; Adair, 2013; van Klinken *et al.*, 2016). However, there are no available biological control agent for the species.

1.7.1 Physical or Mechanical control

Physical or mechanical control of *L. ferocissimum* in Australia includes five techniques (*viz.* winching; pulling; bulldozing, stick raking and blade ploughing and machine-based cut stump). These methods are best used when *L. ferocissimum* plants are not fruiting to avoid fresh seeds being deposited into disturbed soils (Noble & Rose, 2013).

Winching and pulling provides the lowest impact where disturbance is a concern in removing large plants in fragile or difficult to access areas. After this technique is used, to control the remaining stumps of roots (to minimize regrowth) other techniques such as cut stump techniques are employed. Pulling or plucking techniques are usually used in low- to medium- infested sites, thus reducing impacts on the surrounding native vegetation (Brougham *et al.*, 2006; Noble & Rose, 2013; Noble & Adair, 2016). The pulling technique involves using commercially developed mechanical equipment to physically remove individual *L. ferocissimum* plants, with as much of the roots as possible. Bulldozing is useful in less sensitive environments with moderate to heavy *L. ferocissimum* infestation and causes exclusive damage to native vegetation and the landscape. This technique also requires an immediate follow-up application of herbicides to any remaining roots. The mechanical cut stump technique involves the application of herbicide immediately after cutting the plant near its base (Noble & Rose, 2013). A case study in Ravensthorpe Golf Club, in an area of 100m x 50m, where they used the combined methods of mechanical plucking and burning the debris of the shrub, reported a 90% mortality of the shrub, but there was regrowth, mostly under native trees. This could have been from the seed bank the shrub produces (Noble & Rose, 2013).

1.7.2 Chemical control

Chemical control is mostly used when the plant is not under stress and is actively growing. The process includes foliar spraying, cut stump application, stem injection, stem scrape (also known as frilling), basal bark application and soil-root zone application (Brougham *et al.*, 2006; Adair, 2013; Noble & Rose, 2013). Mature *L. ferocissimum* has shown strong resilience to foliar spraying due to its deep root system (see also Adair, 2013). The cut stump technique allows for the destruction and removal of *L. ferocissimum* debris (Adair, 2013). Soil-root zone herbicide technique can be effective but often results in significant non-target damage and is not suitable in environmentally sensitive areas or near waterways (Paterson *et al.*, 2014). Herbicides remain in the soil for a long time and can end up leaching into a water table (Noble & Rose, 2013; Noble & Adair, 2016). In Esperance on the south coast of Western Australia, chemical control was used on a large weed population 100% mortality of the shrub was achieved (Noble & Adair, 2016). The chemical (Tebuthiuron pellets) applied on the root zone was reported to have off-target effects on native biodiversity but effects were negligible (Noble & Rose, 2013).

1.7.3 Biological control

Biological control (Biocontrol) of weeds is the maintaining of alien invasive plants' densities at lower densities through the introduction, management and releases of alien insects, predators, mites or pathogen to give permanent control (McFadyen, 1998). Biological control of weeds involves the selection, importation and establishment of specialist phytopathogens or herbivores into a new environment (Van Driesche *et al.*, 2008).

The success of biological control depends on three main variables that need to be carefully planned when implementing control of the weed. These variables are: the damage each candidate biological control agent can do to the target plant; the ecology of the agent, which determines the population density achieved in the new environment, and the ecology of the weed, which indicates whether the total damage is significant in reducing its population (McFadyen, 1998; Van Driesche *et al.*, 2008). Also host-natural enemy environment matches including fixed effects on how bottom up and top down factors affect both host and natural enemy.

In 2016 the Tasmanian Government proposed that Australia's Invasive Plants and Animals Committee (IPAC) list *L. ferocissimum* as a target species for biological control in Australia (Noble & Adair, 2016). Adair (2013) suggested that a detailed survey for potential biological control agents be conducted in its native origin (South Africa) to ascertain organisms with potential for biological control. Adair (2013) concludes that the presence of only one native congener in Australia, reduces the potential for non-target damage making it an excellent candidate for biological control.

1.8 Natural enemies of Lycium ferocissimum

There is a paucity of natural enemies of *L. ferocissimum* in Australia, with herbivory levels on leaves, stems and fruits being less than 1% (Noble & Adair, 2016). Adair (2013), found twelve phytophagous arthropods associated with the plant, but all the species were polyphagous, whilst many were known pests posing a threat to agriculture. During the literature reviews, Adair (2013) highlighted that three monophagous insects occur on

L. ferocissimum: *Cassida lycii* (Sp.) Nov., *Cassida distinguenda* Spaeth and *Cassida melanophthalma* Boheman, all belonging to Chrysomelidae: Cassidinae occur in South Africa.

The distribution of *C. melanophthalma* has been recorded in Namibia and South Africa, whereas the distribution of *C. lycii* and *C. distinguenda* is restricted to South Africa (Borowiec *et al.*, 2001). However, *C. melanophthalma* was also found to be hosted in *Azima tetracanth* of the Salvadoraceae. *Cassida lycii* was named after its host plant *L. ferocissimum* (Borowiec *et al.*, 2001). Tortoise beetles have successfully been used as biocontrol agents for example Cripps (2013), reported success in biocontrol of *Cirsium arvense* (Californian thistle) using *Cassida rubiginosa*.

The feasibility study conducted in Australia recorded for the first time a new species of psyllid (*Acizzia* sp.) on *L. ferocissimum* (Adair, 2013). This was the first time a psyllid was seen idle or feeding on *Lycium* in Australia. The psyllid was found to be widespread in the state of Victoria and occurring in high densities on *L. ferocissimum*, where they caused considerable damage by distortion of foliage, stunting growth, chlorosis and contraction of internodes and causing stem death (Noble & Rose, 2013; Noble & Adair, 2016). In the plant's native range, a little is already known about its associated natural enemies according to Adair (2013). In the native range, fifteen phytophagous arthropod species are known to be hosted by southern African *Lycium,* with the most diverse group being Eriophyiidae with five species, followed by Hemiptera (4 species), Lepidoptera (3 species), Coleoptera (2 species) and lastly Diptera (1) (Adair, 2013).

A rust fungus (*Puccinia rapipes*) (Figure 1.4) has also been recorded on the plant and may be used as a potential biological agent in Australia (Berndt & Uhlmann, 2006; Adair, 2013; Noble *et al.*, 2014; Noble & Adair, 2016). Adair (2013) also indicated that there are six

Puccinia species recorded on *Lycium* species and they require further investigation to reveal the compatibility of other rusts with *L. ferocissimum*. Rust fungus causes yellowing of the leaves on *L. ferocissimum,* reducing its ability to produce more food through photosynthesis and making the plant lose its leaves (Figure 1. 4).

Figure 1. 4. *Puccinia rapipes* pathogen on *Lycium ferocissimum* Miers (Solanaceae) in South Africa (Adair, 2013; Photo Courtesy: Alan Wood, 2015).

Although there are some insects on *L. fercocissimum* in Australia, none offer feasible biological control options. Little is known about the insects associated with the plant in South Africa. However, we know from other faunal studies within the family Solanaceae that there should be a rich insect fauna with the *L. ferocissimum*. Extensive native range studies should therefore be conducted to increase our understanding of the insects associated with the plant and potentially increase the options for biological control.

The aims and objectives of this thesis were to:

- Conduct extensive surveys of *L. ferocissimum* throughout its range in South Africa;
- Determine the population size and extent of *L. ferocissimum* in South Africa;
- Investigate the morphological variation of *L. ferocissimum* within the native range and invaded range and examine the potential for using genetics to ground truth field identification and alleviate current confusion of the morphological characteristics;
- Investigate genetic diversity of *L. ferocissimum* in the native region and compare to the invaded region to ensure genetic compatibility;
- Identify any insect or fungal species that might be considered as potential biological control agents;
- Determine the most damaging of the potential biocontrol agents;
- Determine a rearing protocol of identified/ potential agents and conduct host specificity testing for prioritised candidate biological control agents.

This thesis aims to contribute to the biological control programmes against *L. ferocissimum* with the ultimate goal of reducing the negative consequences of the invasive species in Australia.

Chapter 2: *Lycium ferocissimum* Miers (Solanaceae) morphological characteristics across native and invaded region

2.1 Introduction

Biological control of weeds involves a series of sequential steps, two of the most important on confirming the identity, and origin of the target weed (Gaskin *et al.*, 2005; Goolsby *et al.*, 2006b; van Klinken & Raghu, 2006; Hill & Coetzee, 2008). In most cases, identifying the correct target weed can be based on species-specific morphological characteristics. Unfortunately, there is considerable morphological variability within the genus *Lycium*, causing confusion in delimiting and identifying species within the genus (Venter, 2000; Levin *et al.*, 2007, Chapter 1). In *Lycium ferocissimum* Miers (Solanaceae) morphological variations are not well-documented, making infield identifications difficult (Venter, 2000; Levin *et al.*, 2007, Chapter 1).

As described in Chapter 1, *L. ferocissimum* has broad distribution in South Africa with two allopatric populations, one in the Eastern Cape Province and the other in the Western Cape Province (Figure 2. 1,Venter, 2000). Within these two populations the shrub is distributed over three biomes: fynbos, grassland and savannah, and within these biomes the plant grows in a variety of different environments including coastal, inland, valleys, riverside and plains (Mucina & Rutherford, 2006). This distribution of the shrub means it can exhibit morphological plasticity across sites (Milton Pers. Comm. 2017). These morphological differences are probably the reason that the morphological characteristics of *L. ferocissimum* are not agreed upon within the literature (Haegi, 1976; Venter, 2000; Levin *et al.*, 2007; Adair, 2013; Noble & Adair, 2016). Further, it has been suggested that *L. ferocissimum* can hybridise with closely and morphologically similar relatives *Lycium afrum* (L.) and *Lycium horridum* (Thunb.) (Venter, 2000; Chapter 1) effectively contributing to the difficulty in identifying the species, as hybrids may exhibit characteristics from both parents.

Identification of plant species has been improved with the development of genetic characterisation (for example, Gaskin *et al.*, 2005; Paterson *et al.*, 2009). Genetic studies have made it possible also to determine the origin of invasive weed populations. This has significant implications in biological control programmes, as matching the target weed's centre of origin as closely as possible has been shown to improve the success of biological control agents (also see, Goolsby *et al.*, 2006a; Paterson *et al.*, 2009; Paterson, 2010). However, genetic characterisation can be both expensive and time consuming, therefore, having accurate morphological data remains important and complimentarry when trying to identify plant species. This is particularly important if comprehensive plant surveys are being conducted across large climatic and geographic regions and/or native range host specificity surveys are being conducted on the species in question (Witt, 2004; Goolsby *et al.*, 2006b, see also Urbaneja-Bernat *et al.*, 2019).

Additionally, with regard to selecting biological control agents it has been shown that plant morphological attributes may affect the performance of biological control agents, and therefore, should be considered when surveying for natural enemies (Harms & Grodowitz, 2011; Sutton *et al.*, 2017; Martin *et al.*, 2018a). For example, morphological studies conducted on *Spathodea campanulata* Beauv (Bignoniaceae) showed that plants growing in the West African (native range) and Pacific Islands (invaded range) had more sparsely pubescent leaves than plants from East/Central Africa (native range), making West African plants a priority for biological control agent surveys (Sutton *et al.*, 2017). Therefore, ensuring that plants in the invaded range have morphological characteristics similar to those in the native range is important. The aim of this study was to sample the known distribution of *L. ferocissimum* in South Africa to accurately determine the morphological characteristics of the plant across its native range. This was done to allow for in-field host specificity testing and ensuring collected insects were collected from *L. ferocissimum*. This was done by collecting morphological data from all species assumed to be *L. ferocissimum*. Additionally, the study aimed to determine whether there were morphological differences between the two disjunct populations in South Africa and Australia. Finally, the study compared the morphological attributes of the plant in the native range as well as the invasive distribution in Australia.

2.2 Methods

2.2.1 Surveys

Surveys for *L. ferocissimum* were conducted across South Africa during 2017 and 2018 and over a 2500 km stretch of the invasive distribution in Australia in during 2019 (see Chapter 1, and Table 2. 1, Chapter 2). In South Africa, the plant distributions were obtained from South African herbarium records, the South African National Biodiversity Institute online database (SANBI, 2016) available literature (Figure 2. 1; Venter, 2000) and consultations with relevant experts. Plant populations within distributions were then located via roadside surveys. Surveys were conducted at fifty-three sites across the two populations of *L. ferocissimum* in South Africa (Figure 2. 1; Figure 2. 2; Table 2. 1).

Sites were chosen where *Lycium* sp. plants could be accessed. At visited sites *L. ferocissimum* identifications were initially made following Venter's (2000) taxonomic description. At the initial sampling, it was unknown whether the plant identification was correct as there was overlap with species description within literature. If a sampled species had morphological features not described in the literature, it was noted. At a later stage the identifications were confirmed with the help of Tony Dold (Selmar Schonland Herbarium, Albany Museum, Makhanda) and additional field guides (e.g. Coates Palgrave, 2005). Once the plant identifications were confirmed, repeated surveys at various times of the year were conducted to ensure all aspect of the plant's morphology were sampled. At each sampling event, a site description, including soil type, climate type and associated vegetation, was recorded. Additionally, an individual of the *L. ferocissimum* population sampled was tagged and its GPS co-ordinates recorded (Figure 2. 1). Plant morphology data were then taken for this plant and the insects associated determined (Chapter 4). A genetic sample was taken from the measured plant as well as from ten surrounding plants growing within 100 meters of the tagged individual (Chapter 3). In Australia, 21 sites were sampled between Queensland and Canberra using the same morphometric analysis (Figure 2. 2; Table 2. 1).

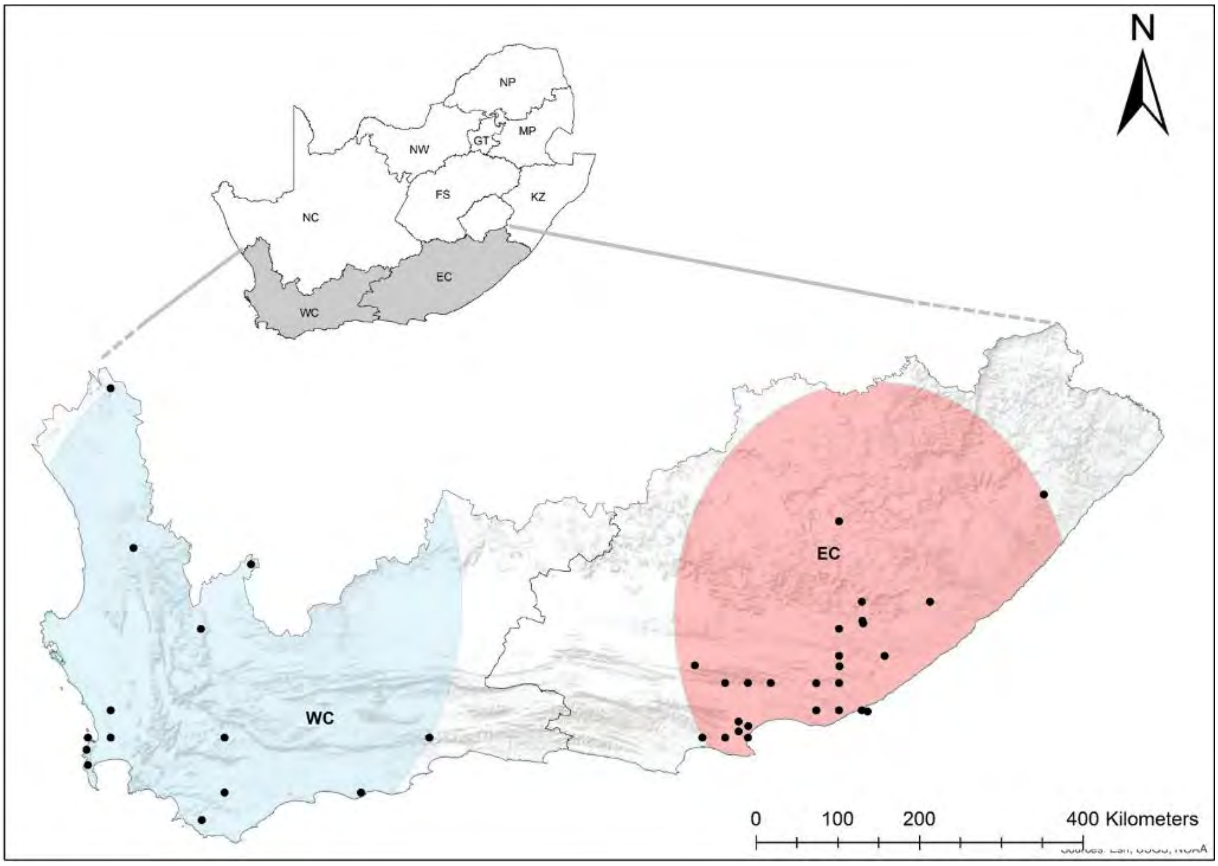

Figure 2. 1. Black dots indicate sampled *Lycium ferocissimum* Miers (Solanaceae) sites in the Eastern and Western Cape provinces of South Africa. Blue area indicates the known approximated distribution of *L. ferocissimum* in the Western Cape Province and the redthe approximate distribution in the Eastern Cape Province (Venter, 2000; SANBI, 2016).

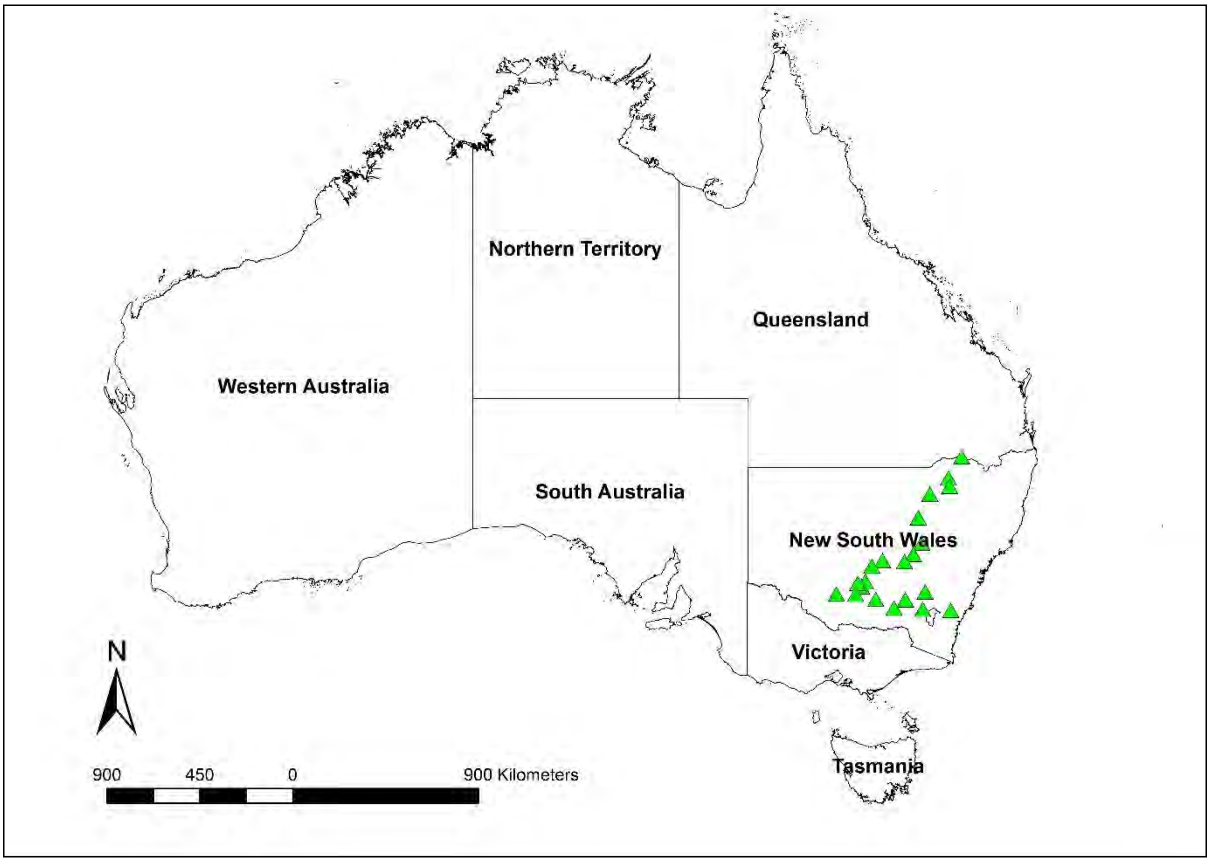

Figure 2. 2. Green triangles indicate sampled *Lycium ferocissimum* Miers (Solanaceae) sites from Queensland to Canberra, Australia.

Table 2. 1. Date and duration of surveys of *Lycium ferocissimum* Miers (Solanaceae) conducted within the native distribution.

Date	Duration	Country, Province	Report Authors
24/02/2017-08/03/2017	13 days	South Africa, Eastern Cape	EV Mauda, LD Chari, GD Martin
17/03/2017-23/03/2017	7 days	South Africa, Eastern Cape	EV Mauda, LD Chari
09/05/2017-02/06/2017	5 days	South Africa, Western Cape	EV Mauda, LD Chari, GD Martin
10/10/2017-12/10/2017	3 days	South Africa, Eastern Cape	EV Mauda, LD Chari, K Ireland
16/10/2017-21/10/2017	6 days	South Africa, Western Cape	EV Mauda, LD Chari, K Ireland, GD Martin
16/11/2017-17/11/2017	2 days	South Africa, Eastern Cape	EV Mauda, LD Chari
15/05/2018-23/05/2018	9 days	South Africa, Western Cape	EV Mauda, LD Chari
03/03/2019-09/03/2019	7 days	Australia, Queensland-Canberra	EV Mauda, S Raghu

2.2.2 Morphological characteristics

To investigate plant morphological features across the sampled populations, the following plant parameters were measured: plant height and breadth, flower characteristics, leaf and fruit measurements, stem diameter. These measurements were taken based on Venter, (2000) and Coates Palgrave (2005) identification guides.

2.2.2.1 Plant height, width and stem diameter

To minimise variability due to measurement error the shrub height was standardised and measured as the highest leaves on the shrub in relation to the ground. Width was taken as the widest horizontal spread of the plant. Due to the plant being dense and highly branched, stem diameter was recorded at approximately 100 mm from the ground to standardise the measurement. In multi-stemmed plants the thickest stem was used for measurement of the diameter (see also, Bouriaud *et al.*, 2005; Sillett *et al.*, 2010; Sumida *et al.*, 2013).

2.2.2.2 Flower characteristics

The number of flowers on each sampled shrub was estimated, giving a measure of flower abundance in the native region and invaded region. At each sampled shrub, a mature reproductive flower was randomly selected and dissected to show a transverse section of the flower and a high-resolution photograph with scale bars was then taken (Figure 2. 3). The photographs of the flowers were used to determine the flower dimension as well as to identify flower shape (tubular and bell), sexuality (bisexual and unisexual) and colour. All measurements were taken from the dissected flowers (Figure 2. 3a & b). The shrub's flowers are typically purple (Venter, 2000; Levin *et al.*, 2007; Noble & Adair, 2016), but this project sought to determine colour variations across the two provinces and across different climatic zones. Flower sexuality was recorded to determine whether the shrub had both stamens and pistils, as some *Lycium* sp. contain only one sex organ (Haegi, 1976).

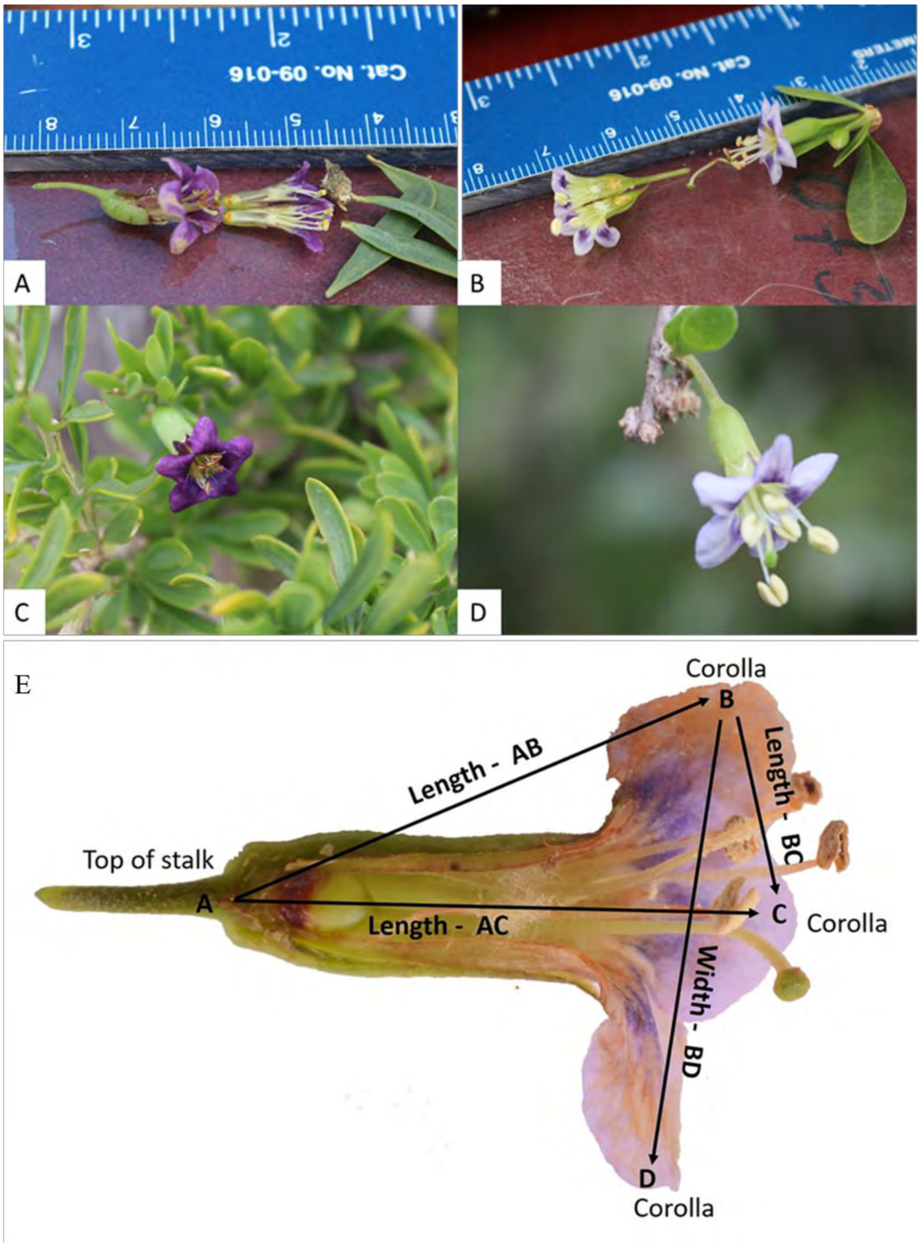

Figure 2. 3. *Lycium ferocissimum* Miers (Solanaceae) flower showing pistil and stamens dissected on a scale bar (A) and external view of the *Lycium* sp. flower (C); *Lycium ferocissimum* flower internally showing pistil and stamens dissected on a scale bar (B) and external view of flower (D), and (E) the transverse section of the flower with letters A, B, C and D representing different measuring points (AB = length from stalk to the highest point of corolla; BD = width of the corolla; BC = length between the corolla petals and AC = length from the stalk to the corolla petals).

2.2.2.3 Leaf measurements

The leaf shape is mainly obovate, broadly or narrowly so, and sometimes elliptic. Both shapes can occur on the same plant. This characteristic has, therefore, no diagnostic value (Venter, 2000). The ratio of length: width is, however, valuable in differentiating between a number of species (Venter, 2000). At each sampled shrub, ten mature leaves (largest leaf in the fascicle) were hand-picked from randomly selected branchlets. The leaf length was measured from lamina tip to the petiole along the lamina midrib and width measured at the widest point of the leaf. The number of leaves per fascicle was also recorded. Selecting matured leaves helps in determining the correct length and width of the plant leaves as new young leaves might still be growing hence true length and width would be compromised (Carvalho *et al.*, 2017).

2.2.2.4 Fruit measurements

Several authors have indicated that shape and size of fruits can be used as an identification characteristic feature in *Lycium* sp. (Milton Pers. Comm. 2017; see also Haegi, 1976; Venter, 2000). Where possible, ten ripe berries were randomly collected from the sampled shrub. Their shape (round or oval) was recorded and the diameter measured (if oval, longitudinal diameter was measured). Unripe berries were avoided or not selected as they might still be growing, and their shape might change with time (Figure 2. 4). Unfortunately, no fresh fruits were measured in Australia at the time of sampling.

Figure 2. 4. *Lycium ferocissimum* Miers (Solanaceae) ripe fruit

2.2.2.5 Herbarium specimens

Herbarium specimens were taken from every *L. ferocissimum* sampled/tagged during the surveys. Herbarium specimens were inspected at the Selmar Schonland Herbarium at the Botany Department, Rhodes University to confirm in-field identifications. Voucher specimens collected from the field sites were pressed and mounted on herbarium papers which were deposited in the Selmar Schonland Herbarium in the Albany Museum, Makhanda (formerly Grahamstown).

2.2.3 Differences in populations

To determine if morphological differences existed between the plants growing in the two populations in South Africa as well as between the plants in Australia the plant morphology was compared across the three populations.

2.2.3.1 Statistical analysis

Basic statistics were conducted on plant morphology measurements using STATISTICA v.10 (Hill & Lewicki, 2007). ANOVA's ($P < 0.05$; significance) was conducted between South African *L. ferocissimum* and Australian *L. ferocissimum* populations.

To determine whether morphological characteristics could be used to differentiate *L. ferocissimum* in the two disjunct population, and to compare the South African populations and Australian populations, a Principle Component Analysis (PCA) in the statistical programme *R* using Factoextra and GGPlot 2 packages was used. Where ANOVA showed significant differences, Fisher LSD tests were used to make post-hoc comparisons of plant characteristics. Differences in flower shape, sexuality and internal structure (stamens and pistils) visibility between the clades were tested for significance using Chi-squared analyses ($\chi 2$; significance level, $p < 0.05$). Analysis of Similarity (ANOSIM) was used to investigate whether there were any morphological differences in *L. ferocissimum* across the two South African Provinces and Australian sampled populations. Non-metric multidimensional scaling (NMDS) was used to visualise the differences in morphological variants across all sampled sites. The BEST analysis function in PRIMER was used to determine factors "best explaining" variation in plant morphological features across the native and invaded region. BEST works by maximising a rank correlation between the respective resemblance matrices of explanatory (factors) and response variables (morphological features).

2.3 Results

2.3.1 Surveys

In the native region of the 53 sites, 18 were confirmed as *L. ferocissimum* in the Western Cape Province, with 5 found to be hybrids or unknown *Lycium* sp. (Chapter 3). In the Eastern

Cape Province 21 were confirmed *L. ferocissimum,* 8 hybrids and/or unknown *Lycium* sp. and one *Lycium oxycarpum* (Chapter 3).

2.3.2 Morphological characteristics

2.3.2.1 Plant height, width and stem diameter

The combined plant height, width and stem diameter showed no distinct differences between the two *L. ferocissimum* populations in South Africa and the Australian populations (ANOSIM; Global R = 0.014, $P = 0.38$). Similarly, the NMDS analysis showed that there was an overlap in measurements between South African populations and the Australian populations (Figure 2. 6). In addition, South African plants did not differ in their stem diameter, but both had significantly wider stems than plants sampled in Australia (Table 2. 2).

2.3.2.2 Flower characteristics

In South Africa, both asexual and bisexual flowers were recorded on *L. ferocissimum* plants, whilst only bisexual flowers were recorded in Australia (Table 2. 3). Similarly, tubular and bell-shaped flowers were recorded within the South African populations, whilst only bell-shaped flowers were recorded in Australia (Table 2. 3). Bell-shaped flowers were more common in South Africa than tubular flowers, only 23% of flowers sampled were tubular in shape (Table 2. 3). Flower length (AC) was greater in the two South African populations than with the Australian plants (Table 2. 3; Figure 2. 5), whilst flower length (AB) was only greater in the confirmed *L. ferocissimum*, but not in unknown *Lycium* sp. populations which comprised hybrids and other species of *Lycium* that have not been taxonomically identified (Table 2. 3; Figure 2. 5). Flower width (BD) was significantly greater in Australian plants than the South African populations (Figure 2. 5). There were also differences in flower shape ($\chi2$=23.72, df = 2, $P < 0.0001$), sexuality ($\chi2$=22.22, df = 2, $P < 0.0001$) and internal structure (stamens and pistils) ($\chi2$=19.03, df = 2, $P < 0.0001$) amongst plants from the two allopatric populations and those collected from Australia (Table 2. 2). Flowers on Australian plants were significantly wider (width BD), but shorter (length AC, AB, BC) than the flowers from South African *L. ferocissimum* species (Table 2. 3). There were significant differences in flower measurements between *L. ferocissimum* and unknown *Lycium* species collected from South African sites (Table 2. 2). Apart from the three floristic dimensions, shape and sexuality, there were no significant differences between the South African populations and the Australian population plant morphology parameters (Table 2. 2; Table 2. 4).

Table 2. 2. Floral characteristics and average values of plant morphology characteristics of South African populations and Australian population. Chi-squared tests ($\chi 2$) were used to compare the frequency of flower traits amongst the populations, whilst ANOVAs were used to investigate differences in mean values of plant characteristics. Fisher's LSD analysis was used to show post-ANOVA differences in means, with different letters (in superscript) denoting a statistically significant difference at $P < 0.05$ and significance values were indicated in bold.

	Lycium ferocissimum		***Lycium* species unknown**	**ANOVA and $\chi 2$ tests**
	South Africa	**Australia**		
Floral characteristics				
Sexuality	Bisexual (34 sites), Unisexual (3 sites)^{a}	Bisexual (6 sites)^{a}	Bisexual (4 sites), Unisexual (6 sites)^{b}	**$\chi 2 = 16.42$, d.f. = 2, $p < 0.001$**
Flower shape	Bell (29 sites) Tubular (8 sites)^{a}	Bell (6 Sites)^{a}	Bell (2 sites), Tubular (8 sites)^{b}	**$\chi 2 = 15.6$, d.f. = 2, $p < 0.001$**
Length AC (mm)	17.0 (±3.9)^{a}	11.8 (±2.3)^{b}	17.8 (±5.2)^{a}	**$F_{2,65} = 46.0$, $p < 0.001$**
Width BD (mm)	9.4 (±2.0)^{a}	10.8 (±2.6)^{b}	8.4 (±1.7)^{a}	**$F_{2,65} = 58.6$, $p < 0.001$**
Length AB (mm)	12.7(±3.2)^{a}	8.8 (±1.0)^{b}	10.8 (±2.3)^{a}	**$F_{2,65} = 49.8$, $p < 0.001$**
Length BC (mm)	8.0 (±2.4)^{a}	7.4 (±2.0)^{b}	8.2 (±2.8)^{a}	**$F_{2,65} = 54.7$, $p < 0.001$**
Stem characteristics				
Diameter (mm)	63.9 (±46.0)^{a}	28.5 (±16.0)^{b}	68.3 (±32.5)^{a}	**$F_{2,65} = 11.1$, $p < 0.001$**
Plant characteristics				
Height (m)	1.80 (±0.80)	2.0 (±0.8)	2.0 (±0.6)	$F_{2,65} = 0.5$, $p = 0.642$
Width (m)	2.5 (±1.1)	2.4 (±1.0)	3.1 (±0.90)	$F_{2,65} = 1.6$, $p = 0.214$
Leaf characteristics				
Leaves per fascicle	7.5 (±2.1)	7.1 (±1.0)	8.4 (±2.2)	$F_{2,65} = 1.7$, $p = 0.192$
Length (mm)	28.1 (±8.6)^{a}	21.0 (±5.5)^{b}	24.8 (±4.6)^{a}	**$F_{2,65} = 4.0$, $p = 0.022$**
Width (mm)	8.1 (±3.8)	8.6 (±2.2)	5.0 (±2.3)	$F_{2,65} = 1.3$, $p = 0.290$

Table 2. 3. Floristic features of unknown *Lycium* sp. and *Lycium ferocissimum* Miers (Solanaceae) in the Eastern Cape (EC) and Western Cape (WC) Cape provinces of South Africa and invaded region Australia.

Lycium **populations**	**Flower Sexuality (Bisexual/Unisexual) and Sites**	**Flower Shape (Bell/Tubular) and Sites**	**Average Flower measurements, mm (SE)**			
			Length AC	**Width BD**	**Length AB**	**Length BC**
L. ferocissimum (SA)	Bisexual (EC1, EC7, EC8, EC10, EC11, EC12, EC13, EC15, EC16, EC17, EC18, EC19, EC20, EC24, EC25, EC3, WC4, WC9, WC10, WC13, WC14, WC15, WC17)	Bell: (EC1, EC7, EC8, EC10, EC11, EC12, EC13, EC15, EC16, EC17, EC18, EC19, EC20, EC24, EC25, EC3, WC4, WC9, WC10, WC13, WC14, WC15, WC17)	16.30 (±0.70)	9.60 (±0.40)	12.30 (±0.60)	7.30 (±0.30)
	Unisexual (EC22, WC12)	Tubular (EC5, EC14, EC22, WC6, WC11, WC12, WC16)	20.60 (±1.40)	9.30 (±0.60)	14.90 (±1.60)	10.30 (±1.50)
Unknown *Lycium* sp. (SA)	Bisexual (EC6, EC26, WC2)	Bell (EC23, EC26)	13.00 (±0.00)	8.50 (±0.50)	8.50 (±0.50)	6.50 (±1.50)
	Unisexual (EC2, EC3, EC4, EC21, EC23)	Tubular (EC2, EC3, EC4, EC6, EC21, WC2)	18.30 (±2.20)	8.30 (±0.90)	11.70 (±1.00)	8.70 (±1.40)
L. ferocissimum (Australia)	Bisexual	Bell	11.80 (±0.30)	10.80 (±0.30)	8.8 (±0.10)	7.40 (±0.20)

Table 2. 4. Characterisation of leaves, stems and overall plant dimensions of unknown *Lycium* sp. and *L. ferocissimum* in South Africa and Australia.

***Lycium* sp.**	**Average Stem diameter, mm (SE)**	**Average Plant height, m (SE)**	**Average Plant width, m (SE)**	**Average leaves per fascicle (SE)**	**Average leaf length and width, mm (SE)**	
					Length	**Width**
L. ferocissimum (SA)	24.80 (±3.70)	1.80 (±0.10)	2.5 (±0.20)	7 (±0.40)	25.80 (±2.10)	7.60 (±0.70)
Unknown *Lycium* sp. (SA)	31.30 (±11.10)	1.90 (±0.20)	3.3 (±0.90)	8 (±1.20)	23.00 (±3.30)	5.40 (±0.80)
L. ferocissimum (Australia)	29.00 (±3.50)	1.98 (±0.17)	2.4 (±0.22)	7 (±0.22)	20.90 (±1.09)	8.60 (±0.49)

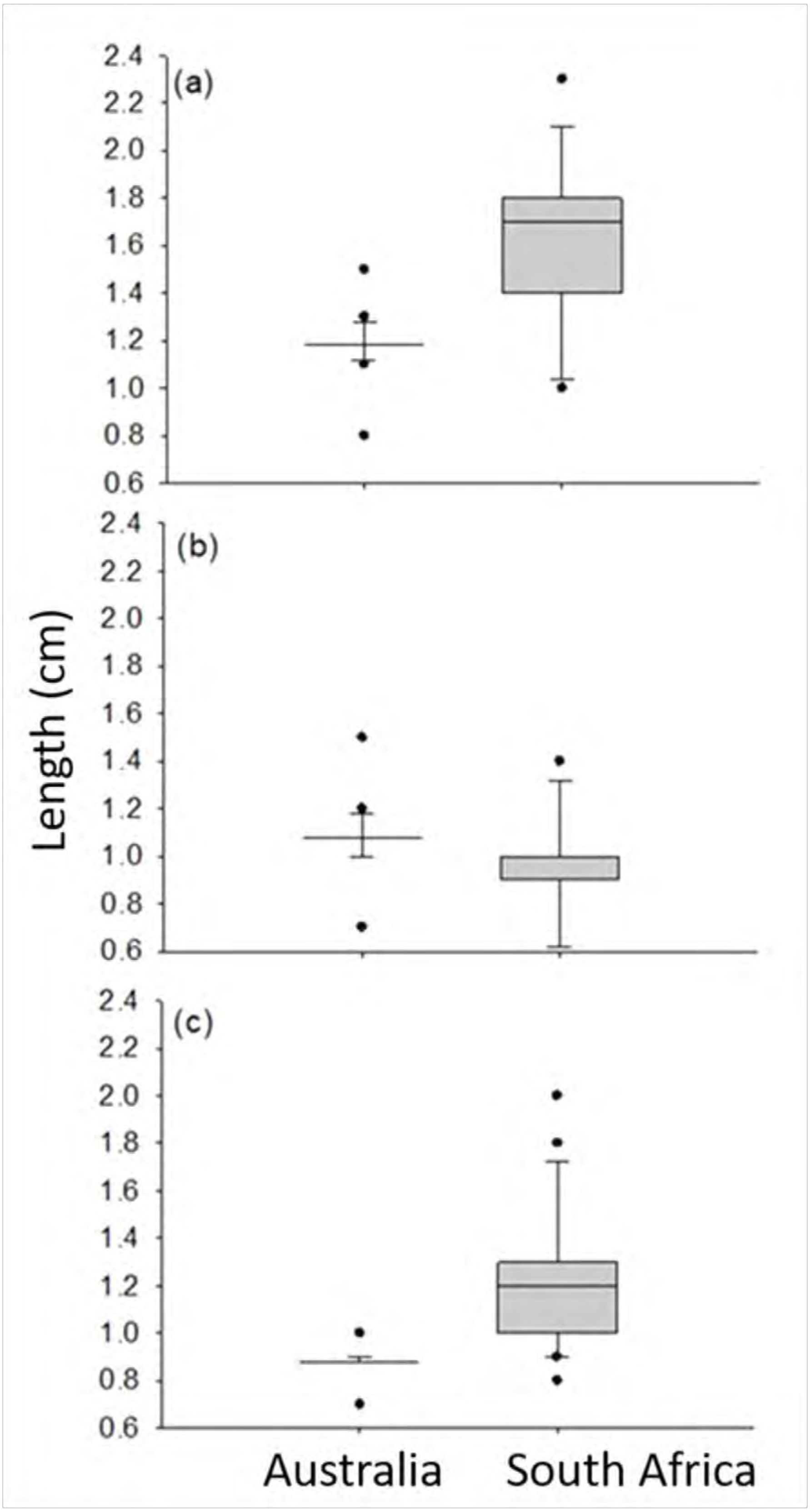

Figure 2. 5. Boxplots illustrating the differences in (a) Flower length-AC, (b) Flower width-BD and (c) Flower length-AB characteristics between the *Lycium ferocissimum* Miers (Solanaceae) in South African populations and those collected from Australia. The boxplots represent the probability

densities in credibility intervals of 95, 75 and 25% for the length measurements. All differences were statistically significant based on ANOVA (*P* < 0.05).

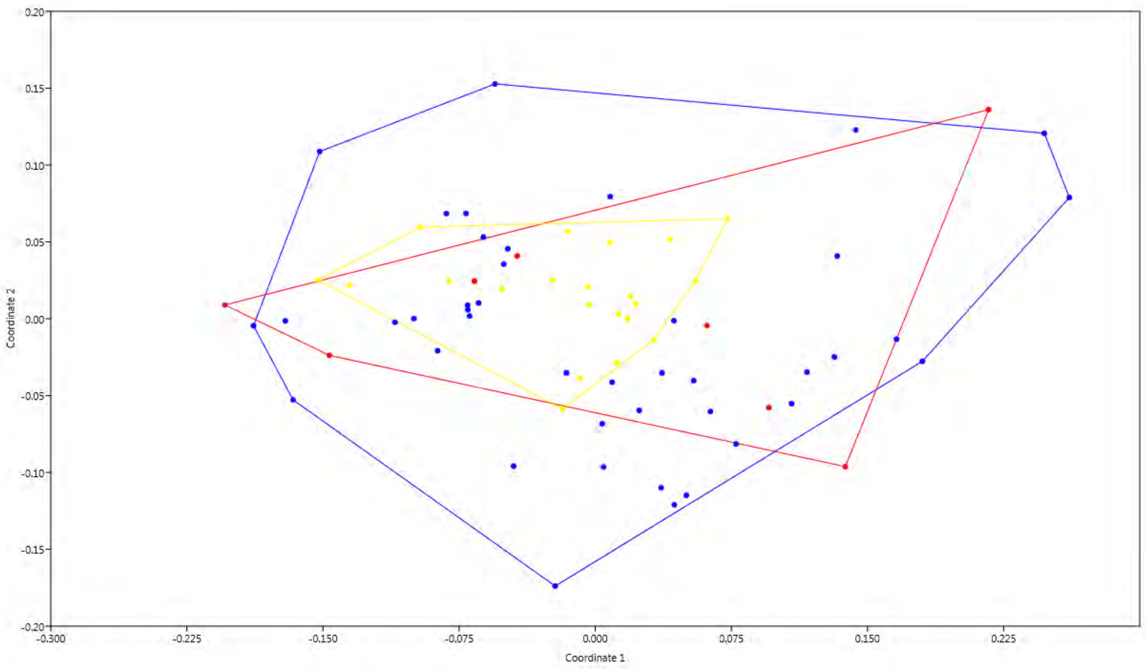

Figure 2. 6. Non-metric multidimensional scaling showing relatedness of *Lycium ferocissimum* Miers (Solanaceae) in South African [*Lycium* sp. (red) and *L. ferocissimum* (blue)] populations and in Australian (yellow) populations based on several measured plant morphological characters (Stress = 0.1).

2.3.2.3 Leaf measurements

There was no significant difference between leaves lengths of *L. ferocissimum* and the unknown *Lycium* species in South Africa or between the two allopatric populations (Figure **2. 7**). However, the leaves of *L. ferocissimum* were much longer on plants from South Africa than on those collected from Australia. The BEST analysis showed leaf length amongst other variables measured on the plant constituted 33% of the variation between South African population and Australian population structure.

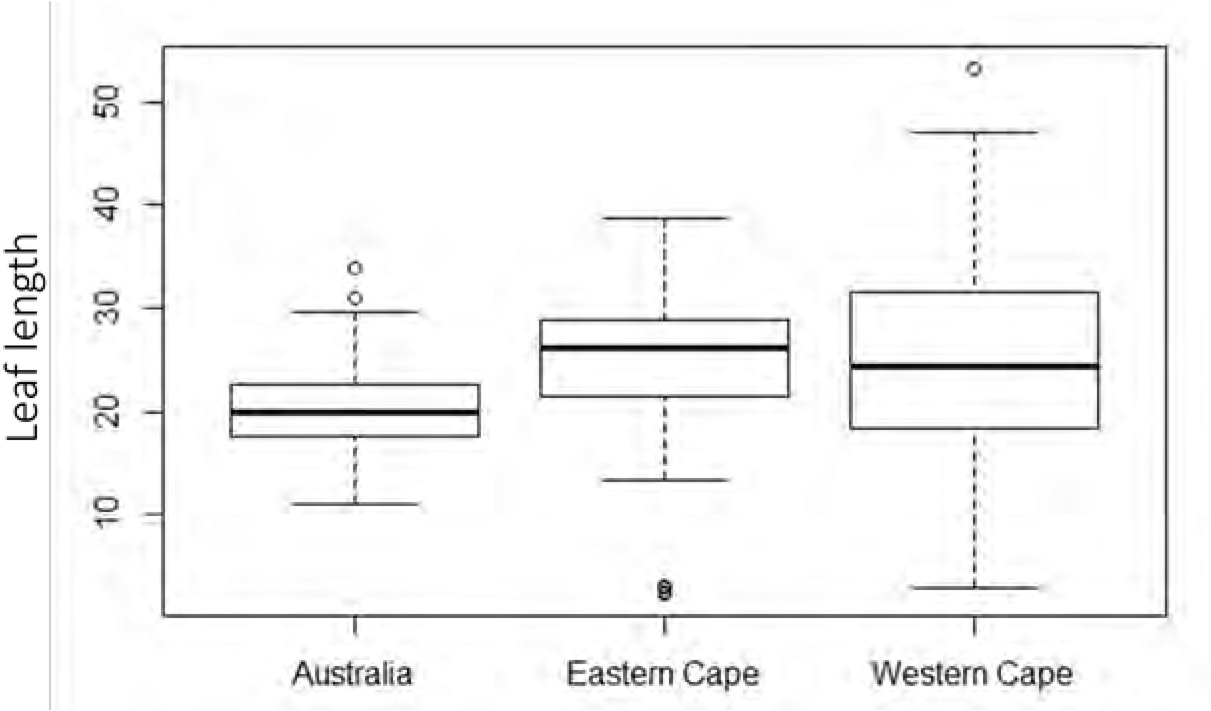

Figure 2. 7. Boxplots illustrating *Lycium ferocissimum* Miers (Solanaceae) leaf length differences among South African populations (Eastern Cape and Western Cape provinces) and Australian populations.

2.3.3 Australian populations vs South African populations

The principle component analysis showed that there was an overall overlap between the two South African populations and Australian population regardless of the area in which the shrub was growing. The non-metric dimensional scaling and PCA together confirmed no difference among the populations when using leaf length and leaf width as a measure of traits (Figure 2. 8; Figure 2. 9).

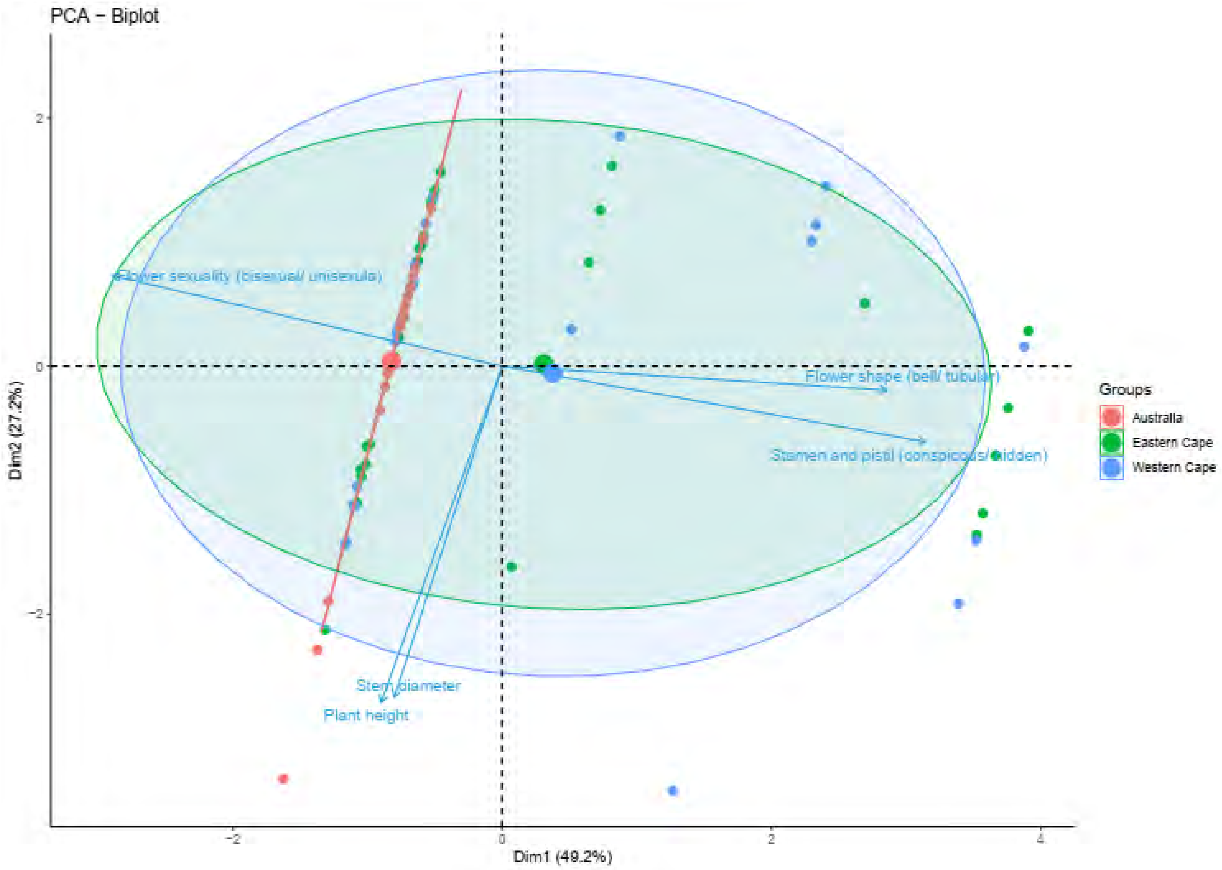

Figure 2. 8. Principle component analysis showing no difference in Australian *Lycium ferocissimum* Miers (Solanaceae) population and South African *L ferocissimum* populations (Eastern Cape and Western Cape provinces).

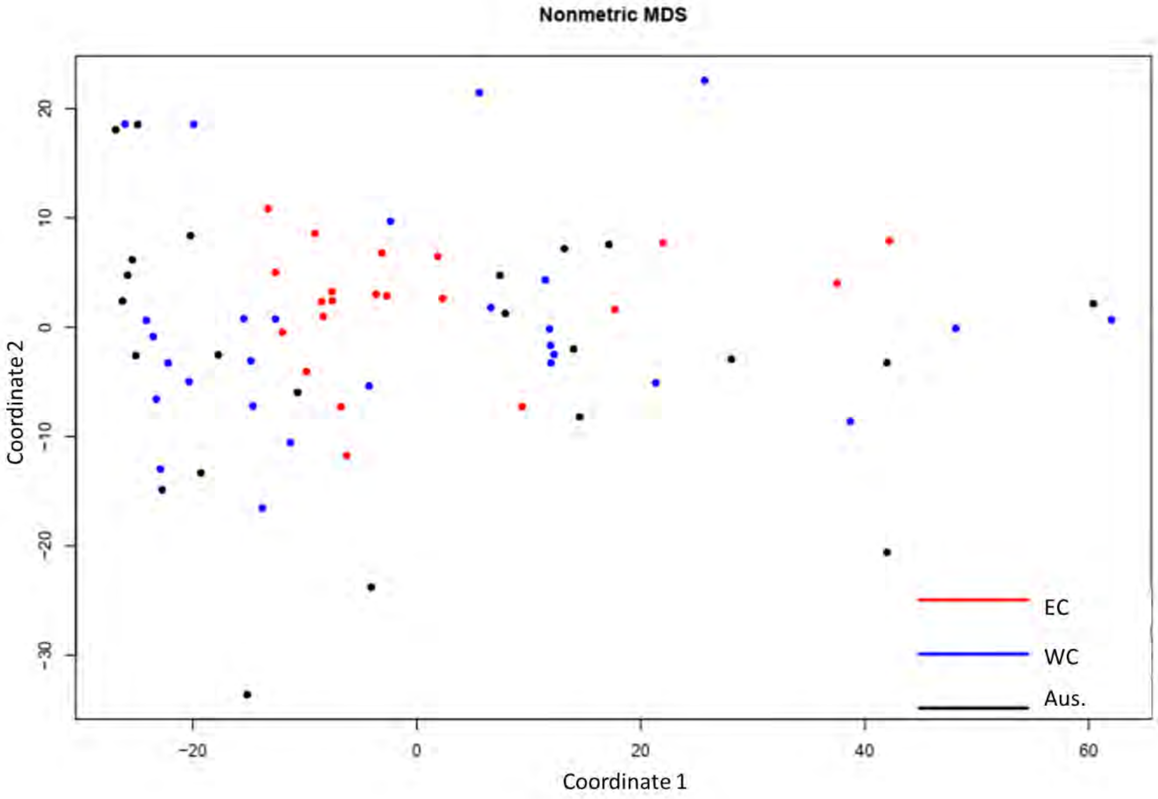

Figure 2. 9. Non-metric multidimensional scaling of Australian *Lycium ferocissimum* Miers (Solanaceae) and South African *L. ferocissimum* (Eastern Cape and Western Cape provinces).

2.4 Discussion

This study provides baseline plant morphology data for *L. ferocissimum* from across the native range in South Africa. Until now there has been conflicting information regarding the plant's morphology within the literature, making in-field identifications difficult. This study contributes towards clarifying three uncertainties regarding the plant's morphology including: (1) conflicts within morphological descriptions in the literature; (2) delineating between *Lycium* species based on plant morphology; (3) morphological differences associated with *L. ferocissimum* and its hybrids and undescribed *Lycium* species. Additionally, this chapter showed morphological differences between *L. ferocissimum* populations invading Australia and the plants in South Africa.

Due to phenotypic plasticity, plants of the same species may have different morphological characteristics, depending on the environment in which they grow (Gratani, 2014). Therefore, plants within the same population may vary under different environmental conditions, making it difficult to delineate or group according to morphology, especially if there are morphologically similar species within the genus. Due to the conflicting literature and the geographic separation of the two populations of *L. ferocissimum* in South Africa it was initially assumed that there would be measurable morphological differences between the plants (R. Dean & S. Milton Renu-Karoo Veld Restoration, South Africa pers. com. 2017). Specifically, this assumption was made because the plants in the Western Cape Province grow in a Mediterranean type climate dominated by Fynbos, while those in the Eastern Cape Province grow in a more arid dry Albany Thicket environment (Rutherford *et al.*, 2012). However, no clear morphological differences could be identified between the South African populations, suggesting limited plasticity. This further suggests that morphological traits are a good indicator to identify *L. ferocissimum* in South Africa across the large disjunct distribution. This is not uncommon as Schweingruber & Poschlod, (2005), argue that despite a certain morphological plasticity in plants, there are only a few species where ecotypes are morphologically different. It should be noted that no individual characteristic was diagnostic, and that the combination of features is usually required for accurate identification.

Based on the literature, which suggested *L. ferocissimum* shared both morphological features, habitat and distribution with other co-existing *Lycium* species, it was suspected that delineating between species could potentially only be confirmed by genetic analysis. Initial investigations of the literature and herbarium specimens suggested that three *Lycium* species (namely *Lycium amoenum* Dammer, *Lycium afrum* L. or *Lycium horridum* Thunb.) had similar morphological characteristics with *L. ferocissimum* and were reported to be found in the same regions as *L. ferocissimum* (see Venter, 2000; Manning & Goldblatt, 2012). However, no *L. afrum* and *L. horridum* were recorded during the surveys. The specimens in this study that were tentatively identified as being *L. afrum* or *L. horridum* turned out to be an unidentified *Lycium* species or *L. ferocissimum* hybrids (Chapter 3). The other *Lycium* species regularly encountered was *Lycium oxycarpum* Dunal. Fortunately, the literature and other identification keys used during the study were good in differentiating the *L. oxycarpum* in the Eastern and Western Cape provinces. The flowers of *L. oxycarpum* are long and tubular with a purple colouration on the inside. The length of the flower is almost double that of *L. ferocissimum* in both provinces. Flowers in both provinces are independent of each other, although sometimes, depending on where they are growing, they may occur in a cluster of two. *Lycium oxycarpum* berries in both provinces are oblong-elliptic and most are red, with some exception in the karoo area were the fruits are orange to red (see also, Venter, 2000; Coates Palgrave, 2005). Other *Lycium* species were also uncounted, namely *Lycium cinereum* Thunb. and *Lycium schizocalyx* C.H. Wright, and were easily identified in the field by morphological characteristics.

Venter, (2000) in the taxonomic revision of *Lycium* based on morphological features and meiotic chromosomes (Spies *et al.*, 1993) suggests that *L. ferocissimum* readily hybridises with *L. afrum* and in some cases with *L. horridum* (see also, Manning & Goldblatt, 2012). Morphological hybrids which were found during the study were distinguishable among each other, but the parent species could not be identified. Contradictorily, Venter (2000) suggests that the hybrids could be distinguished morphologically from the parent species amongst which they were found, indicating that the hybrids exhibited characteristics of both parents, but these characteristics are not expressed uniformly in all individual hybrids that were encountered. The hybrids that were encountered in our study showed variations in their morphological features, with some exhibiting *L. ferocissimum* characteristics more than the other unknown species, in some areas exhibiting more features of the unknown *Lycium* species than that of *L. ferocissimum*. In Chapter 3, we showed using molecular techniques that there was evidence of *L. ferocissimum* hybridising with another species of *Lycium* which was neither *L. afrum* nor *L. horridum,* based on comparisons to GenBank sequences. Herbarium records also show that most of the species previously recorded and identified as the two species were later changed to *Lycium ferocissimum* Miers during the revision of the genus (Selmar Schonland Herbarium in the Albany Museum, Makhanda). The two species (*L. afrum* and *L. horridum*) that are morphologically supposed to hybridise with *L. ferocissimum* were not found during this study despite the extensive repeat surveys.

During surveys plants identified as *L. ferocissimum* hybrids and those identified as unknown species showed variations in flower colour, with most of the unknown species having deep white throat in the corolla and some species, including hybrids, having dark purple flowers with longer corolla tube; some with stamens conspicuous and some hidden. Furthermore, the unknown *Lycium* species could not be identified or placed anywhere using molecular techniques (Chapter 3), suggesting that the species is undocumented. The difference in morphological characteristics of unknown species and hybrids in the two populations further suggests that there might be two or more distinct species hybridising with *L. ferocissimum*. The unknown *Lycium* species hybridising with *L. ferocissimum* remains undetermined.

The Australian populations showed similar morphological characteristics to South African populations and morphological plasticicity may be maladaptive to *L. ferocissimum* in the two regions despite being different to the native environment. However, some differences in flowered morphology were measured, with Australian flowers having shorter, wider flowers. The differences in flower structure between South African and Australian forms are interesting and may be the result of a number of scenarios. Firstly, that only plants containing the genetic material for shorter, bell-shaped and bisexual flowers were introduced into Australia. This is unlikely due to the close genetic match between the Australian and South African plants, and that no flowers have been recorded in South Africa that are as small as the flowers found in Australia. There is also the option that it is a result of phenotypic plasticity, but this was not supported by any of the other morphological attributes measured (Gratani, 2014). Additionally, the plants measured in Australia were from a wide geographic area and therefore growing under different environmental conditions, suggesting that some of the plants would have been similar to South African plants measured if phenotypic plasticity were occurring. Finally, that there is selection pressure occurring in Australia that favours shorter and wider flowers. The Increased Competitive Ability (EICA) hypothesis suggests that as a result of reduced herbivore pressure plants can diminish their allocation of resources to herbivore defences and focus on reproduction and growth (Blossey & Notzold, 1995). The reduction in the number of herbivores (Adair, 2013), associated with flowers and fruits in Australia may have resulted in *L. ferocissimum* being able to produce wider flowers. This may assist in attracting greater numbers of pollinators and promoting the spread of the species via sexual reproduction in the country. Unfortunately, flower, fruit numbers and pollinators were not considered during this study to test the hypothesis.

Overall, few morphological differences were detected across *L. ferocissimum* plants in their native and introduced ranges; this should facilitate the establishment of any foliar and/or stem-boring biological control agents. While morphological differences in flower shape and size were identified across the two countries, they are unlikely to impact on any biological control agents released, unless flower-feeding agents are prioritized. Furthermore, genetic analyses have further confirmed and identified that *L. ferocissimum* samples in South Africa are the true representative of the invasive populations in Australia (Chapter 3) and supported the morphological description that were found during the study. These findings have implications when starting and developing a biological control programme against the invasive species, hence providing information on the insects associated with shrubs and where to search for and prioritise candidate biological control agents.

Chapter 3: Genetic matching of *Lycium ferocissimum* Miers (Solanaceae) populations in the native range South Africa, with the invasive populations in Australia

3.1 Introduction

Several authors have described *Lycium ferocissimum* as having morphological attributes that are shared by species within the genus *Lycium*, making identification of the species based on morphology unreliable (Chapter 2; see also Venter, 2000; Coates Palgrave, 2005; Noble & Adair, 2016). Both Minne *et al.* (1994) and Venter (2000) suggested that the taxonomy of *Lycium* species in southern Africa should be revised using molecular techniques.

Identification uncertainties pose significant challenges to the *Lycium ferocissimum* biological control programme as the practice of weed biological control is based on the close relationship between the target plant and the phytophagous insects and pathogens associated with it. In addition, to ensure that the correct species is being studied, determining where the species was introduced from also has important implications for weed biological control. Goolsby *et al.* (2003) highlighted the importance of accurate plant taxonomy in biological control programmes when they showed that populations of an eriophyid mite collected for the biological control of *Lygodium microphyllum* (Cav.) R. Br. (Lygodiaceae) from plants from the source of the invasive population performed better than those collected on plant genotypes from other parts of the plant's native distribution (see also Hubbard, 2016). Urban *et al.* (2011) sums it up "For biological control, the practical value of plant taxonomic studies is to pinpoint the native home of the target weed, to facilitate exploration for the best-adapted, and possibly most effective natural enemies".

One of the challenges in determining the genetic structure/diversity and identity of the target weed arises when that species experiences interspecific and intraspecific hybridisation. Venter (2000), when observing chromosome numbers and ploidy levels of the African

Lycium species, suggested *L. ferocissimum* was able to hybridise with other species, including *Lycium afrum* and *Lycium horridum*. Paul *et al.* (2010) suggested that if a species experiences any form of hybridisation in its native region, it has a high chance of hybridising in the invaded region provided there are close relatives of the species (see also Ward *et al.*, 2008). In Australia, little is known about the shrub's biology or even if the shrub hybridises with the other *Lycium* species such as the indigenous *L. australe* or the commercially grown *Lycium barbarum* L. "Gojiberry". Levin *et al.* (2007), using amplified exons of the nuclear Granule-bound starch synthase I (GBSSI or waxy) gene in the evolutionary relationship of the tribe Lycieae, included the assumed Australian indigenous species *Lycium australe* F. Muell and *L. ferocissimum*. *Lycium australe* was found to be in the same clade with the southern Africa species, *Lycium tenue* Willd. and *Lycium gariepense*, which nested within a clade with twelve southern African *Lycium* species but not in the same clade as *L. ferocissimum*. *Lycium australe*has an overlapping distribution with *L. ferocissimum* across parts of Western Australia, South Australia, Victoria and New South Wales (Adair, 2013). Likewise, *Lycium afrum*, of African origin, has been introduced and is naturalised in Australia (Randall, 2007).

To successfully implement a biological control programme against *L. ferocissimum* in Australia, greater taxonomic clarity of both the native and invaded plant populations is required. Therefore, *L. ferocissimum* was sampled across South Africa and Australia, and molecular screening (three chloroplast genes, one nuclear gene) was conducted to: (a) confirm the genetic identity of the invasive lineage, and (b) assess genetic structuring across the native and introduced ranges. This allowed for insects and pathogens to be collected from the correct target plant as well as from plants closely matched to those in Australia (Chapter 4). Additionally, it provided insights into potential problems should any erroneous results be found in other stages of the biological control programme, such as varying insect performance on test plant species.

3.2 Methods

3.2.1 Surveys

Surveys for *L. ferocissimum* were conducted across South Africa and Australia (Chapter 2; Figure 3. 1), as sampling a wider area is regarded as more important than sampling within the populations (see Muirhead *et al.*, 2008). Plant distributions in South Africa were obtained from herbarium records, the South African National Biodiversity Institute online database (https://www.sanbi.org/), and literature (e.g. Venter (2000). Plants were subsequently located during roadside surveys, and provisionally identified as *L. ferocissimum* using morphological keys (Chapter 2; Figure 3. 1). In addition, samples of *L. cinereum, L. tenue, L. schizocalyx, L. afrum* and *L. oxycarpum* collected in South Africa were tested to determine their genetic relatedness to *L. ferocissimum* and *L. australe*.

The Australian distribution data of *L. ferocissimum* was obtained from the Atlas of Living Australia (https://www.ala.org.au/) and personal communications with researchers working on the species in Australia (Raghu S. Principal Research Scientist, CSIRO Australia & Ireland, K. Extension Coordinator - Australian Fungicide Resistance Extension Network, Asutralia). Leaf samples for genetic analyses were obtained from up to twelve individual plants sourced from each of the Interim Biogeographic Regionalisation for Australia (IBRA) bioregions invaded by the shrub (https://www.environment.gov.au/parks/nrs/science/bioregion-framework/ibra/index.html). In addition, gojiberry samples from Australia were tested to determine their genetic relatedness to *L. ferocissimum* and *L. australe*.

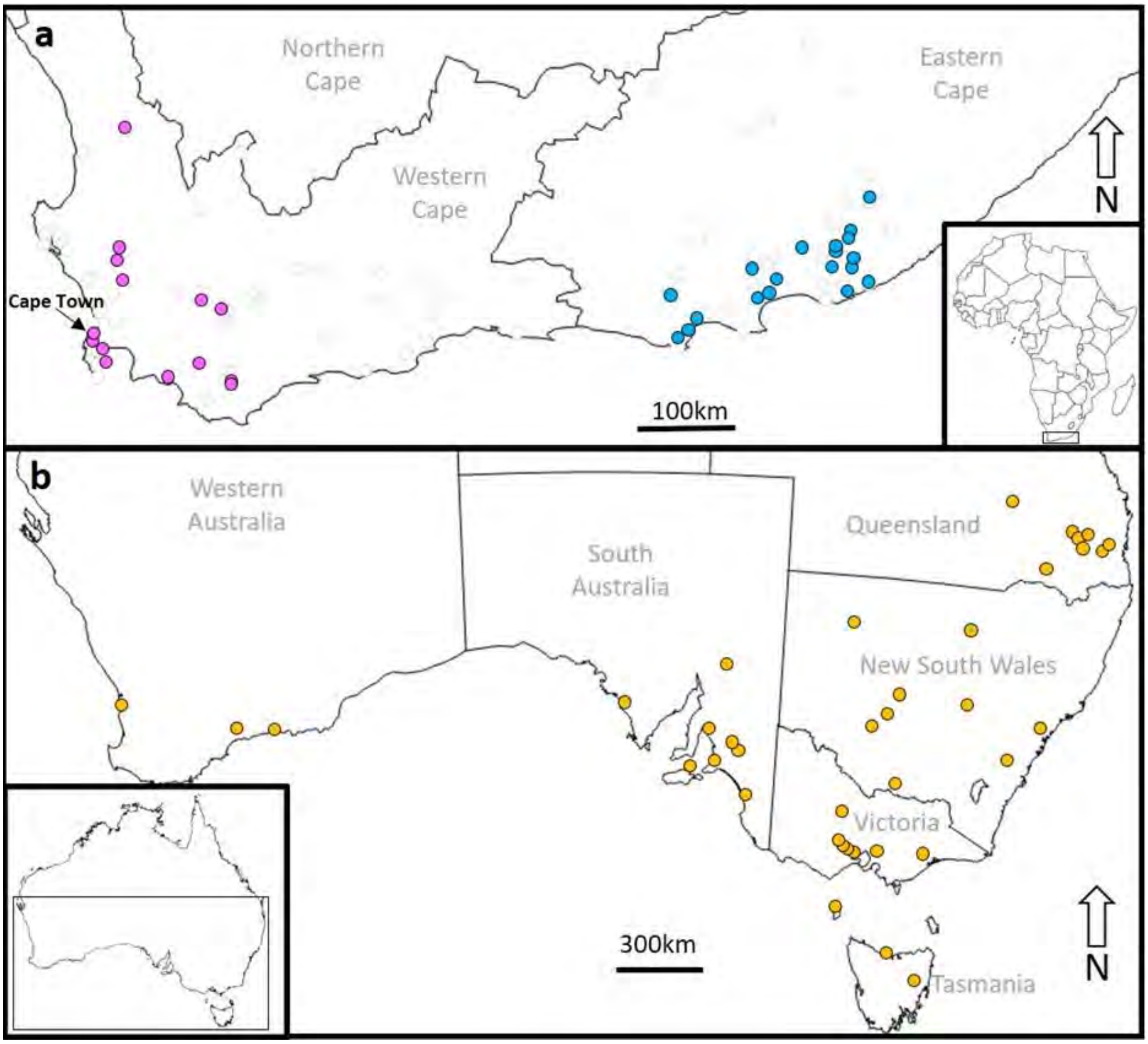

Figure 3. 1. *Lycium ferocissimum* Miers (Solanaceae) distribution sampling sites across (a) South Africa (blue = Eastern Cape, purple = Western Cape), and (b) Australia (yellow).

3.2.2 Genetic Characterisation

3.2.2.1 Native Range sampling

At each site, a single *L. ferocissimum* was identified and tagged with a metal plant tag. Leaf samples for subsequent molecular analysis were taken from tagged plants and from up to ten surrounding *L. ferocissimum* plants growing within 100 metres of the tagged individual. Selecting additional random plants helps reduce the chance of missing the most important genotypes within the populations. The shoot material of growing plants was collected using a pair of scissors and placed into small collecting envelopes. Plant material was dried in silica gel to preserve the quality of the genetic material until shipped to Australia for molecular analysis.

3.2.2.2 DNA extraction and amplification

Total genomic DNA was extracted from material collected at 42 South African and 49 Australian sites, sampled (Figure 3. 1) using the cetyl trimethyl ammonium bromide (CTAB) extraction method, followed by spin column purification. Where possible, 10 individual samples, representing distinct plants, were extracted per site. DNA was extracted from dried leaf samples (see also Doyle & Doyle, 1987). The extracted DNA was stored at -20ºC for subsequent use.

Four DNA regions were amplified: three chloroplast regions (trnH – psbA intergenic spacer; maturase K and the trnT – trnL) and one nuclear gene (GBSSI) (see Levin *et al.*, 2006). The chloroplast region (trnH – psbA intergenic spacer) was amplified using the primers trnHf and psbA3-f (Sang *et al.*, 1997; Tate & Simpson, 2003). For maturase K, matk-1RKIM-f and matK-3FKIM (Kuzmina *et al.*, 2012) was used; for the trnT-trnL intergenic spacer primers trnL-c and trnL-f were used, and for the nuclear gene GBSSI, *waxy*F and *waxy*2R was used (Miller *et al.*, 1999; Levin *et al.*, 2006).

The polymerase chain reaction (PCR) contained 12 µl of each of the primers (12 µl), 7.6 µl deionised water, 2.0 µl 5x buffer (Bioline), 0.24 µl each of 10 mM forward and reverse primer, 0.08 µl Taq polymerase (Bioline) and 2 µl of DNA template. Cycling conditions for the chloroplast genes involved an initial denaturation step (95ºC for 3 minutes) followed by 40 cycles of 95ºC for 20 seconds; 50ºC for 30 seconds and 72ºC for 45 seconds with a final annealing step of 72ºC for 10 minutes. Gel electrophoresis was used to confirm the amplification of the DNA. A touchdown procedure was used to amplify GBSSI following the protocols used by Levin & Miller (2005). The thermal cycler program was used in a touchdown procedure with an initial denaturing at 94°C for 4 minutes; 14 cycles at 94°C for 30 seconds, 57°C-51°C decreasing one degree every two cycles for 1 minute, ending with an extension at 72°C for 10 minutes.

3.2.2.3 Gene sequencing

Total genomic DNA was extracted from dried *Lycium* leaf samples collected at 42 sites in South Africa and 49 sites in Australia, using the CTAB extraction method followed by spin purification. DNA samples were sequenced in both directions by Macrogen (Korea). To select the best model of sequence evolution for each region JModeltest v2.1.2, was used (Darriba *et al.*, 2012). The three plastid regions were concatenated into a single dataset; as individuals, they exhibit low levels of sequence divergence and there should be no convergence among regions of the plastid genome, as they cannot assort independently, or recombine (McCulloch *et al.,* in review). MrBayes 3.2.6 was used to reconstruct some of the phylogenetic relationships in the *Lycium* species (Huelsenbeck & Ronquist, 2001). The concatenated chloroplast dataset was partitioned by DNA region, allowing independent estimation of parameters for each partition. Four Markov chains were run for five million generations, with chains sampled every 200 generations.

(i) Molecular identification of Lycium *samples*

Sequencing was used to determine whether all samples collected from Australia and South Africa were *Lycium ferocissimum* and if there were other species co-occurring within the sampled populations. Sequences were edited in Geneious v11.1.5 (https//:www.geneious.com; Kearse *et al.*, 2012), and aligned with additional South African and Asian *Lycium* species (downloaded from GenBank) using the MUSCLE plugin (Edgar, 2004). Having samples from South Africa and Australia made it possible to determine how closely they were related by matching the South African native *L. ferocissimum* genotypes with the invading Australian *L. ferocissimum* genotypes.

(ii) Genetic variation and structuring within Lycium ferocissimum

Gene sequencing was used to determine genetic variation within *L. ferocissimum* and, if significant genetic diversity was found in South African populations, it would suggest the presence of cryptic species within *L. ferocissimum*. If there was genetic structuring within the populations, it could also be used to deduce the origin of the Australian lineages. A high level of genetic diversity within the Australian population may indicate multiple introductions of the shrub from different regions of South Africa.

3.3 Results

3.3.1 Population genetics of Lycium ferocissimum

3.3.1.1 DNA extraction and gene sequencing

More than 20 ng /µl of high-quality molecular weight DNA suitable for conventional gene sequencing was extracted. Six haplotypes of *L. ferocissimum* in South Africa were matched to the GenBank sequences. Additionally, a *Lycium* sp. haplotype that could not be matched to any of the accessioned GenBank sequences was found. Bayesian analysis of the samples identified two well-supported clades (labelled clade A and clade B; Figure 3. 2), with each clade containing multiple *Lycium* species.

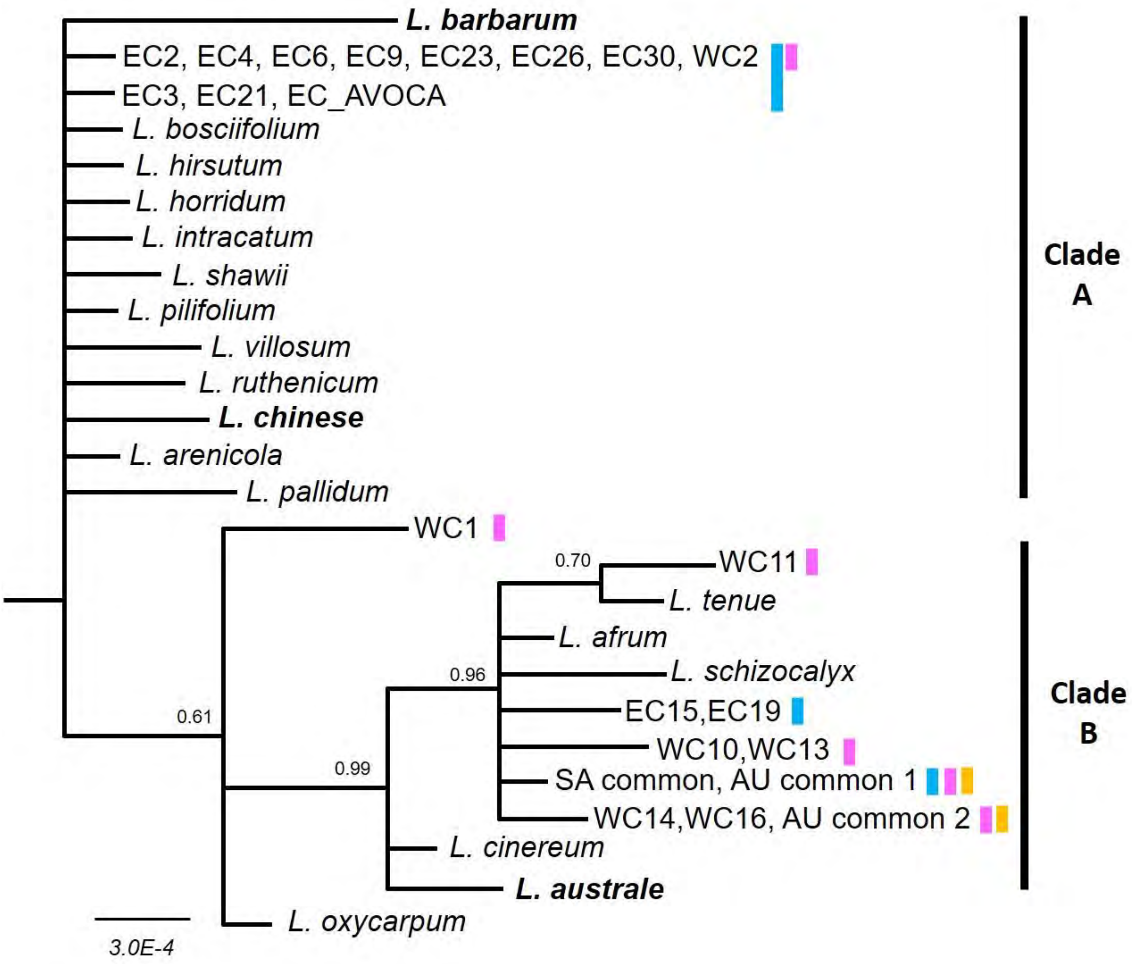

Figure 3. 2. Bayesian phylogeny based on three concatenated chloroplast regions (trnH – psbA intergenic spacer, maturase K, trnT-trnL intergenic spacer). Posterior probabilities are noted above each node. Outgroups have been removed for diagrammatic clarity. Coloured bars indicate the geographic region where the haplotype was found (blue = Eastern Cape (EC), purple = Western Cape (WC), yellow = Australia; see Figure 3. 1). The most common South African haplotypes (SA common) were found at EC1, EC5, EC7-8, EC10-14, EC16-18, EC20, EC22, EC24-25, WC3-4, WC6, WC9, WC12, WC14-15, WC17, and matched the *Lycium ferocissimum* Miers (Solanaceae) accessions on GenBank (McCulloch *et al.,* in press).

Twenty-four (EC1, EC5, EC7-8, EC10-14, EC16-18, EC20, EC22, EC24-25, WC3-4, WC6, WC9, WC12, WC14-15, WC17) South African samples (SA common 1) and all the Australian *L. ferocissimum* (AU common 1) were identical in all three chloroplast genes and matched the *L. ferocissimum* accessions from GenBank (Figure 3. 2). Seven samples from the South African populations (EC15, EC19, WC10, WC11, WC13, WC14 and WC16) did not match any of the sequences of *Lycium* on GenBank; however, these samples were included in a well-supported *L. ferocissimum* clade (clade B) (Figure 3. 2). Two of the Western Cape

Province sites (WC14 and WC16) shared a haplotype which is also common in the Australian populations.

Lycium samples from eleven sites in South Africa grouped into clade A rather than clade B (Figure 3. 2). Furthermore, these assumed *Lycium* sequences did not match any sequences of *Lycium* species on the GenBank database, and their phylogenetic position and morphology (Chapter 2) also indicated that they were not *L. ferocissimum* but another *Lycium* species (Chapter 2) and these species were classified as *Lycium* species unknown (Chapter 2; Table S1. 1). One site in the Western Cape Province had a unique chloroplast haplotype, and the placement of this sample was not well resolved (Figure 3. 2; Table S1. 1).

The GBSSI results showed that there were six haplotypes identified within the over 300 *L. ferocissimum* samples sequenced from across South Africa and Australia. However, only one haplotype (haplotype 5) was common between South Africa and Australia, with other haplotypes typically restricted to only a single site (Figure 3. 3; Table S1. 1). The relationship among haplotypes was not well resolved and the nuclear and chloroplast phylogenies were not completely similar.

Specimens from chloroplast clade B were included in a single clade in the GBSSI phylogeny which included the *L. ferocissimum* accessioned in GenBank; however, this clade was not well supported (PP = 0.70; Figure 3. 2; Figure 3. 3) and *Lycium afrum* L., *Lycium cinereum* Thunb. and *Lycium schizocalyx* C.H. Wright, were also included in the chloroplast phylogeny (Figure 3. 2), but *Lycium tenue* Willd. and *Lycium australe* F. Muell, were not included in the Bayesian phylogeny based on nuclear GBSSI gene (Figure 3. 3). *Lycium* species other than *L. ferocissimum* in the chloroplast clade A formed a clade in the GBSSI phylogeny with *L. tenue*, *Lycium gariepense* A.M. Venter and *L. australe;* EC9 was most closely related to *Lycium oxycarpum* Dunal (Figure 3. 2; Figure 3. 3).

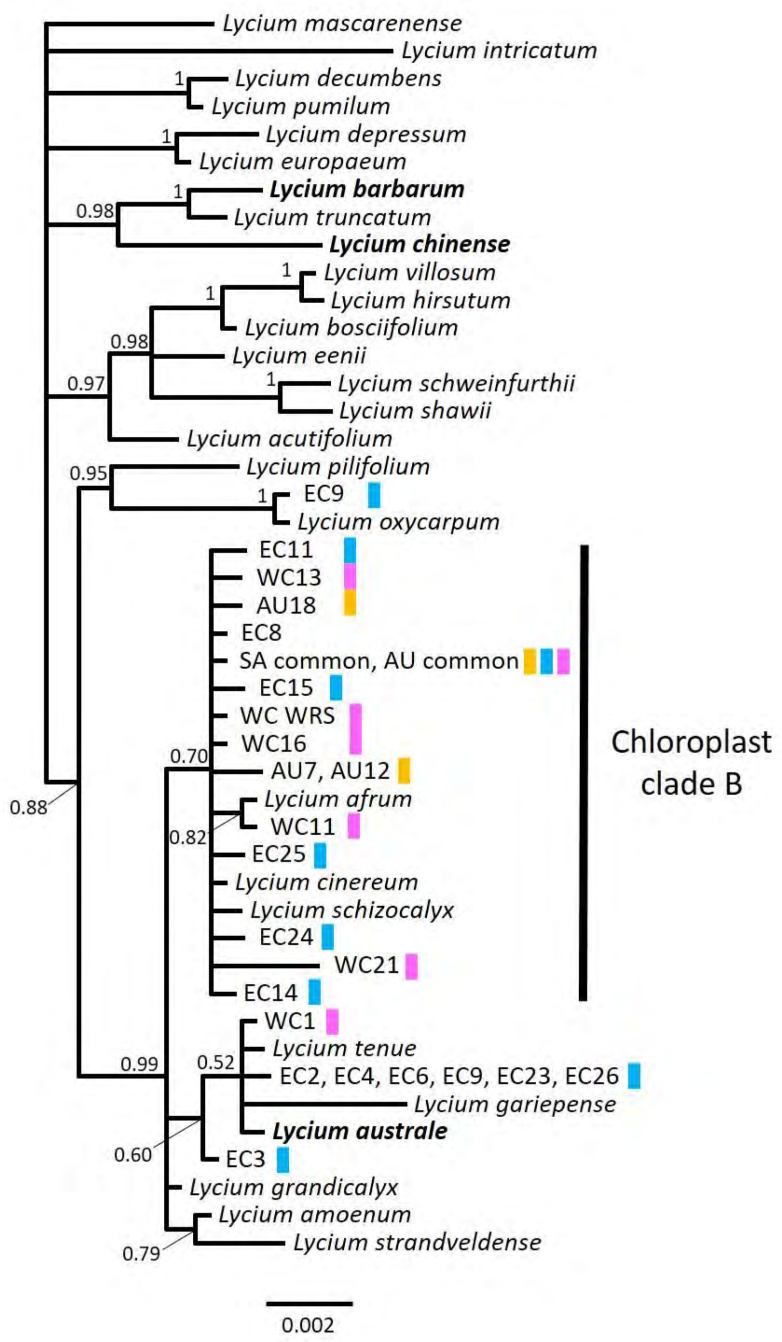

Figure 3. 3. *Lycium ferocissimum* Miers (Solanaceae) Bayesian phylogeny based on the nuclear GBSSI gene. Posterior probabilities are noted above each node. Outgroups have been removed for diagrammatic clarity. Coloured bars indicated the geographic region where the haplotypes were found (blue = Eastern Cape Province, purple = Western Cape Province, yellow = Australia). Chloroplast clade B refers to the *L. ferocissimum* clade in Figure 3. 2. SA common, found in clade B matched the GenBank sequences and AU common was also found in clade B matching the *L. ferocissimum* GenBank accessions (McCulloch *et al.,* in press).

Using the four gene regions (GBSSI, trnT, trhH-psbA and trnL), it was found that *L. australe* was included in clade B (Figure 3. 2) with a number of African *Lycium* species:

L. cinereum, L. tenue, L. schizocalyx, L. afrum and *L. oxycarpum*. The gojiberry samples tested tested were identified as *L. barbarum*, rather than *L. chinense* species not genetically close to *L. ferocissimum* (Figure 3. 4).

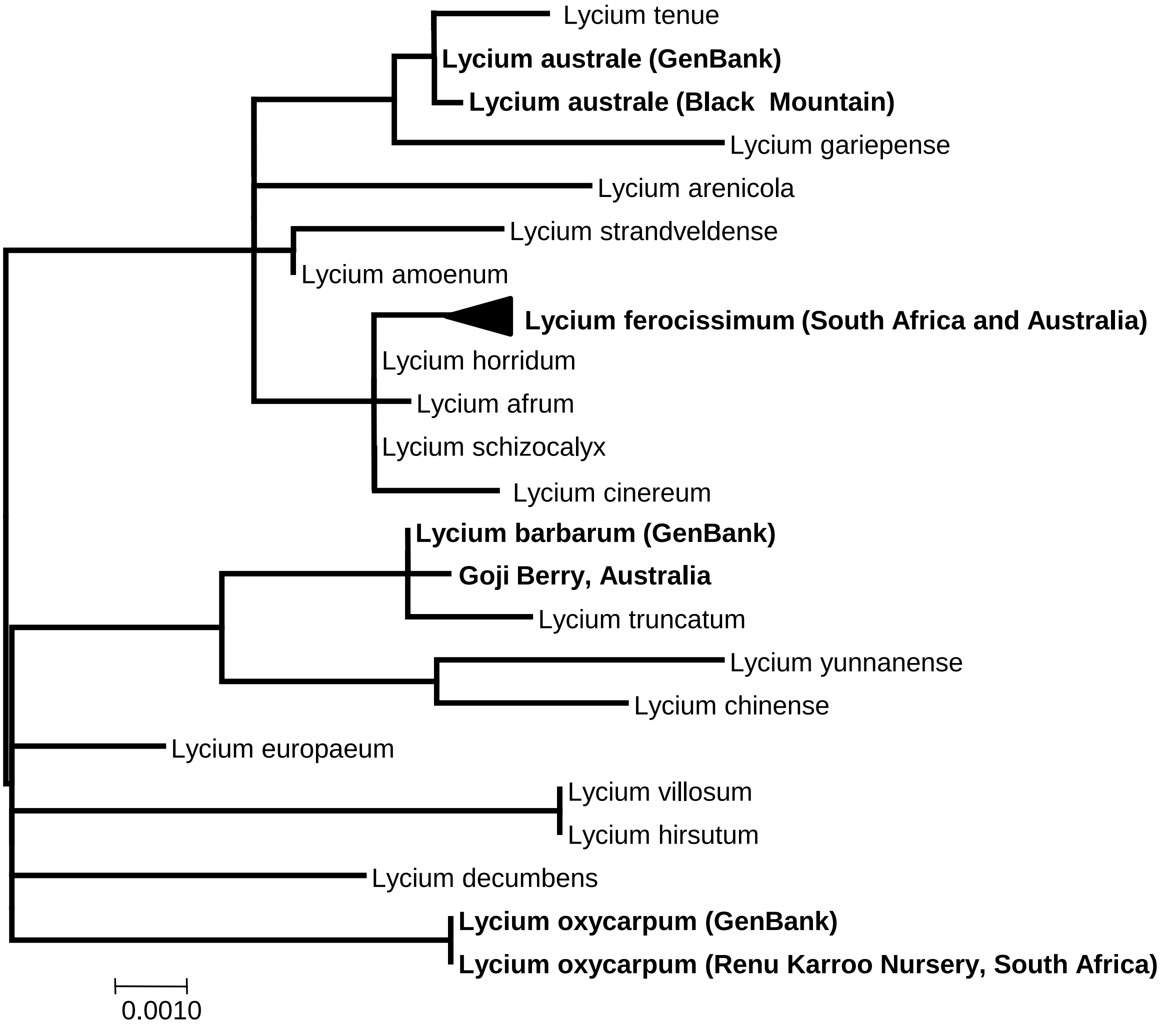

Figure 3. 4. Maximum likelihood phylogeny of *Lycium* using four genes (GBSSI, trnT, trnhH-psbA and trnL). Species of interest are represented in bold.

3.3.2 Lycium ferocissimum *distribution in South Africa and Australia*

All specimens from Australia were confirmed as *L. ferocissimum* and matched the GenBank sequences for *L. ferocissimum* (Table S1. 1; Figure 3. 5). Most South African specimens were confirmed *L. ferocissimum;* however, some specimens selected for analysis, primarily in the Eastern Cape Province, were found not to be *L. ferocissimum*. The molecular analysis suggested that there are at least four distinct species of *Lycium* (not accessioned in GenBank) in addition to *L. ferocissimum* (Table S1. 1; Figure 3. 5).

Lycium ferocissimum across Australia and South Africa had six distinct haplotypes identified from over 300 specimens sequenced (Figure 3. 6). The genetic differences between haplotypes were high, providing evidence that there might be cryptic species occurring in the South African populations. One haplotype (haplotype 5) was found in most of the plants across South Africa and Australia (Figure 3. 6). Three haplotypes (haplotypes 1, 3 and 4) were found only in the Eastern Cape Province. Two haplotypes (haplotypes 2 and 6) were found only in the Western Cape Province and sporadically in New South Wales, South Australia, Victoria and Tasmania (Figure 3. 6). So far, the results suggest that there were multiple introductions in the Australian lineages from the Western Cape population, South Africa, however, Figure 3. 6 indicates that the haplotype (haplotype 5) invading Australia is found in the entire native region distribution.

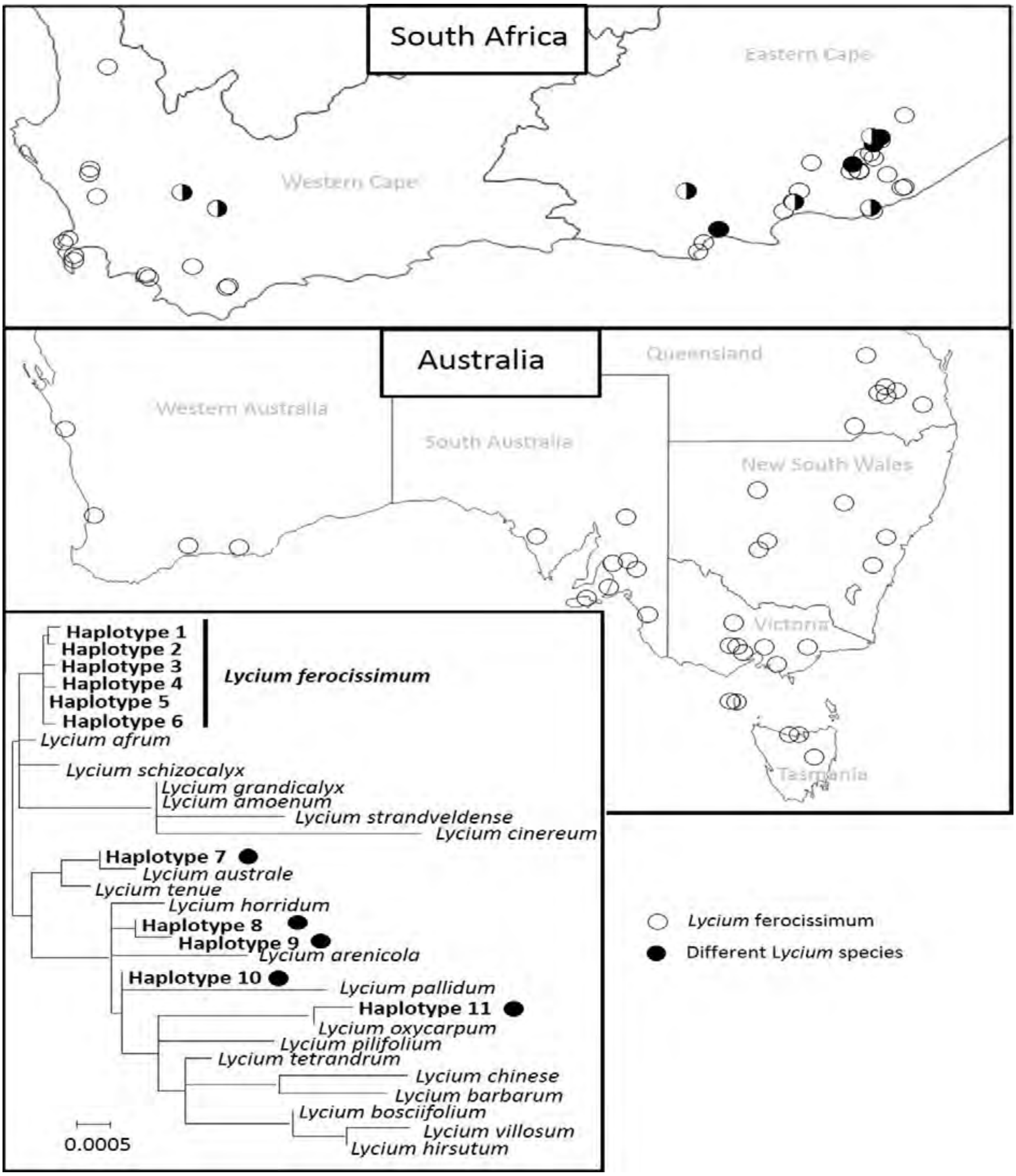

Figure 3. 5. The distribution of *Lycium ferocissimum* Miers (Solanaceae) (open circles), unknown *Lycium* species (black circles) in South Africa and Australia. Insert: Maximum likelihood phylogeny of *Lycium* based on four genes (GBSSI, ITS1, trnT and trhH-psbA).

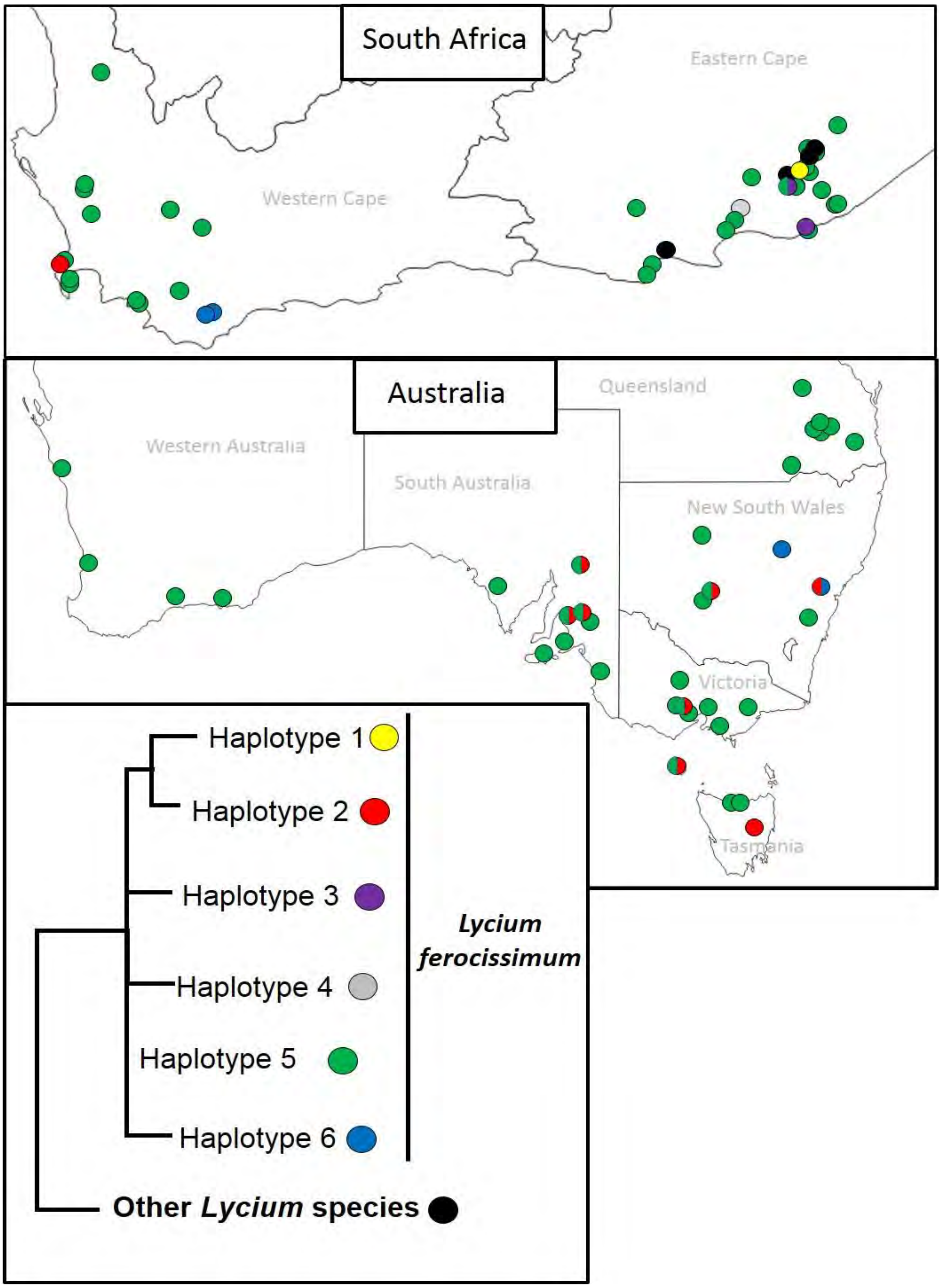

Figure 3. 6. The distribution of *Lycium ferocissimum* Miers (Solanaceae) haplotypes across South Africa and Australia. Insert: Maximum likelihood phylogeny of *Lycium* based on four genes (GBSSI, ITS1, trnT and trnhH-psbA).

3.4 Discussion

3.4.1 Genetic variation across Lycium *samples collected in South Africa*

Plants sampled at a number of sites in both the Eastern and Western Cape were confirmed as *L. ferocissimum*, using haplotype data from the nuclear and chloroplast regions. Additionally, the genetic analysis confirmed the taxonomic identity of most of the samples collected, however, several specimens, particularly from the Eastern Cape Province, were from *Lycium* species not contained in GenBank. Several of these plant species may represent an unknown *Lycium* species, as heterozygous bases were identified in some of the GBSSI sequences, though further investigation is required. In addition, at a few sites in South Africa (EC2, EC3-6 and WC2) both *L. ferocissimum* and an unidentified *Lycium* species were found within the same population (Table S1. 1). These results emphasise the importance of having a correct identification for the target plant (in this case *L. ferocissimum*) in the field when conducting biological control surveys (Chapter 2; Chapter 5). Having the right identification of the target weed both in native and invaded range increases the chance of finding the most effective biological control agent and reduces the possibility of collecting biological control agents from the wrong plant species (Chapter 4; Chapter 5).

3.4.2 Genetic variation across Lycium *samples collected in South Africa and Australia*

All the specimens collected in the invaded range in Australia were *L. ferocissimum*. There was also no evidence of hybridisation of *L. ferocissimum* and Australian indigenous species (*L. australe*), or with commercially planted gojiberrysfrom. However, in the native range we found support for Venter's (2000) study which suggested that *L. ferocissimum* is able to hybridise with close relatives in South Africa (Chapter 2).

In the native range (South Africa) we found high haplotype diversity of *L. ferocissimum* across all sites, with the Eastern Cape Province material being more diverse than that of the Western Cape Province insect surveys also showed that the Eastern Cape Province had more insect diversity collected from *L. ferocissimum* than was the case in the Western Cape Province (Chapter 4). Australia had far less genetic diversity of *L. ferocissimum* across all sampled sites, a phenomenon commonly observed in invasive plant species (Ward *et al.*, 2008). Invasive species often have low genetic diversity (Williams *et al.*, 2005; Cassey *et al.*, 2018). For example, Paterson *et al.* (2009) showed that the genetic diversity of *Pereskia aculeata* Miller (Cactaceae) invading South Africa (invaded region) was low compared to the native region, and was probably the result of a single introduction. However, if multiple introductions occur, genetic diversity may be increased within invasive populations (Minne *et al.*, 1994; Williams *et al.*, 2005). For example, Sutton *et al.* (2017) showed that the genetic diversity of invasive populations of the African tulip tree, *Spathodea campanulata* Beuv. (Bignoniaceae) was high in the invaded region, probably because of multiple introductions of the species from different native range populations. Weeds with high genetic diversity might be more difficult to control through biological control than those that have low genetic diversity providing support that *L. ferocissimum* should make an excellent candidate for biological control.

Two of the haplotypes (haplotypes 2 and 6) were found only in the Western Cape Province and Australia, suggesting a high probability that some of the plants were introduced in Australia from the Western Cape Province. However haplotype 5 a common haplotype throught South Africa and found in the invaded regionas well. The finding also suggested that, like other invasive weeds [for example, *L. camara* (Ray & Quader, 2014), *Phragmites australis* (Paul *et al.*, 2010) and *S. terebinthifolius* (Mukherjee *et al.*, 2012)], *L. ferocissimum* in Australia shows signs that it might have been introduced multiple times by the presence of more than one haplotype in the populations sampled. However, more work needs to be done to access the genetic structure of the populations both in South Africa and Australia.

3.4.3 Implications for biological control

The findings outlined in Chapter 2 showed that it is difficult to distinguish between plants identified as *L. ferocissimum* and those identified as a different *Lycium* species. It is important to correctly identify the plant species to the right taxonomic level if we are to find an effective biological control for the plants (Chapter 3; Chapter 4; see also Goolsby *et al.*, 2006a; Paterson *et al.*, 2009; Madeira *et al.*, 2016; Sutton *et al.*, 2017).

Confirmation that several of the plants putatively identified as *L. ferocissimum* in the field using morphological characters were from different (unidentified) *Lycium* species is cause for concern in the context of surveying for candidate biological control agents. Whereas floral traits, such as shape and sexuality, may assist to distinguish *L. ferocissimum* from other *Lycium* species, they cannot do so with complete accuracy. Furthermore, flowers are not always present, and our analyses highlighted that identification based on other morphological characters is not reliable. To overcome this problem, surveys for potential biological control agents were conducted on tagged and genetically confirmed *L. ferocissimum* plants. Further, it was deemed prudent to keep specimens and colonies of candidate biological control agents collected from different plants separate, until their identity as *L. ferocissimum* was confirmed. Local adaptation is widely believed to lead natural enemies to be more damaging to plants from populations with which they have co-evolved locally (Kniskern & Rausher, 2001; Chapter 5).

Results of a recent investigation into the rust fungus *Puccinia rapipes* Berndt & Ulmann, a candidate biological control agent found on *L. ferocissimum* in South Africa, are consistent with this conjecture. In this study an isolate of this rust fungus collected from the Western Cape Province was significantly more pathogenic on *L. ferocissimum* plants from Australia, than an isolate from the Eastern Cape Province (Ireland *et al.*, 2019a). Future studies should employ next-generation-sequencing approaches, such as genotyping-by-sequencing (Burrell *et al.*, 2015), to reconstruct the invasion history of *L. ferocissimum* in Australia more accurately to test this proposition further. Furthermore, the genetic structure of *L. ferocissimum* needs to be investigated to pinpoint the original introduction of the shrub. Other methods that could help in determining if hybridisation occurs within the species need to be explored, since we know from the literature that the shrub has potential to hybridise in the native region.

Chapter 4: Insect surveys in South Africa for the potential biological control of *Lycium ferocissimum*

4.1 Introduction

Biological control of plants within the Solanaceae has been succesfull, with more than seven agents established on four *Solanum* weed species in South Africa, New Zealand and USA (Olckers, 1999; Winston *et al.*, 2014). However, biological control programmes on Solanaceae are often complicated because many species are important crops such as *Solanum melongena* L. (eggplant/aubergine), *Solanum tuberosum* L. (potato), *Solanum lycopersicum* L. (tomato), *Capsicum* spp. (pepper) and *Lycium barbarum* L. (goji berries). For example, during host specificity trials conducted in quarantine for *Solanum elaeagnifolium* Cavanilles (Solanaceae), potential agents fed on non-target congeneric species - in particular, cultivated *S. melongena* and several indigenous South African *Solanum* species (Olckers, 1996, 1999). Usually, this would have terminated the programme, however, further extensive quarantine and native range host specificity trials clarified the ecological host range of the agents and they were deemed safe for release (Olckers, 1996; Olckers *et al.*, 2002). This programme was particularly time consuming and required multiple repeated native range surveys (Olckers *et al.*, 2002). The programmes would have significantly benefited from structured, long-term, native range study conducted at the initiation of the programme.

Many biological control programmes have been based on limited and *ad-hoc* surveys that looked only for the most damaging and abundant insect species, resulting in varied outcomes (Goolsby *et al.*, 2006b). It is suggested that biological control programmes can be significantly assisted by a comprehensive understanding of the natural enemies associated with the target plant in the region of origin (Syrett *et al.*, 2000; Goolsby *et al.*, 2006b).

Having a greater suite of potential agents to choose from allows researchers to make informed decisions about the most appropriate agent for the target weed, ultimately reducing the risk of introducing unsuitable, ineffective agents (Harris, 1973; Goolsby *et al.*, 2003, 2006b; Raghu *et al.*, 2006). Furthermore, there is an increasing trend in weed biological control science to prioritise species before their introduction into quarantine facilities to save expense, space and time (Briese, 2005; Raghu *et al.*, 2006; Morin *et al.*, 2009).

In a feasibility study done on the insects associated with *L. ferocissimum* in Australia, only 12 phytophagous arthropods were found, unfortunately most were generalist species, many known as agricultural pest species and posing a threat to agriculture (Adair, 2013). Unfortunately, little is known about *L. ferocissimum* in its native range including the shrub's associated insects. Previous studies have found that among the *Lycium* species in southern Africa there were approximately fifteen phytophagous arthropods associated with the species (see Adair, 2013).

There is limited or no information about potential biological control agents as most species during previous studies were generalist. Chapter 2 highlighted the first step of conducting a biological control programme, which was to ensure that the correct plant species was identified in both the native and invaded range. During *L. ferocissimum* surveys conducted across the plant's known native range in South Africa, chapter 2 indicated that plant populations were found in both the Eastern and Western Cape provinces of South Africa. The two populations are separated by a 200 km geographic barrier, a combination of the Cape Fold Mountains and the Knysna Forest (Venter, 2000). Once the populations had been correctly identified, insect surveys could be conducted. Chapter 3 investigated and confirmed the origin of the shrub, matching the haplotypes in South Africa with the invading haplotypes in Australia and confirming that insects collected were indeed from the correct species. Genetic matching of species in the native region with those in the invaded region is important in establishing a successful biological control programme (see also Goolsby *et al.*, 2006a; Paterson *et al.*, 2009; Gaskin *et al.*, 2011; Paterson & Zachariades, 2013).

The current study aimed to identify herbivorous insects and phytopathogens associated with the plants in the Eastern and Western Cape provinces of South Africa. In addition to identifying the insects, comparisons were made between richness, diversity, and community composition of the populations. The study also aimed to identify the insects that warrant further investigation as potential biological control agents. The prioritised agents' biology and host specificity were studied further (Chapter 5).

4.2 Methods

4.2.1 Insect surveys

In Chapter 2 it was determined that *L. ferocissimum* is divided into two separate populations in South Africa, namely the Eastern and Western Cape Province. Additionally, a number of survey sites were identified and established within the two *L. ferocissimum* populations. At each of these sites, surveys for phytophagous insects were carried out intermitantly over a two-year period. To account for seasonal variations, collections were done at least twice during both winter and summer months. Searches were timed to ensure uniformity across all sites; three researchers searching for five minutes each. Leaves were scrutinised for both ecto- and endophages, whilst stems were dissected to find borers. Leaves with leaf-mining larvae were collected and stored in well aerated plastic containers until they developed into adults. Fruits and flowers with any signs of mining were kept in dark well aerated chambers to allow any developing larvae to complete their development. After active searching, a beating method was used to sample cryptic species or those that were difficult to catch. A single person held a beating tray (50cm x 50cm), whilst another person using a stick, beat the branches above the tray to knock off insects. Beating was carried out for five minutes at each site. All insects were collected and killed in the ethyl acetate killing bottles. Insects were then preserved in 70 % alcohol or pinned. Using identification guides (e.g. Scholtz & Holm, 1985; Picker *et al.*, 2004), insects were identified to the lowest possible taxa and then sent to the National Collections of Insects - Agricultural Research Council (ARC-PPRI) Biosystematics for full species identifications, confirmations and accessioning in the National Insect Collection. Remaining voucher specimens are housed at the Centre for Biological Control (CBC), Rhodes University and referred to by Rhodes University (RH) accession numbers e.g. RH1117.

To establish sampling representativeness and adequacy, a sample-based rarefaction (accumulation) curve was compiled from the abundances of insect species collected during each sampling occasion, using the software Estimates 9 (Colwell, 2005). The rarefaction curve was created using the analytically calculated S_{est} (number of species expected; *Mao Tau*). The non-parametric Michaelis-Menten (*MMMean*) estimator and the incidence-based coverage estimator (*ICE*) were used to evaluate sample size adequacy (Chazdon *et al.*, 1998; Toti *et al.*, 2006). When the observed rarefaction curve (S_{est}) and the estimators *(MMMean* and *ICE*) converge closely at the highest observed richness, the richness estimates can be considered representative (Longino *et al.*, 2002).The analyses did not include insects that were encountered only once as they were considered "passer-by" and did not have any effect on the plant.

To investigate the differences in species richness between the Eastern and Western Cape provinces, rarefaction curves of the two provinces were plotted with their 95% confidence intervals. Any overlap in the confidence intervals was taken to reflect no significant differences at $p < 0.05$ (Colwell, 2005). To assess the insect species diversity between the two regions, the Shannon diversity index (H`) was calculated using Estimates 9

(Colwell, 2005). The Shannon diversity index combines species richness with equitability (evenness) with which individuals are distributed among sites and is less sensitive to rare species (Shannon & Weaver, 1949). Student's t-tests (significance level $p < 0.05$) were used to investigate the significance of differences in Shannon diversity indices between Eastern and Western Cape provinces, South Africa.

Analysis of similarity (ANOSIM), conducted in PRIMER software package (Clarke & Warwick, 2001), was used to infer differences in insect assemblages between the two regions. A Bray-Curtis similarity measure was used to calculate the similarity matrix, and non-metric multidimensional scaling (nMDS) was used to visualise differences in assemblages between the two regions (Clarke & Warwick, 2001). To ensure that the representation of the data in two dimensions was appropriate (i.e. not misleading), the stress score was interpreted following recommendations by Clarke (1993). Values below 0.20 are considered acceptable stress values, but the lower the values the more acceptable the outcome. Solutions with stress values above 0.30 require cautious interpretation (Clarke, 1993). Additionally, a scree plot, with stress versus number of dimensions, was plotted to ensure that the reduction of the dimensions (to two) in the insect community data was appropriate.

Indicator Species Analysis was used to identify insect species which drove the differences in the clustering into the two assemblages (Eastern and Western Cape provinces); i.e. those characterizing the assemblages of the two provinces. This method combines relative frequency and relative abundance of species between groups (provinces; Dufrêne & Legendre, 1997). The resulting indicator values were tested for significance ($p < 0.05$) using a randomisation test (1000 iterations). Indicator Species Analysis was conducted using the "labdsv" package (Roberts, 2010) in the statistical software R (v 3.0.1) (R Development Core Team 3.0.1., 2013).

4.2.2 Prioritisation of identified insects

To identify insects warranting further investigation as potential biological control agents, they were prioritised on the following attributes: prevalence, occurrence, distribution, host specificity and available literature. Prevalence of insect taxa was estimated as the percentage of total abundance of each species (at each site), per province. Prevalence was calculated as:

$$\frac{Number\ of\ conspecific\ insects\ at\ the\ site}{Total\ number\ of\ insects\ at\ the\ site} \times 100$$

The distribution of each taxon was described as the number of sites where an insect species was collected. Historical host range information of each taxon was obtained from the literature, and museum reports. This information was used to estimate the potential level of specificity of each taxon i.e. monophagous, polyphagous or oligophagous. Furthermore, comparisons of prevalence, distribution and host specificity were made between the two populations. Mann-Whitney non-parametric tests were used to investigate the differences between the two *L. ferocissimum* populations. Insects initially prioritised as potential biological control agents were then cultured in the laboratory to undergo further host specificity testing and to determine their basic biology, which helps in maintaining laboratory colonies (Chapter 5).

4.3 Results

4.3.1 Insects collected

1315 insects belonging to 96 (morpho) species were collected from 53 sites across the Eastern and Western Cape provinces of South Africa. The analytically calculated number of species (S_{est}) did not converge with the two richness estimators (*ICE* and *MMMean*), suggesting that insects were under-sampled (Figure 4. 1). However, the cumulative species sampling curve approached the asymptote and sampling can be considered adequate although not exhaustive (Figure 4. 1). The non-overlapping confidence bands of the rarefaction curves suggested there was no significant difference in projected species richness between the two provinces (Figure 4. 2). The diversity on the basis of Shannon index was significantly higher in the Western Cape Province (Figure 4. 3; Mann-Whitney, $U = 451.50$, $z = -2.59$, $P = 0.01$).

There were differences in insect species composition between the Eastern and Western Cape provinces; however, there was some overlap (Figure 4. 4). Out of the 96 insect species collected across all sites, 18 were collected from both provinces. The ANOSIM analysis revealed distinct assemblages between the two provinces, but with a very low level of separation (Global $R = 0.271$, $P < 0.001$). Eight indicator species were identified across the two provinces. *Cassida distinguenda* Spaeth (Chysomelidae), *Cleta eckloni* Mulsant (Coccinellidae) and *Epilachna* sp.1 were associated with the Eastern Cape Province sites (Indicator values = 0.37, $P < 0.01$; 0.34, $P < 0.01$ and 0.27, $P= 0.03$, respectively), whilst *Apalochrus* sp.1, *Ceratitis* spp., *Epilachna* sp.2, Homoptera spp. and *Panchycnema* sp.1 were significantly associated with the Western Cape Province sites (Indicator values = 0.13, $P = 0.05$; 0.34, $P < 0.01$; 0.14, $P = 0.05$; 0.18, $P = 0.04$ and 0.23, $P < 0.01$, respectively).

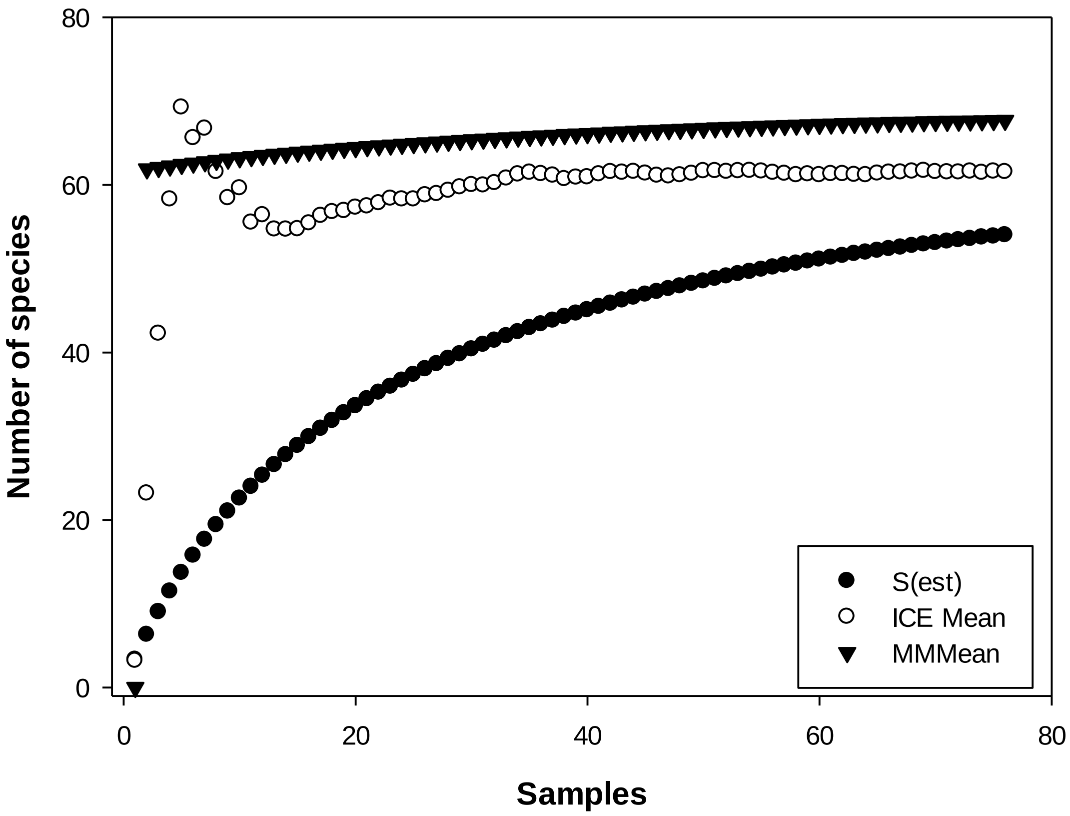

Figure 4. 1. Sample-based rarefaction curves indicating observed number of species (S_{est}; *Mao Tau*), incidence-based coverage estimator (*ICE*) and Michaelis-Menten mean (*MMMean*) richness estimators of all insects collected of *Lycium ferocissimum* Miers (Solanaceae) across surveyed sites (Eastern and Western Cape provinces combined; 76 samples from 53 sites).

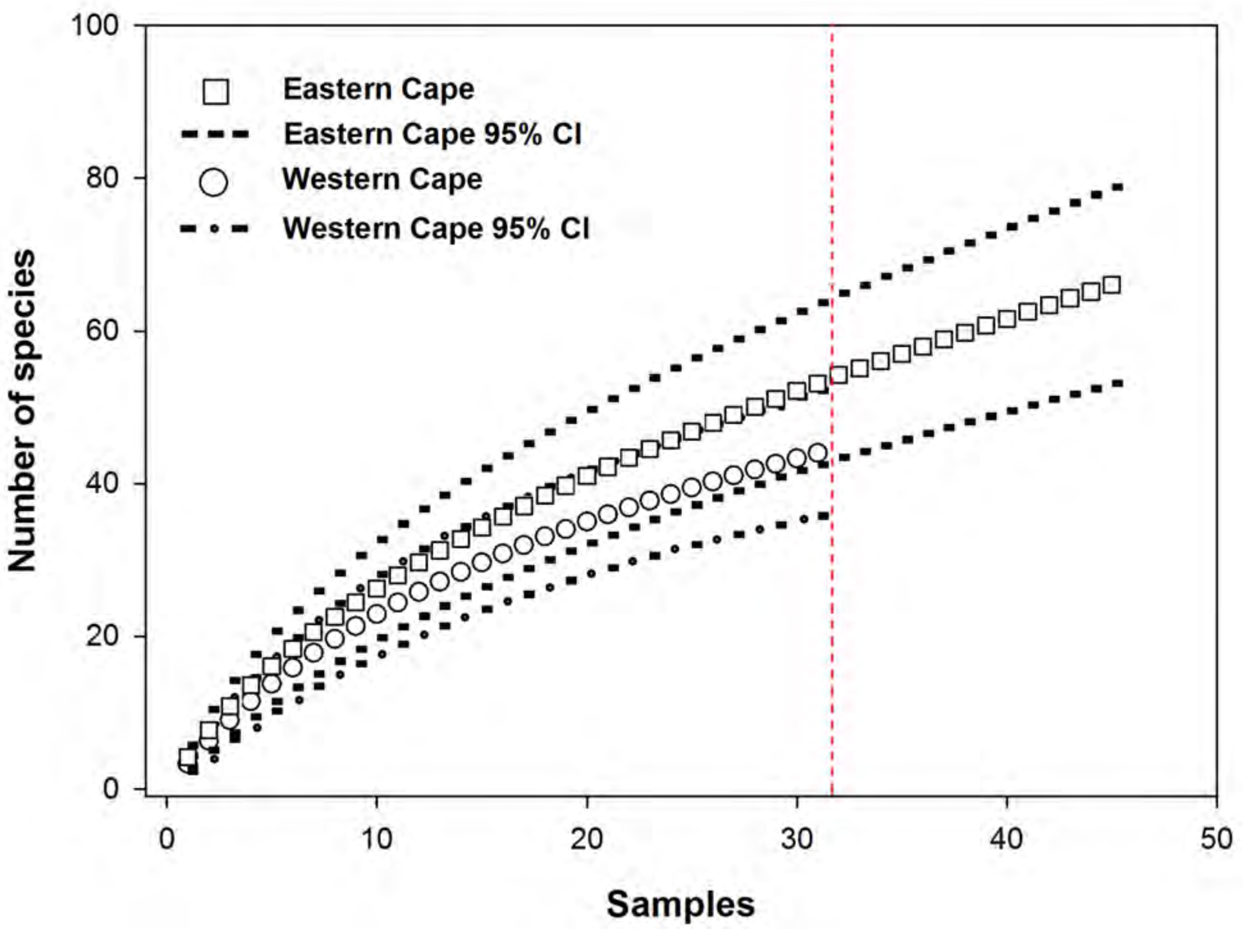

Figure 4. 2. Sample-based rarefaction curves indicating observed number of insect species (S_{est}). Species richness should be compared when the number of samples is equal in all provinces (i.e. approximately 33 samples (Red line). The open shapes represent the 95 % confidence interval. Where confidence intervals overlap, the differences in species richness are not significant at $P > 0.05$.

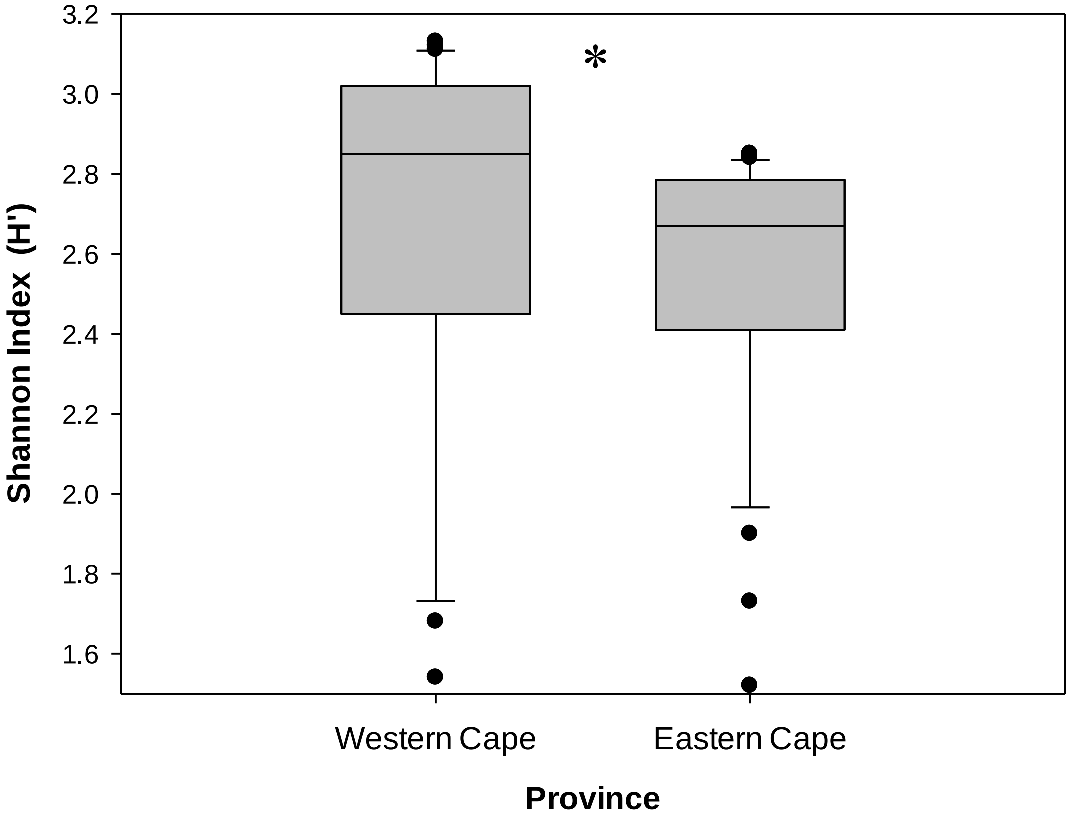

Figure 4. 3. Average values of Shannon diversity index (H') representing the diversity of insects collected from *Lycium ferocissimum* Miers (Solanaceae) in the Western and Eastern Cape provinces of South Africa. Asterisk (*) represents a significant difference ($P < 0.05$) in diversity between the two provinces.

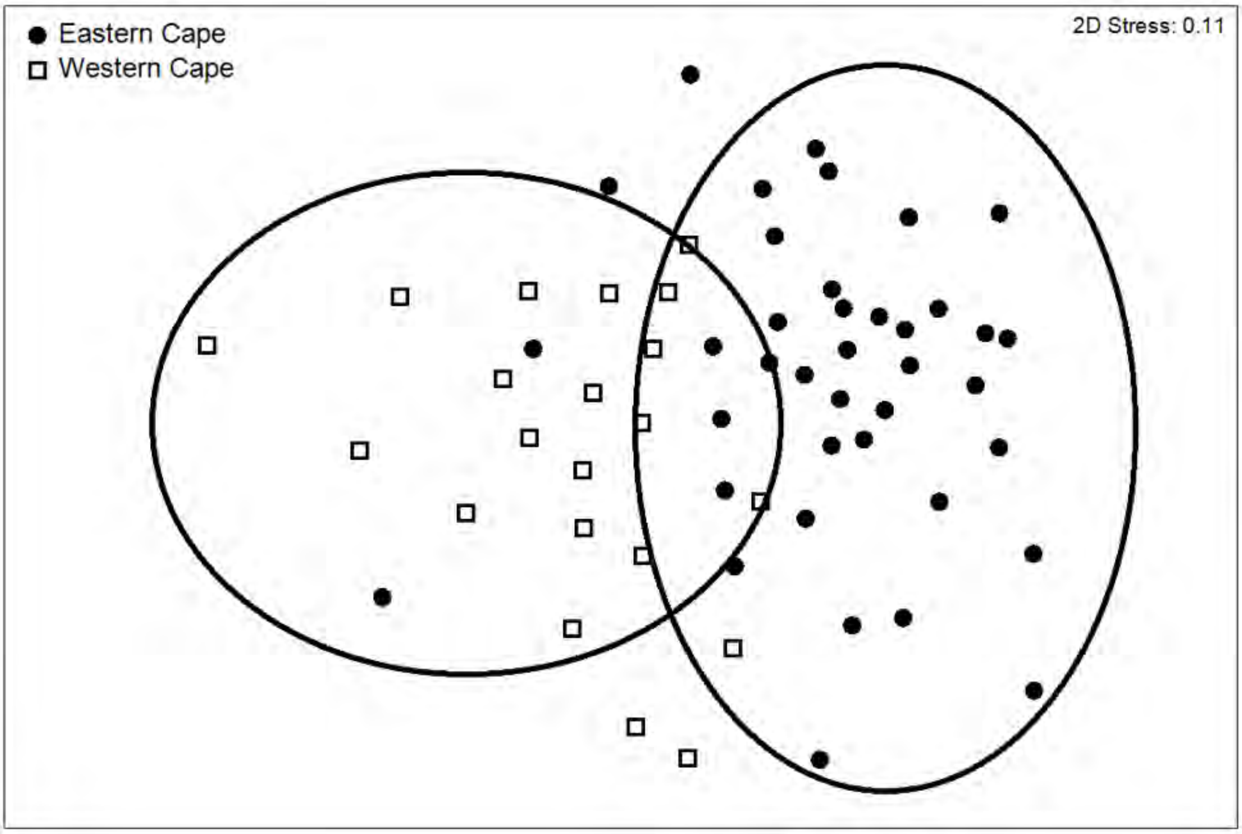

Figure 4. 4. Non-metric multidimensional scaling representation of herbivorous insect species collected from *Lycium ferocissimum* Miers (Solanaceae) the two populations in South Africa, the Eastern (black circles) and Western Cape provinces (open squares). Data were ordinated using the Bray-Curtis similarity matrix.

A greater number of insect species were collected from the Eastern Cape Province (995 insects, 55 species), than from the Western Cape Province (320 insects, 41 species). There was a generally lower prevalence and insects had more limited distributions in the Western Cape Province than in the Eastern Cape Province (Figure 4. 5), but only the differences in distribution were statistically significant (Mann-Whitney, $U = 287.50$, $z = 3.00$, $P < 0.01$). Insects in the Eastern Cape Province had a substantially wider distribution, with at least 12 species occurring in more than 16 % of the sites, than in the Western Cape Province. There was no difference in overall prevalence between the two provinces, probably because there was greater variation in prevalence values in the Eastern Cape Province (Mann-Whitney, $U = 454.50$, $z = 0.77$, $P = 0.44$; Figure 4. 5).

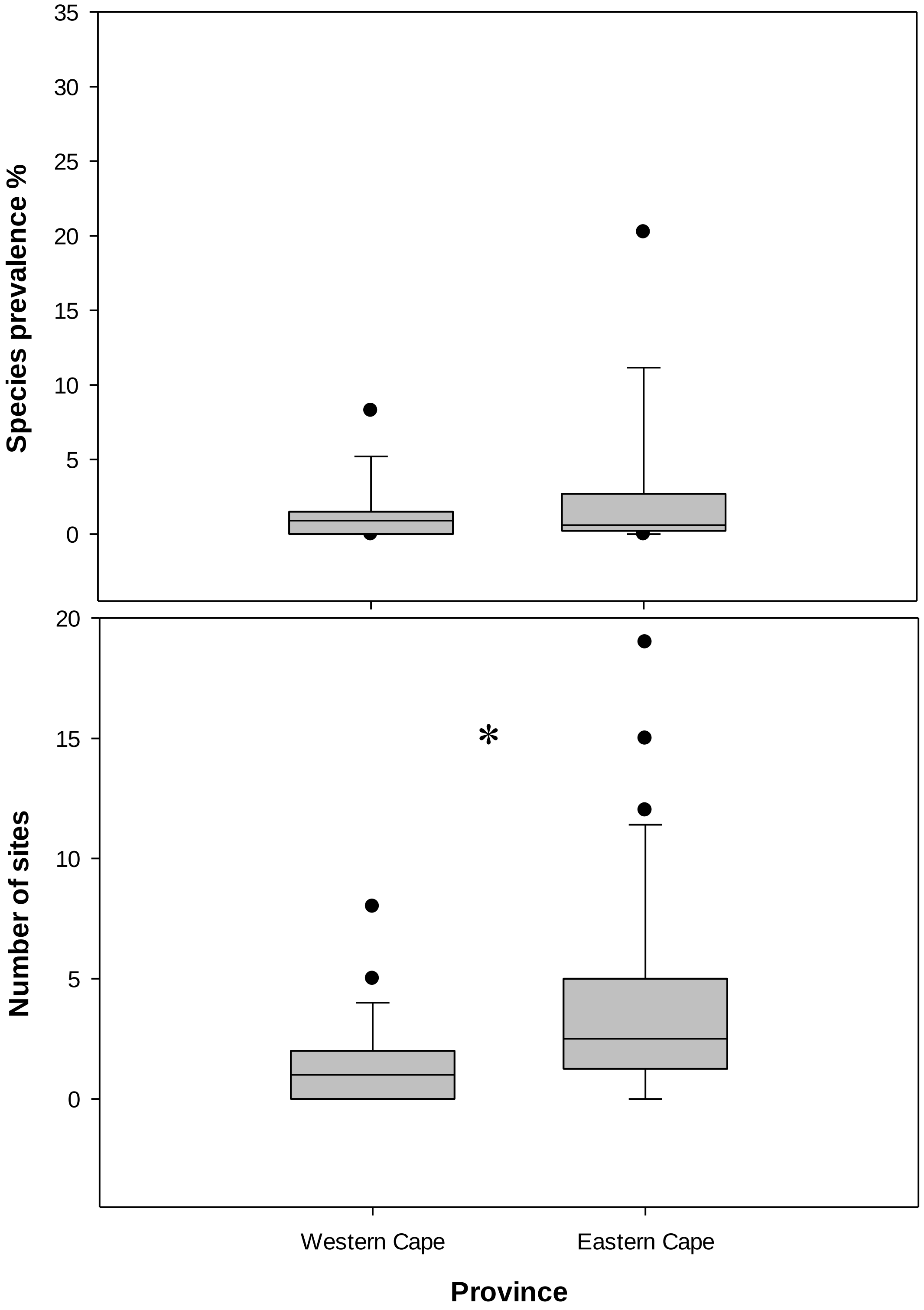

Figure 4. 5. Species prevalence and numbers of sites where insect species occur (distribution), on *Lycium ferocissimum* Miers (Solanaceae) in the Western and Eastern Cape provinces of South Africa. Asterisk (*) represents a significant difference in number of sites between the two provinces.

When insect species prevalence was categorised based on feeding guilds, foliage-feeding insects were more prevalent in the Eastern Cape Province, but fruit- and flower-feeding insects were more prevalent in the Western Cape Province (Figure 4. 6). Regarding the number of sites at which insects were recorded (distribution), foliage-feeding insects were found at more sites in the Eastern Cape Province than in the Western Cape Province (2.40; Mann-Whitney, *U* = 287.80, z = 3.10, *P* < 0.01; Figure 4. 7). Fruit feeders were found at more sites in the Western Cape Province than in the Eastern Cape Province, whilst flower feeders were found at more sites in Eastern Cape Province than in the Western Cape Province (Figure 4. 7).

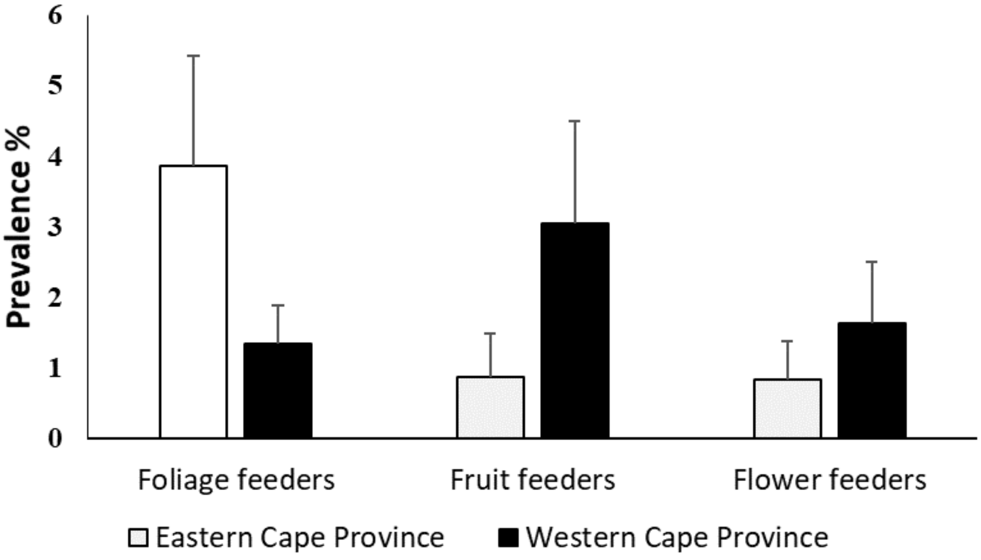

Figure 4. 6. Average (with standard deviation) species prevalence, expressed as the percentage of total abundance of each species found on *Lycium ferocissimum* Miers (Solanaceae) in the Eastern and Western Cape provinces. Prevalence was contrasted for each insect feeding guild.

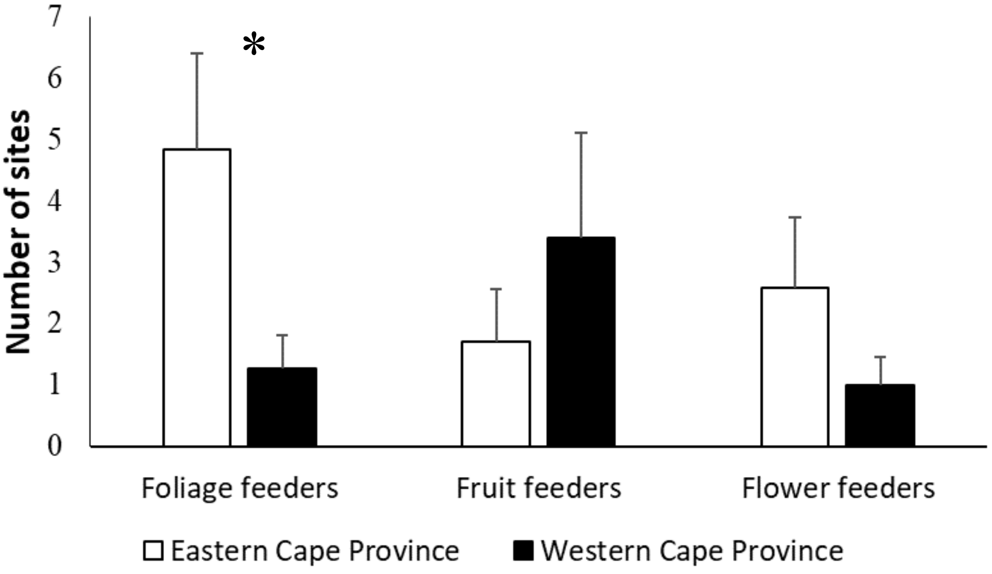

Figure 4. 7. The average distribution expressed as the number of sites at which each insect species was observed on *Lycium ferocissimum* Miers (Solanaceae) in each of the two provinces. Asterisk (*) represents a significant difference in number of sites between the two provinces.

4.3.2 Prioritisation

Insect species with widest distribution (at least 10 sites across both provinces) included *Cassida distinguenda* Spaeth (Chrysomelidae), *Cleta eckloni* Mulsant (Coccinellidae), *Neoplatygaster serietuberculata* Gyllenhal (Curculionidae), *Pseudambonea capeneri* Schuh (Miridae), *Chnootriba* spp. and *Malachiinae* spp. Three of these species were also amongst the most prevalent species by abundance: *C. eckloni*, *P. capeneri* and *Chnootriba* sp. The following insect species were the most geographically widespread across the native range: *C. distinguenda*, *C. eckloni*, *N. serietuberculata*, *P. capeneri*, *Chnootrib*a spp. and *Malachiinae* spp. The mite species (*Aculus* sp. or *Aculops* sp.), *C. distinguenda*, *C. eckloni and N. serietuberculata* were also amongst the most prevalent herbivores on *L. ferocissimum* (Table 4. 1).

Table 4. 1. Insects associated with *Lycium ferocissimum* Miers (Solanaceae) in the Eastern and Western Cape provinces of South Africa. An 'X' indicates unidentified taxa. An asterisk (*) represented insects that were further prioritised for host specificity testing (Chapter 5). (see also Table S1.2 and Table S1.3).

Family	Species
COCCINELLIDAE	*Cleta eckloni* Mulsant *
	Cleta sp. 1 1
	Cleta sp. 1 2
	Epilanchna sp.1
	Exochomus flavipes
	Scymnus sp.
	Cheilomenes lunata Fabricius
	Cheilomenes sulphurea Olivier
	cf. *Nephus* sp.
	Sticholotidini sp.
	Chnootriba sp.
	Oenopia cinctella
	Hippodamia variegate Goeze
	Micraspis sp.
MIRIDAE	*Pseudambonea capeneri* Schuh
	Schuhistes lekkersingia Menard
	Miridae nymphs
CHRYSOMELIDAE	*Cassida melanophthalma* Boheman
	Cassida distinguenda Spaeth *
	Cassida reticulipennis Borowiec & Świętojańska
	Macetes sp.
	Cryptocephalus nr *liturellus* Suffrian
	Aphthona sp.
	Near *Phyllotreta* sp.
	Epitrix sp.
	Peploptera dorsata Lacordaire
	Smaragdina nr *terminata* Lacordaire
	Xenoomorphus sp.
	Decaria abdominials Jacoby, 1899
	Chrysomelinae sp.
	Sulcobruchus longipennis
	Adoxini sp.
	Psylliodes calcaratum Bryant
	Monolepta bioculata Fabricius
EURYTOMIDAE	Eurytomidae sp.
TENEBRIONIDAE	*Alleculinae* sp.
SCARABAEIDAE	*Pachycnema* sp.
	Scarabaeidae sp.
CURCULIONIDAE	*Neoplatygaster serietuberculata* Gyllenhal *
	Sciobius sp.
	Tychius sp.
	Lixini sp.
MEMBRACIDAE	*Beaufortiana comuta* Distant
PENTATOMIDAE	*Antestiopsis thunbergi*
	Pentatomidae sp.
	Pentatomidae sp. nymph
MELYRIDAE	*Nepachys ramicomis* Boheman
	Rhadalinae sp.
	Apalochrus sp.
CERAMBYCIDAE	*Hylomela sexpunctata* Fabricius
ANTHRIBIDAE	Anthripidae sp.
ZYGAENIDAE	*Neurosymploca concinna* Dalman, 1823
ANTHICIDAE	*Anthelephila* sp.
COREIDAE	*Acanthocoris spinosus* Spinola
PYRRHOCORIDAE	*Cenaeus pectroralis* Stål

BUPRESTIDAE	*Anthaxia* sp.
THRIPIDAE	*Thrips simplex* Morison
	Frankliniella schultzei Trybom
TEPHRITIDAE	Tephritidae sp.1
	Tephritidae sp.2
	Ceratitis sp.
ENCYRTIDAE	Encyrtinae sp.1
	Encyrtinae sp.2
	Encyrtinae sp.3
	Encyrtinae sp.4
CHALCIDOIDEA	*Brachymeria* sp.
ELATERIDAE	Elateridae sp.
MUSCIDAE	Muscidae sp.
SYRPHIDAE	*Allograpta* sp.
	Syrphidae sp.
CICADIDAE	Cicadidae sp.
COCCOIDEA	Scale insects
APIDAE	*Apis mellifera*
PTEROMALIDAE	Pteromalidae sp.
	Pteromalus puparum
AGAONIDAE	Agaonidae sp.
CHRYSOPIDAE	Chrysopidae sp.
PAMPHAGIDAE	Pamphagidae sp.
ERIOPHYIDAE	*Aceria* sp.
	Aculops sp.
	Aculus sp.
AMPHIPSOCIDAE	Amphipsocidae sp.
AEOLOTHRIPIDAE	Aeolothripidae sp.
X	Diptera sp.
	Fruit flies
	Fruit miners
	Leaf miners
	Hymenoptera sp.

4.4 Discussion

This study revealed a high diversity and abundance of herbivorous insects associated with *L. ferocissimum* in South Africa, with considerable geographic variability. The sampling conducted was broad, repeated and representative of habitats and climatic zones of *L. ferocissimum*. Species accumulation curves showed that sampling was sufficient, although additional sampling might result in the identification of a few new insects. The number of herbivorous insect species collected in this study is considerably greater than the number of insect species collected over three years on the closely related and indigenous *Solanum panduriform* E. Mey (49 species) and *Solanum incanum* L (Solanaceae) (33 species) (Olckers *et al.*, 1995). Completely exhaustive surveys are often not achievable except for species that can be identified easily, such as plants and breeding birds where it is possible to obtain a count of all the species present (Colwell & Coddington, 1994). For other taxa, such as arthropods, it is often not possible to identify all the species, particularly at a large spatial extent (Ugland *et al.*, 2003).

While the simultaneous use of two different insect collection methods, active searching and beating, increased the chances of catching a wider suite of insects, the use of additional methods would have improved catches. For instance, there was a poor representation of leafhoppers (Cicadellidae) in the collections (M. Stiller, pers. comm.). Records show that several genera are common to *Lycium* spp. worldwide (Chapman & Joern, 1990; Menard, 2010; Adair, 2013; Taylor & Kent, 2013), but have yet to be observed on *L. ferocissimum*. Furthermore, several records in the South African National Collection of Insects indicate that a considerable number of leafhoppers are associated with the genus *Lycium* from the Eastern Cape and Limpopo provinces, e.g. *Circulifer* spp., *Nesoclutha* spp. and *Tzitzikamaia* spp. live on *Lycium schizocalyx* (M. Stiller pers. comm.). A D-Vac sampler has previously been used to collect these leafhoppers, hence it is plausible that since a D-Vac was not used in our study, leafhoppers may have been missed (M. Stiller pers. comm.). Sticky traps and emergence traps could have similarly improved sampling methods, but we believe a reasonable representation of insect assemblages was attained by rigorous sampling across time and space. Woody plants typically provide food and habitat for stem- boring or mining insects (R. Staals pers. comm.), but it was odd that no stem miners were identified from all the sampled *L. ferocissimum*. Additional destructive sampling could be attempted to locate stem minors on *L. ferocissimum*.

There were differences in insect prevalence, abundance, distribution and composition between the two sampled provinces. The reason for the difference was not determined but may be related to habitat and/or climatic differences. The Western Cape population is climatically unique in comparison to the rest of South Africa because of its Mediterranean-type climate characterised by warm, dry summers and mild, moist winters (Midgley *et al.*, 2005). The Western Cape populations occur in two of the world's largest biodiversity hotspots, the Cape Floristic Region and the Succulent Karoo (Myers *et al.*, 2000), both of which are recognised for their unique characteristics and habitat types (Mucina & Rutherford, 2006). The province also regularly experiences climate extremes such as droughts and floods (Rouault & Richard, 2003; Araujo *et al.*, 2016). Alternatively, the Eastern Cape population generally receives greater volumes of rainfall over a more extended period, mostly during summer months (Conradie, 2012). The Eastern Cape population occurs in much more complex biomes and vegetation types which are different from those of the Western Cape population and are dominated by Thicket and Nama Karoo vegetation types (Mucina & Rutherford, 2006). Conspecific plants growing in different regions usually experience differing biotic and abiotic pressures, hence they can be expected to have phenotypic and genotypic variations, as is the case with *L. ferocissimum* (Chapters 2 and 3). In turn, the herbivorous insect assemblages associated with the phenotypic and genotypic variants may then differ to suite the plants they are living on (e.g. Denno & Roderick, 1991; Langellotto & Denno, 2004). It is plausible that the geographic separation between the Eastern and Western Cape provinces of is the cause of the different insect assemblages seen in South Africa. This highlights the need to survey the entire distribution of plant species when trying to identify the insects associated with the species. This is particularly applicable to biological control as it ensures most insects are identified, thus allowing researchers to identify species that will be environmentally suitable to the target species being considered.

The second objective of the study was to identify insects that warrant further investigation as potential biological control agents based on the occurrences, abundance, associated damage and available literature. A considerable number of herbivorous insects that were found on *L. ferocissimum* according to the literature are probably monophagous or oligophagous and therefore could be considered as potential biological control agents. However, alternate host plants of insect species are often not known unless they are of economic importance; hence our results only give an indication of specificity based on the literature and any prioritized species would still require host specificity testing to confirm their actual host range. In support of the available literature, observations in the field showed that *C. distinguenda, C. eckloni* and *N. serietuberculata*, were never observed on surrounding vegetation at all sites across South Africa, hence it is plausible that these insects had a strong host preference for *L. ferocissimum* and therefore should be prioritised.

Lycium ferocissimum has already invaded a substantial proportion of the Australian continent. Selecting biological control agents that could establish across the whole invaded range would ensure fewer agents are required to manage the species in Australia. For example, the invasive species *Solanum mauritianum* Scop. (Solanaceae) has a broad distribution in South Africa (Olckers, 1999; Olckers *et al.*, 2002). In 2008 a biological control agent *Anthonomus santacruzi* Hustache (Coleoptera: Curculionidae) was released in an attempt to control the plant (Klein, 2002; Singh & Olckers, 2017). Unfortunately, despite significant release efforts, the agent only established along a small climatically limited area (Cowie *et al.*, 2016; Singh & Olckers, 2017). Retrospective climate matching has showed that regions where the insect had established in South Africa matched to areas where the insects were collected from its native range, while areas where the agent had not established had a less than 50% match to the native range (Cowie *et al.*, 2016). The agent has not had a significant impact on the *S. mauritianum* population in South Africa because it is climatically limited (Cowie *et al.*, 2016). Collecting extensive distribution data of potential biological control agents from the native range can assist researchers in predicting the climates where the insects could survive in the invaded range. Six insect species (*C. distinguenda, C. eckloni,* *N. serietuberculata, P. capeneri, Chnootriba* spp. and *Malachiinae* spp.) were the most geographically widespread across the native range of *L. ferocissimum* and are therefore most likely to have the widest potential distribution in the invaded range of the plant. This however would require further investigation into the insect's physiology.

Foundational biodiversity lists on insects associated with plant species contribute to the understanding of the complex relationships existing between plants and their associated insects. These types of studies are more common in European and American literature and are infrequent in developing countries (Goolsby *et al.*, 2006b). This study provides valuable information towards understanding insect diversity and structure associated with the *Lycium* genus in South Africa, and is likely to prove beneficial to many disciplines beyond biological control. With regard to biological control, this study emphasises the importance of conducting quantitative studies of the insects associated with the target plant. Identifying the available agents across the native range allows researchers to prioritise insects that are suitable to manage the species within the invaded range. Collecting meta-data other than just species identifications allows researchers to further prioritise species before they are introduced into quarantine facilities. Additionally, the data will provide a reference point to other countries considering biological control against not just *L. ferocissimum* but other species within the Solanaceae family.

Chapter 5: Native range studies on the biology, plant damage and preliminary host specificity of prioritised natural enemies of *Lycium ferocissimum* Miers (Solanaceae)

5.1 Introduction

The biological control of weeds using insects involves several steps (Chapter 1; McFadyen, 1998; Olckers, 1999; van Klinken & Edwards, 2002; van Klinken & Raghu, 2006), including an assessment of the insect's host range and impact on the target plant. These steps nearly always occur within quarantine facilities, in the country where the insect is going to be released. The entire life cycle of the candidate biological control agent is studied on the target weed and on plant species (especially plants closely related to the target species) the agent is capable of successfully finding and exploiting (van Klinken & Heard, 2000; Paynter *et al.*, 2015). Host specificity testing is used to describe properties of the insect, which are then used in combination with ecological information to predict where, when, and to what extent non-target attack would occur.

Host specificity testing has been used for decades in biological control studies of weeds and is designed to reveal which agent species collected will be useful for controlling the target weed and how safe the agent would be in its adopted environment if released. Host specificity tests determine if the agent would affect and complete life cycle on non-target indigenous species. Laboratory choice and no-choice tests are often used to affirm the host specificity of species prioritised for biological control, however, some authors suggest that these tests provide inconsistent results that might lead to a candidate agent being dismissed (Wapshere, 1983; Blossey *et al.*, 2018). Briese (1999) argued that field host specificity testing is considered by many as a more realistic method for assessing host range of the agents as they are not restricted in their movement by some form of caging in the laboratory.

Quarantine facilities are also expensive to maintain and manage because environmental aspects such as light, humidity and temperature are nearly always regulated. Therefore, the larger the facility the more expensive and difficult to manage the conditions within them (Buckingham, 1992). In addition to the facility maintenance and running costs, the personnel used to manage facilities need to be highly trained to ensure the safety of the facilities. Quarantine facilities are filter systems used to prevent unwanted organisms accessing the environment and therefore require stringent safety measures (Buckingham, 1992). To ensure the safety levels are maintained and enforced most practicing biological control nations require their quarantine facilities to meet minimum safety requirements which are verified by a larger governing body. Due to the cost of running and maintaining quarantine facilities and the minimum safety requirements enforced on them, there are usually a limited number of facilities within the countries practicing weed biological control. For example, South Africa has only five functioning quarantine facilities (Martin pers. comm.), Australia has over 100 approved quarantine facilities, however, only 4 are doing weed biocontrol work: CSIRO (Brisbane and Canberra), QDAF (Brisbane), Victoria DPI (Melbourne) and NSW DPI (Orange) (Raghu pers. comm.). As such, priority needs to be given to those species that are effective to avoid congestion in these quarantine facilities.

Impact trials conducted under quarantine conditions are difficult to conduct but may provide valuable information on how the insect may damage the plant, should it be released. Raghu *et al.* (2006) however suggest that more time and resources should be invested in assessing the effectiveness of biological control agents in the native range of the invasive weed.

The aim of this chapter was to determine the behaviour, biology and preliminary host ranges of prioritized natural enemies (see chapter 4) in their native range. Information gathered during refinement of the candidates would inform the Australian government/authorities on which insect candidates would be best suitable for importation into quarantine.

5.2 Methods

Biology, and host specificity testing was conducted on the potential biological control agents prioritized in chapter 4, *Cleta eckloni* Mulsant (Coccinellidae: Epilachnini), *Cassida distinguenda* Spaeth (Chrysomelidae: Cassidinae), *Neoplatygaster serietuberculata* Gyllenhal (Curculionidae: Ceutorhynchinae: Hypurini) and *Puccinia rapipes* R. Berndt & E. Uhlmann, sp. nov. (Uredinales). In addition, the impact of *Cleta eckloni* (only recorded from Eastern Cape Province plants) and *Cleta* sp. 1 (only recordedfrom Western Cape Province plants) on plants from the Eastern Cape and Western Cape provinces respectively, was determined. The performance of *C. eckloni* and *Cleta* sp. 1 on the two plant populations was also assessed.

5.2.1 Insect and rust cultures

Cultures of *C. eckloni, C. distinguenda* and *N. serietuberculata* were collected as adults, pupae, larvae and in some instances as eggs from sites across the Eastern Cape Province of South Africa (Chapter 4). To maintain genetic diversity of the cultures, insects were collected from more than one site across the distribution of the shrub. The cultures were kept under laboratory conditions with an average temperature of 22°C and a 12:12 hour day: night light ratio. The plants were changed when necessary. The cultures were used to collect eggs for use in the trials.

In addition, 200 *Cleta* sp. 1 were collected from *L. ferocissimum* plants near the town of Elands Bay (32°18’15.05’’ S; 18°20’33.1’’ E) in the Western Cape Province. Adults were collected using a bush beating technique (Chapter 4) and kept in plastic containers. Adults were brought back to Rhodes University where they were placed into a mesh cage

(60cmx60cmx90cm) onto plants collected from the Western Cape Province. The cultures were kept under laboratory conditions with an average temperature of 22°C and a 12:12 hour day: night light ratio. The plants were changed when necessary. The culture was used to collect eggs for use in the trials.

Puccinia rapipes was collected from two sites in the Western Cape Province (Miller's Point, 34º13'53.18'' S; 18º28'28.35'' E) and one site in the Eastern Cape Province (Makhanda, 33º19'10.56'' S; 26º32'17.16'' E).

5.2.2 Plant cultures

Lycium ferocissimum from both the Eastern and Western Cape provinces were dug up and kept at the Waainek Research Facility (33.314091 S; 26.519683 E), Makhanda, Eastern Cape Province. They were potted in five-litre potting pots that contained 50% potting soil and 50% sand. The soil had been watered to saturation three days before planting. Test plants *Lycium oxycarpum* Dunal., *Lycium barbarum* Mill., *Solanum melongena* L., *Capsicum* sp. and *Solanum lycopersicum* L. were bought from various nurseries across South Africa and kept in the research facility. All potted plants were fertilised with five grams of 3:1:5 slow-release N-P-K Wonder™ fertiliser and MgSO4. Plant cultures were grown under 10% shade cloth before being used. Plants were watered weekly. All were exposed to identical shade, watering and fertilizer regimes.

5.2.3 Insect biology

Two males and two females taken from the founder culture of each insect species were placed in an insect cage with the target plant (60 cm x 60 cm x 90 cm) using a fine paintbrush. The insects were monitored daily for adult oviposition. The purpose of having two pairs of each insect was to increase the chance of reproduction as it was not certain if they could still reproduce and females were still laying viable eggs. Once oviposition occurred, eggs were monitored for hatching and the duration of development of each life-stage was recorded.

5.2.3.1 Cleta eckloni *and* Cleta sp. 1 *development on different* L. ferocissimum *populations*

During the native range surveys two very similar species of *Cleta* were found, one from the Western Cape Province population, *Cleta* sp. 1, and the other from the Eastern Cape Province population, *Cleta eckloni*. Initially, they were thought to be the same species. *Cleta* sp. 1 identity has yet to be confirmed.

Five Newly hatched *Cleta eckloni* larva (collected in the Eastern Cape) were placed onto Eastern Cape and Western Cape provinces *L. ferocissimum* populations. Five plants of each remained insect free, this was replicated five times. In addition, five newly hatched *Cleta* sp. 1 (collected in Western Cape) larva were placed onto Eastern Cape and Western Cape *L. ferocissimum.* The plants were placed into a mesh cage (60cmx60cmx90cm) (Figure 5. 1). Each plant was watered 250 ml every 5 days. The larvae were monitored daily to determine the amount of days until pupation. In addition, larval survival as a percentage survival from eggs adults was also recorded

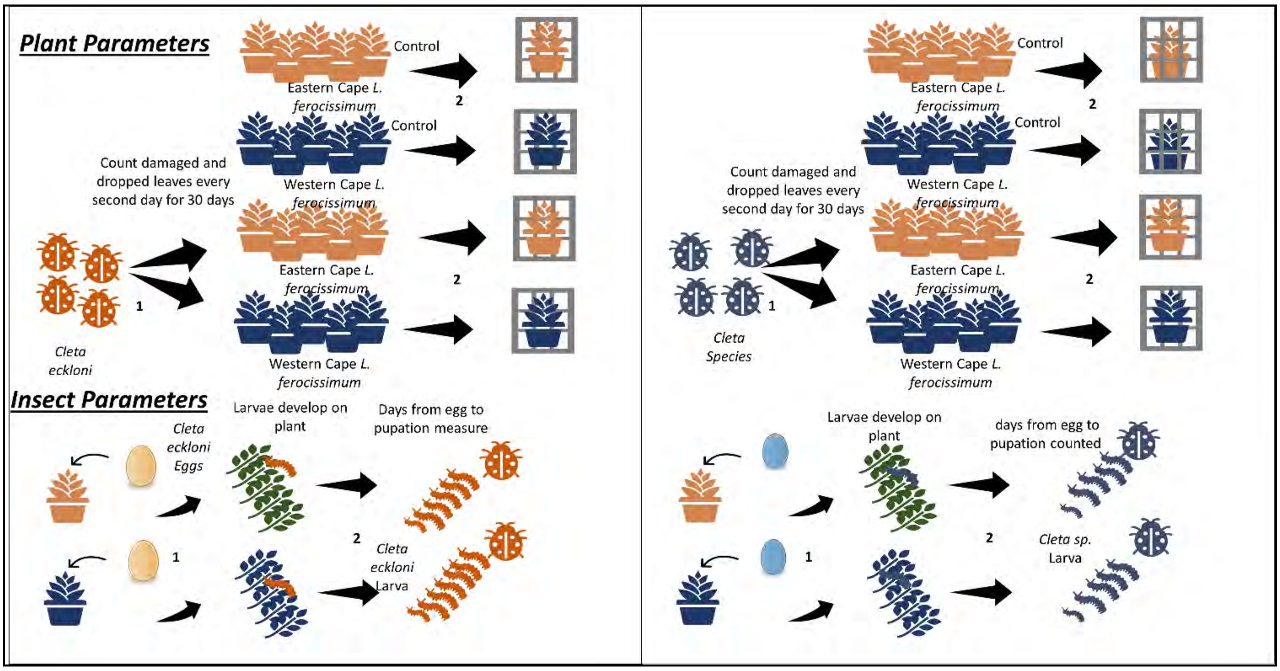

Figure 5. 1. Experimental design; blue plants represent *Lycium ferocissimum* Miers (Solanaceae) from the Western Cape and Orange plants represent plants from the Eastern Cape. Orange insects represent *Cleta eckloni* (Eastern Cape) while Blue insects represent *Cleta* sp. 1 from the Western Cape.

5.2.4 Laboratory host specificity testing

Plants were chosen on the basis of relatedness to *L. ferocissimum* and of economic importance to Australia: namely *Lycium oxycarpum* (Cape honey-thorn), *Lycium barbarum* (gojiberry), *Solanum melongena* (egg plant/aubergine/brinjal), *Capsicum* sp. (pepper) and *Solanum lycopersicum* (tomato). Male-female pairs of both insects were placed on the test plants (twelve and nine replicates on non-targets) and a control plant (fifteen *L. ferocissimum*). The plants were monitored daily for feeding, oviposition and egg hatching. If larvae were recorded their duration of development was recorded. Laboratory host specificity of the rust fungus was done in a high containment facility in CSIRO, Canberra, Australia and results of the experiment have been reported by Ireland *et al.* (2019a).

5.2.4.1 Open field trials

The host range testing of insects on *L. ferocissimum* plants in the open field was conducted to investigate non-target effect of insect candidates. The same plant species used in the laboratory host specificity testing were used for field host specificity. Three sites in the Eastern Cape Province were selected for in-field host specificity, viz. Makhanda (33.31° S; 26.52° E), Salem (33.46° S; 26.48° E), and Kenton on Sea (33.68° S; 26.67° E). At each sampling site, three *L. ferocissimum* plants of the same height and estimated similar age were randomly selected approximately 50 metres away from residential areas. Each of the test plant species was planted approximately 2 m from the target plant. This distance also depended on the amount of shade available and the surrounding habitat. Plants were then dug into the ground and visited weekly. They were watered on a weekly basis with no fertiliser application. Feeding damage, and whether the insect could complete its life cycle on the test plants were monitored.

5.2.4.2 Rust fungi assessment in the field in South Africa

The biology and host specificity of *P. rapipes* was conducted in Australia (Ireland *et al.*, 2019a) but some native range field studies were conducted to confirm the quarantine experiments. Two *L. ferocissimum* plants which were naturally infected with *P. rapipes* orange uredinia spores were identified. These included a mature *L. ferocissimum* plant approximately 1.5m tall in the field and a potted *L. ferocissimum* plant growing under 10% shade cloth in the research facility.

At each of these sites, potted medium-sized (25 – 50 cm) *Lycium* species were placed randomly in a circle at approximately 1-1.5 m from the infected *L. ferocissimum* plant. Three replicate plants of *L. ferocissimum* (Western Cape population), *L. barbarum*, and *L. oxycarpum* were tested at both experimental sites while *L. ferocissimum* (Eastern Cape population) and a single *L. cinereum* were included as one of the infected plants. All *Lycium* species used in the two experimental sites were disease-free at the start of the experiment. Potted *Lycium* species were watered regularly and no additional overhead or supplemental watering was used to encourage infection by rust. Infection by the rust was visually assessed on each plant as the percentage of leaves with at least one uredinia of *P. rapipes*. Assessments of infection were made weekly for the first six weeks, with final assessment made at 11 weeks. The testing was conducted during winter, with dominant westerly winds, and average temperatures ranging from 18.8ºC to 20ºC with temperature extremes of approximately 35°C and 6.2°C recorded at the sites throughout the experiments. Rainfall was experienced twice during the period of experimentation.

5.2.4.3 Cleta eckloni *and* Cleta sp. 1 *impact trials*

Ten similar sized *L. ferocissimum* plants from the Western Cape Province and ten *L. ferocissimum* from the Eastern Cape Province were selected from cultures growing at the Rhodes university mass rearing facility. The total number of leaves on all the plants were counted and recorded and then placed into a mesh cage (60cm x 60cm x 90cm) (Figure 5. 1) Four *Cleta eckloni* (Eastern Cape Province) and four *Cleta* sp. 1 (Western Cape Province) adults were placed on each plant, bar the ten control plants. The plants were examined every second day for the period of 35 days. The total number of leaves damaged over the study period was recorded over the period of the experiement.

5.2.4.4 Statistical analysis

A factorial two-way ANOVA was used to investigate differences in total number of leaves lost between the two types of plant after feeding by the two insect species (statistical significance at $P < 0.05$). The ANOVA was conducted using the statistical programme

Statistica 10.4 (Hill & Lewicki, 2007). The Tukey HSD test was used to investigate significant differences through multiple comparisons ($P < 0.05$).

A two-way factorial ANOVA was used to investigate differences in total larval survival between the two plant types after feeding by the two insect species (statistical significance at $P < 0.05$). Similarly, the pupation and pre-pupation periods of insect species growing on the two plant types were contrasted using two-way ANOVAs on Statistica 10.4.

5.3 Results

5.3.1 Biology and host range of candidate agents

5.3.1.1 Cleta eckloni *Mulsant (Coccinellidae: Epilachnini)* & Cleta sp. 1

Both *Cleta eckloni* and *Cleta* sp. 1 adults and larvae fed on the leaves of *L. ferocissimum*. Both species laid 3-13 eggs in clusters on the top surfaces of the leaves (Figure 5. 2) and rarely laid a single egg only. Eggs were elongate-ovoidal, initially yellow turning brown (Figure 5. 2). They took approximately seven days to hatch. The dorsal and lateral surfaces of the neonate larvae were covered with branched processes (Figure 5. 2). Larvae underwent four to five instar stages, over 20-30 days, to reach pupation (Figure 5. 2). The larvae predominantly fed on the undersides of the leaves, whilst adults predominantly fed on the upper surfaces. The larvae pupated under the leaves or on the stems of the host plants. The pupa remained in the moult of the final instar larval stage (Figure 5. 2). The pupae were well camouflaged on the *L. ferocissimum* stems. Adults were enclosed from the pupae after approximately seven days (Figure 5. 2).

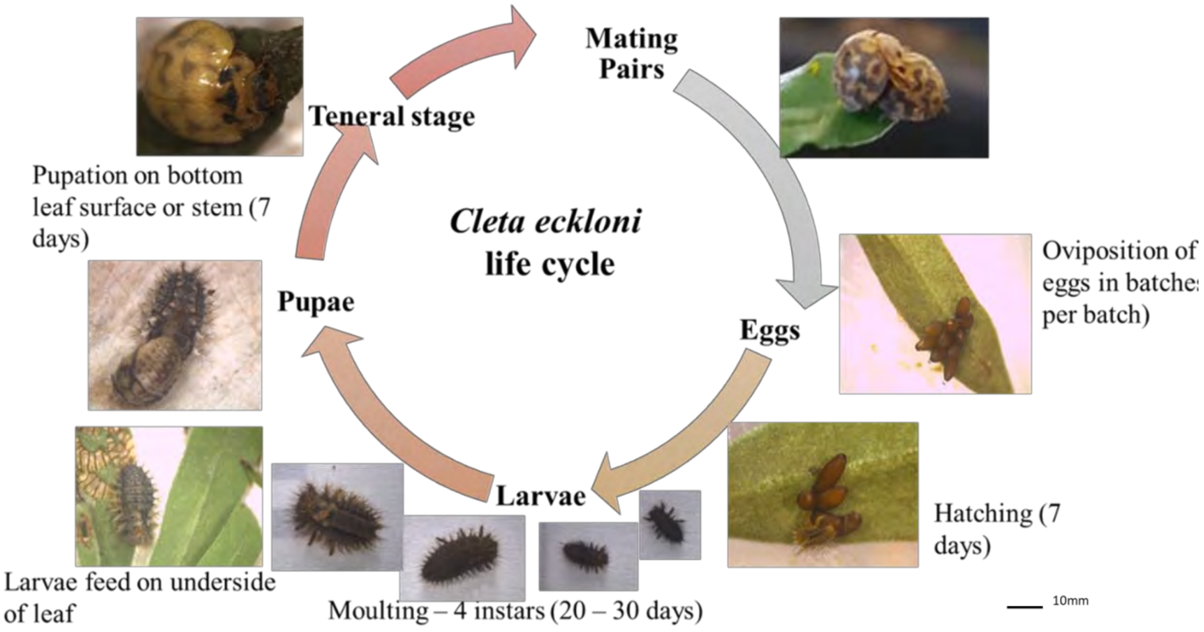

Figure 5. 2. Life cycle of *Cleta eckloni* Mulsant (Coccinellidae) determined through observations of lab reared insects (n = 20)

Preliminary host range testing suggested that *Cleta eckloni* has a narrow host range restricted to the genus *Lycium*. During native range surveys in the Eastern and Western Cape provinces of South Africa *C. eckloni* was not observed feeding on any plant other than *L. ferocissimum*. Adults and larvae fed by scraping the soft tissue of the leaf, chewing it and sucking the juices. The damage by a couple of insects was quite substantial and resulted in the plant's dropping its leaves (Figure 5. 2). During laboratory host range testing, feeding was recorded on *L. oxycarpum* (Table 5. 1). However, field host range testing revealed that *C. eckloni* could potentially feed on *S. melongena* (non-target) as relatively low feeding marks were recorded but could be neglected as it did not have any impact on the health of the plant, and the insect could not complete its life cycle on the plant in the field.

Table 5. 1. Host specificity testing of *Cleta eckloni* Mulsant (Coccinelidae) in the laboratory and during open field host range testing with *Lycium ferocissimum* Miers (Solanaceae) on closely related species of economic value in the Solanaceae family. Results are presented as presence (✓) or absence (X) of different experimental attributes.

	Laboratory test		Open field test	
Closely related plants	**Feeding damage**	**Survival/ Reproduction**	**Feeding damage**	**Survival/ Reproduction**
L. barbarum (N=12)	X	X	X	X
Capsicum spp. (N=9)	X	X	X	X
S. melongena (N=9)	X	X	✓	X
L. oxycarpum (N=12)	✓	X	X	X
S. lycopersicum (N=9)	X	X	X	X
L. ferocissimum (N=15)	✓	✓	✓	✓

5.3.1.1.1 Cleta *spp. survival and development on* L. ferocissimum *populations*

There was no difference in larval survival between either *Cleta* spp on either of the plant populations (Eastern or Western Cape Provincce) (average = 65 ± 24%) (Figure 5. 3).

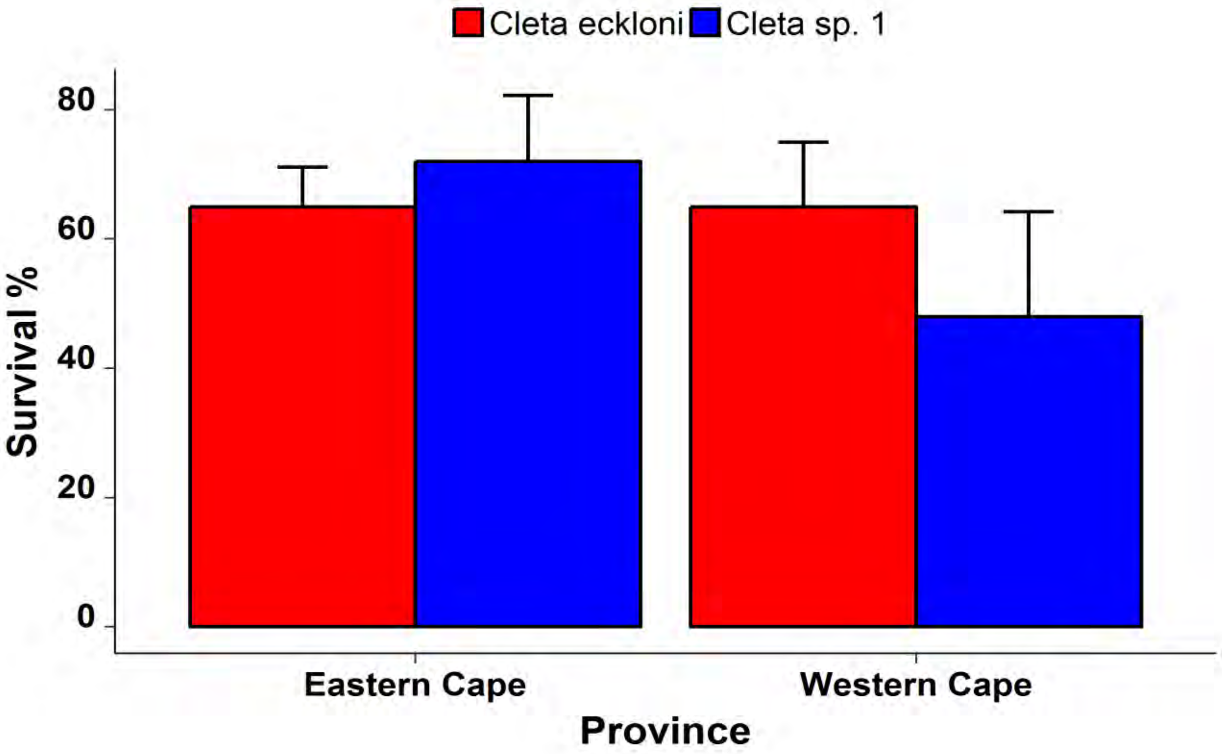

Figure 5. 3. *Cleta eckloni* and *Cleta* sp. 1 larval survival (%) on two populations of *Lycium ferocissimum*, Eastern and Western Cape Province.

The pupation period of *Cleta* sp. 1 was significantly shorter on Western Cape Province plants than on the Eastern Cape Province plants (F $_{(1,\ 29)}$ = 5,4305, *P* = 0,02695; Figure 5. 4). All individuals that pupated managed to develop successfully into adult insects.

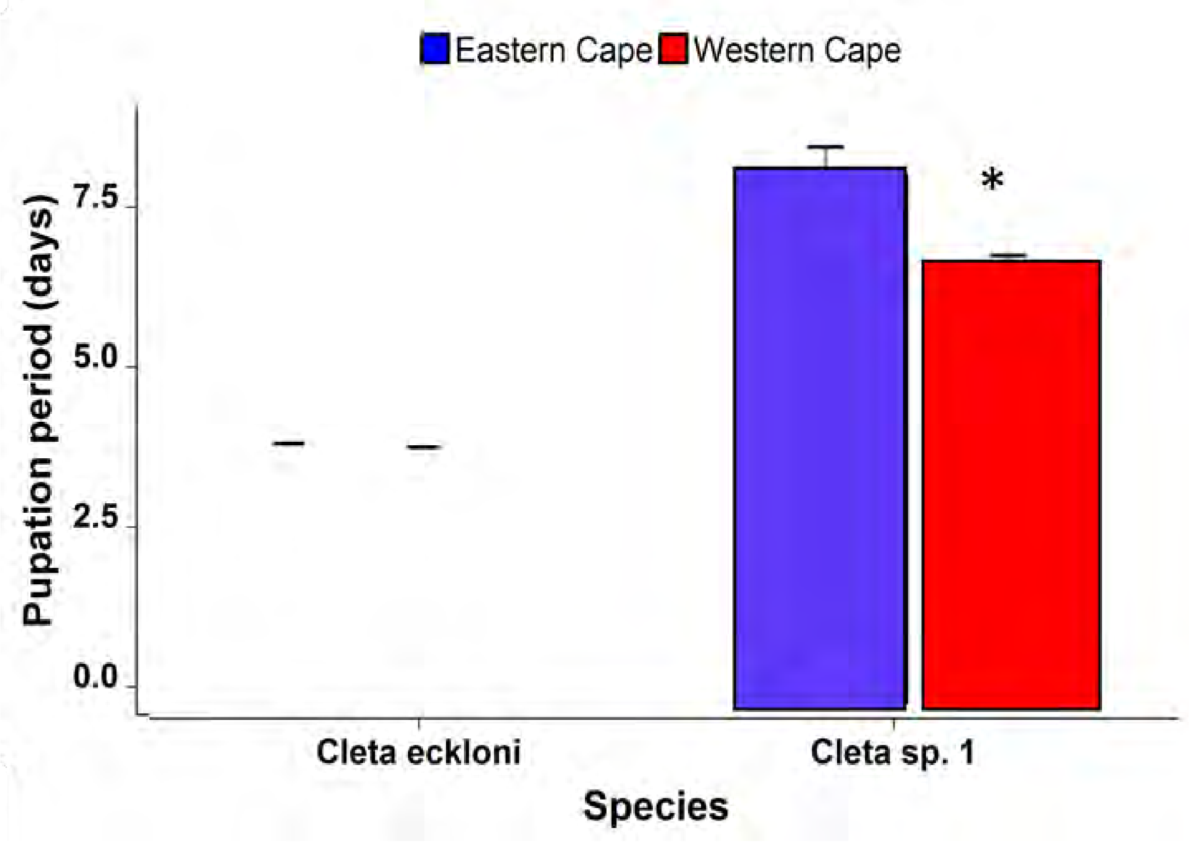

Figure 5. 4. Pupation period (days) of *Cleta* sp. 1 on *Lycium ferocissimum* Miers (Solanaceae) from the Eastern and Western Cape provinces, South Africa. *Cleta eckloni* as control measure on pupation.

There was a difference in the number of days it took *Cleta* spp larvae to develop from eggs to pupation (Figure 5. 5). There were significant differences among both species (F $_{(1,\ 58)}$ = 136,87, *P* < 0.001) and provinces (F $_{(1,\ 58)}$ = 46,55, *P* < 0.001; Figure 5. 5). Both insect species pupated faster on the Eastern Cape Province than Western Cape Province plants (Figure 5. 5). *Cleta* sp. 1 developed faster on plants from the Eastern Cape Province than the Western Cape Province.

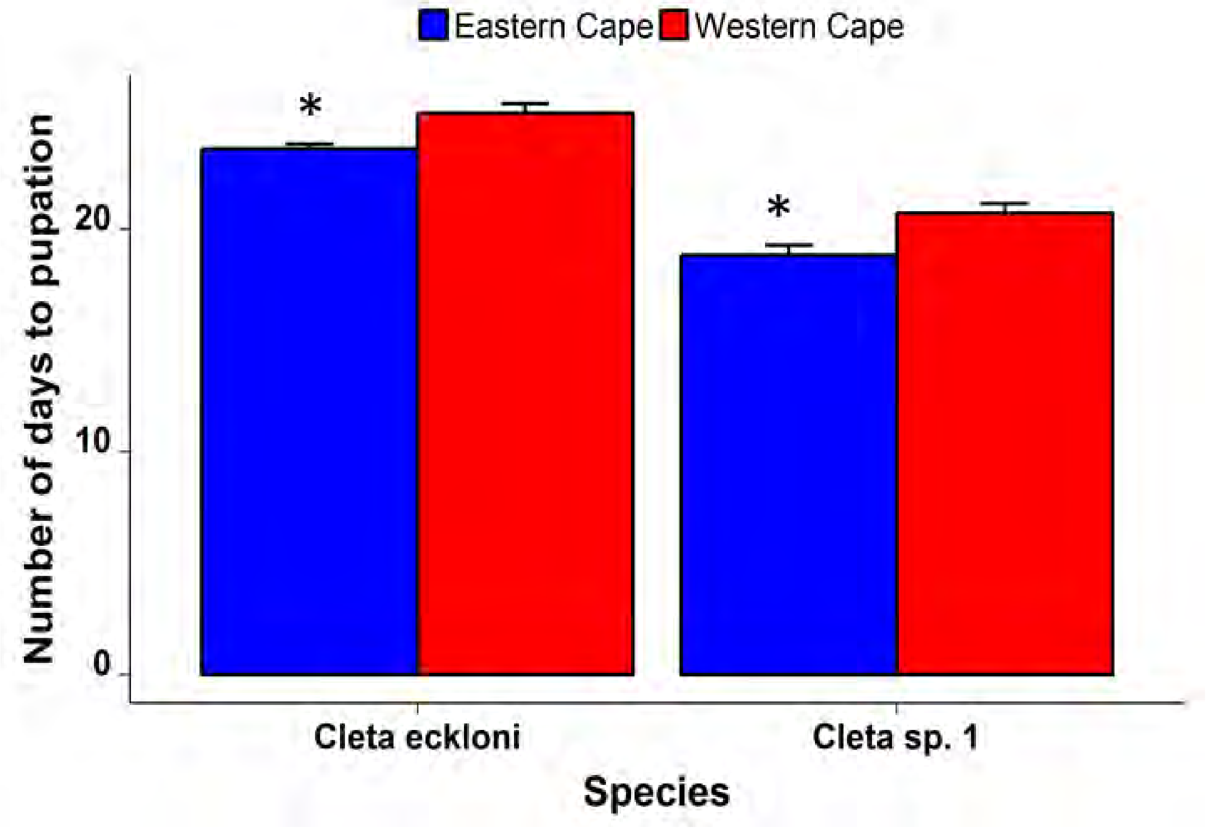

Figure 5. 5. Number of days taken by two incipient insect species (*Cleta eckloni* and *Cleta* sp. 1) to pupate on *Lycium ferocissimum* plants from two provinces of South Africa (Eastern and Western Cape provinces).

5.3.1.1.2 Impact of Cleta *spp. on* Lycium ferocissimum *populations*

When leaf loss was combined for both allopatric populations of *L. ferocissimum* over a period of 35 days there were significant differences across the two provinces ($F_{(1,319)} = 20.99$, $P = 0.000007$) and species ($F_{(2,319)} = 14.04$, $P = 0.000001$). Apart from the control plants, total leaf loss was higher on Western Cape Province plants than on those from the Eastern Cape Province (Figure 5. 6). *Cleta eckloni* caused greater leaf loss than *Cleta* sp. 1 on Western Cape Province plants ($F_{(2, 319)}$, $P < 0.001$; Figure 5. 6). There were no significant differences in total leaf loss caused by the two insect species on the Eastern Cape Province plants.

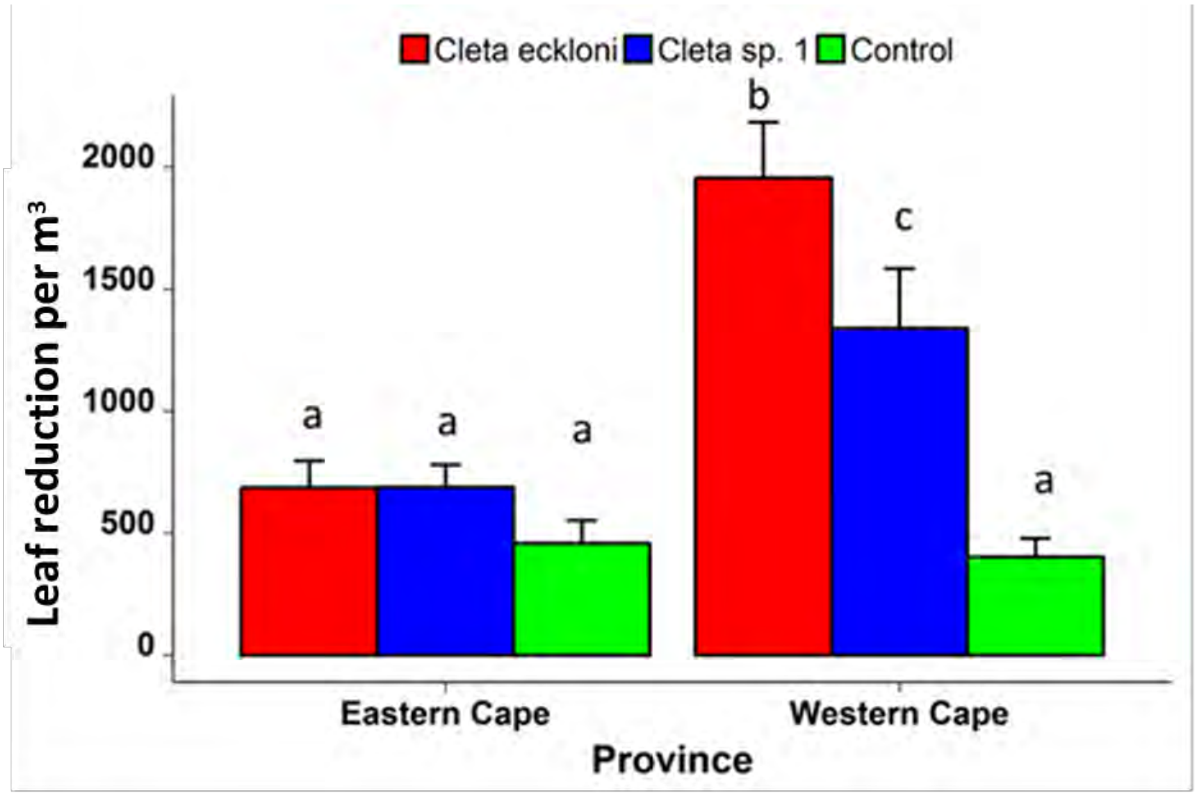

Figure 5. 6. Total number of leaves lost (leaves/m^3) by *Lycium ferocissimum* populations from the Eastern and Western Cape provinces fed on by two similar herbivorous insect species *Cleta eckloni* (red) and *Cleta* sp. 1 (blue).

5.3.1.2 Cassida distinguenda *Spaeth (Chrysomelidae: Cassidinae)*

Cassida distinguenda adults and larvae fed on the leaves of *L. ferocissimum*. Adults fed on leaves, chewing off the leaf edges, whereas larvae fed by chewing, creating holes (Figure 5. 7), ultimately cutting off the top half of the leaf. The damage by a 4-8 insects was significant and resulted the plant's dropping its leaves. Females laid a single egg on the upper leaf surface and rarely laid two eggs on the same leaf. When two eggs were laid on one leaf, the second egg was usually laid on the underside of the leaf. The eggs were yellow-brown and took 8-10 days to hatch. The larvae were light yellow and slightly dorso-ventrally flattened (Figure 5. 7). They used an exuvial-faecal shield for protection against desiccation and predation. Larvae took approximately 30 days to develop into adults, of which most of the time was spent in the last instar. Pupation took place on the bottom side of the leaf.

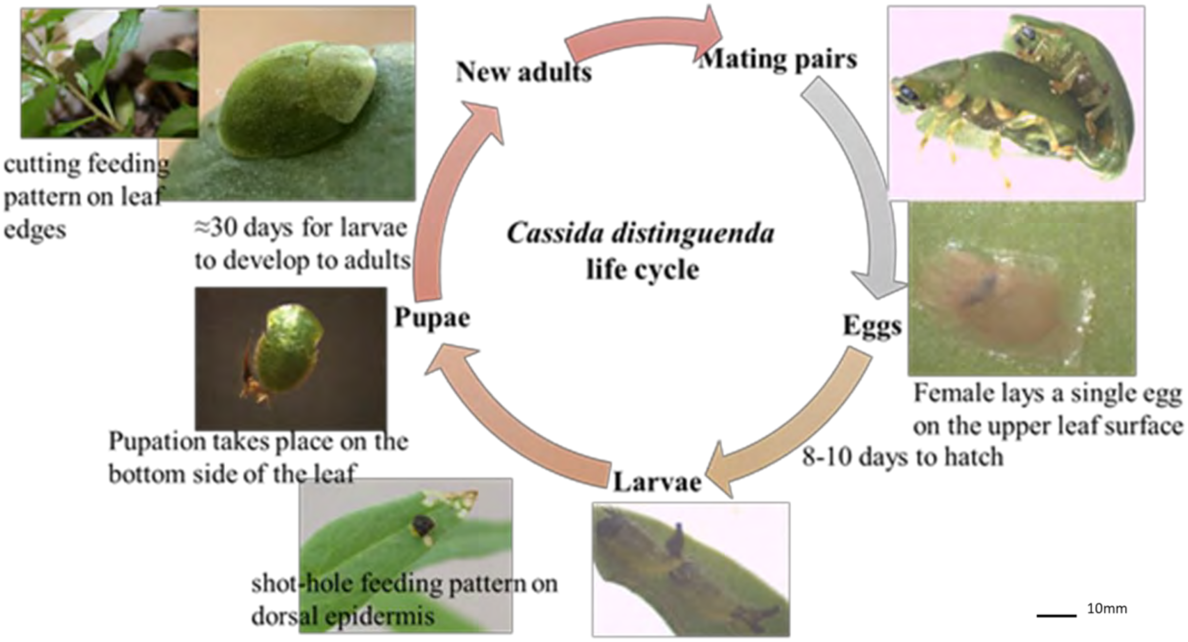

Figure 5. 7. Life cycle of *Cassida distinguenda* Spaeth (Chrysomelidae) on *Lycium ferocissimum* Miers (Solanaceae) in the laboratory (n= 20).

Cassida distinguenda was found to be restricted to *Lycium* species. Adult feeding and oviposition were recorded on *L. barbarum* (an economically important plant in Australia) and *L. oxycarpum,* with 100% survival of eggs to adults. The field results showed that *C. distinguenda* was restricted to feeding on *L. ferocissimum* (Table 5. 2).

Table 5. 2. Host specificity testing of *Cassida distinguenda* Spaeth in the laboratory and during open field host range testing with *Lycium ferocissimum* Miers (Solanaceae) on closely related species of economic value in the Solanaceae family. Results are presented as presence (✓) or absence (X) of different experimental attributes.

	Laboratory test		**Open field test**	
Closely related plants	**Feeding damage**	**Survival/ Reproduction**	**Feeding damage**	**Survival/ Reproduction**
L. barbarum (N=12)	✓	✓	X	X
Capsicum spp. (N=9)	X	X	X	X
S. melongena (N=9)	X	X	X	X
L. oxycarpum (N=12)	✓	✓	X	X
S. lycopersicum (N=9)	X	X	X	X
L. ferocissimum (N=15)	✓	✓	✓	✓

5.3.1.3 Neoplatygaster serietuberculata *Gyllenhal (Curculionidae: Ceutorhynchinae)*

Neoplatygaster serietuberculata adults and larvae (Figure 5. 8 insert) fed on *L. ferocissimum* leaves; adults fed on leaves making shot-holes and larvae mined the leaf tissue (Figure 5. 8). Females oviposited a single egg on the bottom side of a leaf where it joined the petiole. Adults avoided feeding on the leaves that had larvae developing inside. Neonates mined directly into the leaf and remained inside the leaf cuticle, developing throughout the larval instars. This took approximately 23 days (Figure 5. 8). The larvae pupated within the leaf before eclosing as new adults.

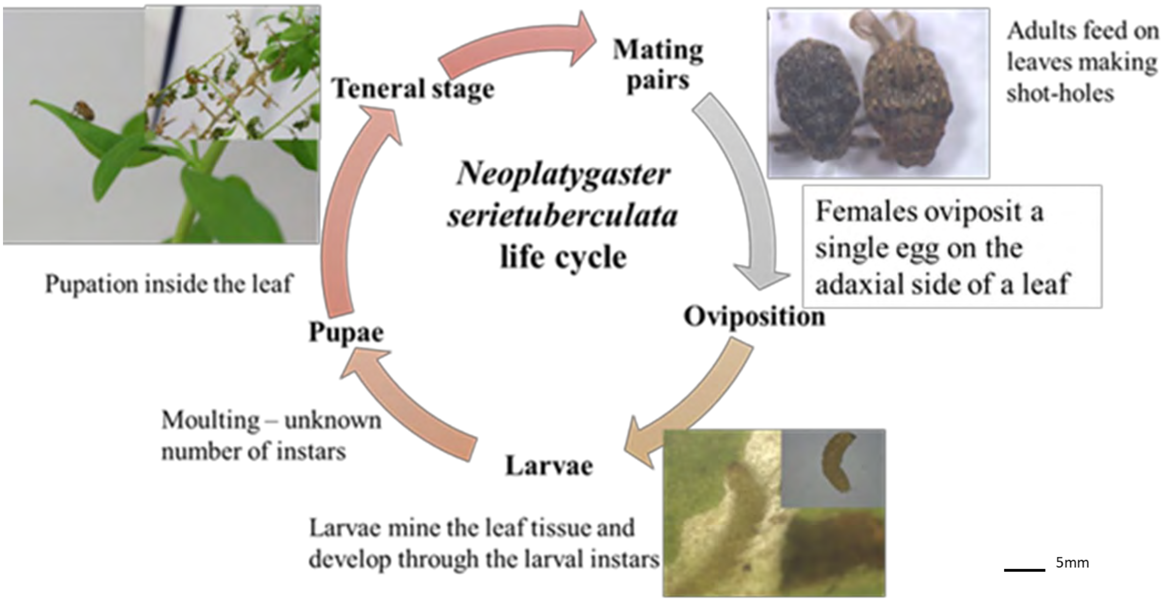

Figure 5. 8. *Neoplatygaster serietuberculata* Gyllenhal (Curculionidae) adult life cycle, male (left) and female (right) adults showing colour and size variations, 1st instar larvae feeding inside the leaf epidermis of *Lycium ferocissimum* Miers (Solanaceae), larvae (insert photo).

Initial host specificity on *N. serietuberculata* showed the weevil to have a narrow host range restricted to the *Lycium* genus. Adult feeding and oviposition was recorded on *L. ferocissimum* and some feeding on *L. oxycarpum* and *L. barbarum*, however, no survival and reproduction were recorded on the two species (Table 5. 3).

Table 5. 3. Host specificity testing of *Neoplatygaster serietuberculata* Gyllenhal in the laboratory and during open field host range testing with *Lycium ferocissimum* Miers (Solanaceae) on closely related species of economic value in the Solanaceae family. Results are presented as presence (✓) or absence (X) of different experimental attributes.

	Laboratory test		**Open field test**	
Closely related plants	**Feeding damage**	**Survival/ Reproduction**	**Feeding damage**	**Survival/ Reproduction**
L. barbarum (N=12)	✓	X	X	X
Capsicum spp. (N=9)	X	X	X	X
S. melongena (N=9)	X	X	X	X
L. oxycarpum (N=12)	✓	X	X	X
S. lycopersicum (N=9)	X	X	X	X
L. ferocissimum (N=15)	✓	✓	✓	✓

5.3.1.4 Puccinia rapipes

Quarantine cultures of the rust fungus were maintained for subsequent use in the life cycle and host range testing. The rust fungus underwent five stages in its life cycle on much older leaves on the plants with teliospore germination (Figure 5. 9). Spermogonia were mostly found on the exposed side of the basidiospores and developed within 14 days of inoculation with basidiospores from teliospore germination. Aecia developed 5-11 days after spermogonia were cross-fertilised when they were glossy and raised, followed by maturation over the course of three weeks (Figure 5. 9). Uredinia developed on the upper surfaces of leaves in the containment facility within 7-14 days. The rust fungi developed more rapidly during spring season and young leaves on *L. ferocissimum* were the first to get damaged by the rust during this time, with older bigger leaves resistant to some degree to the infection by the rust.

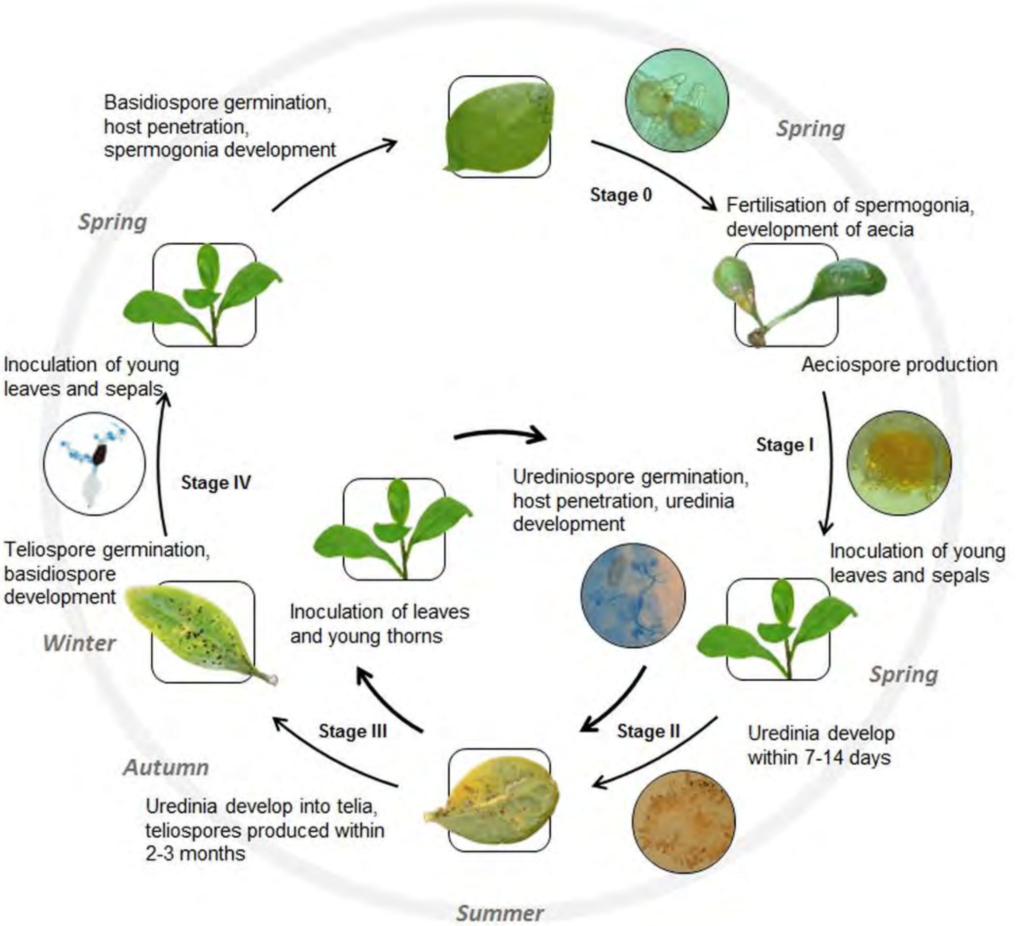

Figure 5. 9. Life cycle of the rust fungus *Puccinia rapipes* on *Lycium ferocissimum* Miers (Solanaceae) with seasonal variations (Ireland *et al.*, 2019a).

Initial host specificity trials showed that *P. rapipes* can spread from infected *L. ferocissimum* to *L. barbarum* (gojiberry) as well as the southern African native *L. oxycarpum*. This is the first record of infection of these hosts by *P. rapipes* under natural field conditions.

After two weeks from the start of the experiments *P. rapipes* was recorded on *L. ferocissimum* from the Eastern Cape population and *L. barbarum* (Table 5. 4, Table 5. 5). *Lycium ferocissimum* and *L. barbarum* were observed to have the highest incidence and severity (observational) of the rust infection at both experimental sites, whilst *L. oxycarpum* had the lowest infection. *Lycium ferocissimum* test plants from the Eastern Cape had 100% of their leaves infected with the rust fungus by the end of 11 weeks. While not tested explicitly, it was observed at the field site that *P. rapipes* infection was recorded on plants in the direct line of the prevailing winds from the infected central plant.

Table 5. 4. *Puccinia rapipes* infections of *Lycium* species at the open field site, as a measure of percent of all leaves on each plant infected with *P. rapipes* uredinia. Replicates *Lycium* species indicated below each species name, with Eastern Cape (EC) and Western Cape (WC) populations indicated. The EC replicate of *L. ferocissimum* indicates the naturally infected plant at the beginning of the experiments surrounded by all other *Lycium* species being tested.

	L. ferocissimum				***L. barbarum***			***L. oxycarpum***		
Week	**EC**	**WC1**	**WC2**	**WC3**	**LB1**	**LB2**	**LB3**	**LO1**	**LO2**	**LO3**
1	100	0	0	0	0	0	0	0	0	0
2	100	0	0	0	0	0	0	0	0	0
3	100	0	0	0	0	0	5	0	0	0
4	100	5	0	0	20	60	40	10	0	0
5	100	10	5	5	40	95	100	20	5	10
6	100	10	5	5	55	95	100	35	50	35
11	100	15	10	10	100	100	100	35	50	35

Table 5. 5. *Puccinia rapipes* infection of *Lycium* species at the 10% shade cloth site, as a measure of percent of all leaves on each plant infected with *P. rapipes* uredinia. Replicates *Lycium* species indicated below each species name, with Eastern Cape (EC) and Western Cape (WC) populations indicated. The EC replicate of *L. ferocissimum* indicates the naturally infected plant at the beginning of the experiments surrounded by all other *Lycium* species being tested.

	L. ferocissimum							***L. cinereum***	***L. barbarum***			***L. oxycarpum***		
Week	**EC**	**EC1**	**EC2**	**EC3**	**WC1**	**WC2**	**WC3**	**LC1**	**LB1**	**LB2**	**LB3**	**LO1**	**LO2**	**LO3**
1	1000	0	0	0	0	0	0	0	0	0	0	0	0	0
2	100	0	0	0	0	0	0	0	0	0	0	0	0	0
3	100	5	5	0	0	0	0	0	5	5	18	12	0	0
4	100	50	20	17	0	0	0	0	20	50	26	20	0	0
5	100	60	55	50	5	30	0	0	90	85	90	20	0	0
6	100	100	100	100	5	30	5	0	100	100	100	25	5	10
11	100	100	100	100	5	35	5	0	100	100	100	25	5	10

5.4 Discussion

All the natural enemies prioritised, were able to complete their development on *L. ferocissimum. Neoplatygaster serietuberculata* had the fastest life cycle under laboratory conditions, while *P. rapipes* took the longest to develop (40-50 days). *Cleta eckloni* larvae and adults did the most visible damage to the plants causing “windowing” (leaving one epidermis and the veins intact) leaving leaves skeletonized and drying up and often defoliating an entire test plant (see Tomaszewska & Szawaryn, 2016 for similar example). Both adults and larvae of *C. distinguenda* damaged the leaves of the target plant. While *N. serietuberculata* adults chewed shot holes in the leaves the larvae mined within the leaf cuticle (pers. obs.). Therefore, all three species show potential to build up large numbers and to inflict substantial foliar damage on the plant should they be released in the invaded range.

There is no available literature on *C. eckloni* as it has not been listed as a potential biocontrol agent or pest of any other plant. There are no formal records of *Cleta* spp. being considered for biological control anywhere, however, *Mada polluta* Mulsant (Coleoptera: Coccinnellidae), a member of the tribe Madaini, has been tested and suggested for biological control of *Tecoma stans* (L.) Jussex Kunth var stans (Bignoniaceae), an invasive plant in South Africa (Madire, 2013). Epilachnini is widely distributed in tropical areas of the world (Gordon, 1975), but *C. eckloni* is native only to South Africa (Jadwiszczak & Wegrzynowicz, 2003). Unlike other phytophagous beetles, Epilachnini do not swallow pieces of leaf fragments, but only scrape the soft tissue, chew it and suck the juices (Howard, 1941). The tribe Epilachnini are traditionally known as feeders on economically important plants belonging to the families Solanaceae and Cucurbitaceae. However, this applies mainly to the economically important species commonly encountered on crops such as *Epilachna varivestis* Mulsant or *Henosepilachna vigintioctopunctata* (Fabricius).

The trials conducted to examine the ability of *Cleta eckloni* and *Clet* sp. 1 to survive on the different populations of the plants showed both species survived equally well on the plants from their own province as well as the plants from the other province. However, the difference times to development was interesting showing that both species were able to develop faster on plants from the Eastern Cape Province. *Cleta* sp. 1 also pupated faster on its own host (plants from the Western Cape).

In addition to different development times, the insects affected the plants from the different provinces differently. Both insects' caused higher leaf drop on plants from the Western Cape Province compared to plants from the Eastern Cape Province. However, *C. eckloni* impact was greater than *Cleta* sp. 1 on Western Cape Province plant population. The results suggest that there is a potential new association, however, further studies would be required to confirm these findings. The theory of new associations suggests that plants and insects that have not co-evolved together may respond differently than to insects that they have evolved together with. It is believed that a new association could result in an increased impact to the target weed (Dennill *et al.*, 1993). The use of the 'new associations' approach assesses potential biological control agents that have not evolved with the invasive plant. The use of a newly associated insect with an invasive plant could prove to be more damaging than a more closely related natural enemy. A reason for this is that the plant may have no adapted defences to the insects means to cause damage (Hokkanen & Pimentel, 1984).

Cassida distinguenda belongs to the subgroup of large species with almost semicircular pronotum. *Cassida distinguenda* is found in the *Cassida litigiosa* group from southern Africa (Coleoptera: Chrysomelidae: Cassidinae) (Borowiec *et al.*, 2001). The insect is the smallest species of the subgroup. Members of the *Cassida* genus are often considered for biological control programmes of various invasive plant species (Goeden *et al.*, 1974; Maw & Steinhausen, 1976; Kleinjan & Scott, 1996) and some of them have been approved and released (e.g. Kok, 2001; Downey *et al.*, 2007). For example in 1995 the bitou tortoise beetle (*Cassida* sp.) was released against *Chrysanthemoides monilifera,* an invasive plant in Australia (indigenous to South Africa), and has since established in areas near the release sites (Downey *et al.*, 2007). Southern African members of the genus *Cassida* represent mostly distinct species groups of endemic taxa, of which *Cassida distinguenda* is restricted to the Cape provinces of South Africa (Borowiec, 2005). *Cassida distinguenda* was also able to complete its life cycle on *L. barbarum* and *L. oxycarpum* during laboratory trials (Table 5. 2), therefore it is suggested that multigenerational experiments as well as paired choice experiments will be further required on *L. barbarum* and other economically and ecologically important plant species, to determine suitability for release in Australia. This is supported by the fact that during the in-field host specificity testing the species did not feed or reproduce on the non-target species, suggesting that the impact seen in the laboratory may be an artefact of the laboratory conditions (Wapshere, 1983; Blossey *et al.*, 2018).

There is currently no evidence that *N. serietuberculata* can complete its life cycle on any species other than *L. ferocissimum*. Additionally, the impact caused by the insect both in the field and in the laboratory was substantial. The loss of photosynthetic tissues is anticipated to result in reduced plant vigour and reproductive output. Extensive leaf damage can cause the shrub to drop all its leaves. The Ceutorhynchinae subfamily is a large group of weevils with just over 1,300 species, that are important as pests and as biological control agents for noxious weeds (Anderson, 2005; Colonnelli, 2005). Together with *Micrelus*, *Neoplatygaster* is one of two major Afrotropical-based genera in the Hypurini tribe (Korotyaev, 2008). Other species in the genus [e.g. *N. venustus* (Faust) and *N. wittmeri* (Colonnelli)] are native to Kazakhstan, Mongolia, Saudi Arabia, Syria, Tajikistan and Uzbekistan. Similarly, weevils in the subfamily Ceutorhynchinae are often specialists that develop on a narrow range of plants within a single family, genus, or species (Anderson, 1993; Colpetzer *et al.*, 2004). Prior to the current study, *N. serietuberculata* has only been collected from leaves of *L. ferocissimum* at two sites in the Eastern Cape Province of South Africa (GBIF, 2017; van Noort & Ranwashe, 2017).

Puccinia rapipes was one of the first species to be prioritise as a biological control agent as it has previously been recorded on *L. ferocissimum* (Adair, 2013). However, laboratory and native range testing has shown the rust not to be host specific, going onto the commercially important *L. barbarum* (gojiberry) (Ireland *et al.*, 2019a). However, five gojiberry growers were contacted in South Africa, and they reported that they had not encountered the rust fungi in their respective farms and did not report any kind of fungi or pest associated with the species. *Puccinia rapipes* has only ever been officially reported on *L. ferocissimum*, despite the wide diversity of *Lycium* species prevalent and in close proximity to the known distribution of *P. rapipes* (Berndt & Uhlmann, 2006), and no other species of *Puccinia* are known to occur on *L. ferocissimum* (Otálora & Berndt, 2018; Ireland *et al.*, 2019a). However, prior surveys for the rust fungus in South Africa were solely focused on *L. ferocissimum* at the time of investigation (Ireland *et al.*, 2019a), and surveys targeted at a wider range of *Lycium* species in South Africa, especially targeting the closely related *L. oxycarpum* in the field, may show that the host range is larger than currently understood.

Prior host specificity testing for other fungal agents has shown that a greater breadth of host range is revealed under ideal laboratory conditions, when compared to natural field infections following agent release (Barton, 2004, 2012). Host specificity testing of *Lycium* species in Australia was conducted under ideal conditions (20°C constant temperature, 14-h artificial photoperiod, very high inoculum concentrations and moist chamber conducive to infection following inoculation) (Ireland *et al.*, 2019a). These conditions may result in observations of a high susceptibility phenotype, which may not reflect the potential or severity of field infections. *Puccinia rapipes* in the laboratory was not host specific when tested on close relatives of *L. ferocissimum* and it was then ideal to conduct further host specificity tests in the native range where the rust occurs naturally and there is no threat of escaping containment. During field host specificity testing in the native range, the rust fungus was also not host specific to *L. ferocissimum* as it also damaged *L. barbarum* and *L. oxycarpum*. The rust fungus shows that as an agent, it is not host specific in the laboratory and in the field, therefore, it can be expected to cause some damage to non-target species if released in Australia or other parts of the world where *L. barbarum* is in close proximity.

This study showed that collecting meta-data on insects, rather than just species identifications, allows researchers to further prioritise species before they are introduced to quarantine facilities, thus potentially saving both time and resources (see van Klinken & Raghu, 2006; Morin *et al.*, 2009). The insect biology and host specificity testing conducted on the three insects during the study provided a reference point to Australia and other parts of the world considering biological control against not just *L. ferocissimum* but also other species within Solanaceae. As these studies were purely for prioritisation there remains a need for further testing both impact and host specificity of the agents. All agents could be considered as good biological control candidates, especially in countries without any native *Lycium* species. Australia has *Lycium australe,* therefore agents more specific than the rust fungus might be required. Based on this prioritisation it is recommended that for Australia priority should be given to *N. serietuberculata* followed by *C. eckloni* and *C. distinguenda*.

Chapter 6: General Discussion

6.1 Introduction

The research conducted in this thesis documented the insects associated with *Lycium ferocissimum* Miers (Solanaceae) in its native range, South Africa, with the intention of prioritizing candidate biological control agents for the shrub in Australia where it is invasive. The results of the study have implications for decision making in the biological control programme against *L. ferocissimum* and possibly other *Lycium* species across the world.

6.2 The importance of plant genotype matching

Classical biological control programmes rely on the correct identification of the target weed. The first step in this thesis was to confirm that the species invading Australia was indeed *L. ferocissimum*, as well as the plant known to be native to South Africa (Chapters 2 & 3). Initial surveys across South Africa showed that *L. ferocissimum* has disjunct populations in the Eastern and Western Cape provinces. Plants growing in different regions have been found to develop phenotypic traits influenced by the environment in which they grow (Gratani, 2014), which might have an impact on the herbivore assemblage (Raghu *et al.*, 2006). Plant genotype matching has important implications for biological control programmes as several studies have shown that it is important to collect agents from the plants which most closely match those in the invaded region (Goolsby *et al.*, 2006a; Paterson *et al.*, 2009; Paterson & Zachariades, 2013; Sutton *et al.*, 2017).

In addition to showing the populations in Australia closely matched to populations in South Africa we found similar morphological characteristics of *L. ferocissimum* between Australia and South Africa (Chapters 2 & 3). However, some differences in flower morphology were observed, with the Australian population having shorter, wider flowers (Chapter 2). Flower characteristics did not differ between provinces in the native region of the weed, but differed from the flowers of plants in the introduced range, where they were bell shaped, bisexual and short stalked. Gratani (2014) showed that plants growing in different environments may show signs of phenotypic plasticity in flower shape. We have thus suggested that there might be a selective pressure in the invaded region that favours shorter wider flowers (Chapter 3), probably due to lack of pollinators from the native region that would normally pollinate the shrub's flowers.

Another important consideration within this study was the potential influence of hybridisation. Several authors (e.g. Spies *et al.*, 1993; Minne *et al.*, 1994; Venter, 2000; Coates Palgrave, 2005; Manning & Goldblatt, 2012) have suggested that *L. ferocissimum* hybridizes with closely related plants within *Lycium*. Whilst we found considerable morphological variation (e.g. plant height) (potentially hybrids) between different populations, making it hard to correctly identify the shrub, we could not be certain that hybridisation occurs as we did not have genetic structure analysis to confirm our findings. Thus, more work is needed on detangling hybridisation and genetic structure of *L. ferocissimum* in the native region. In Australia, however, there was very little morphological difference within *L. ferocissimum* populations. Hybridisation is also less likely in Australia as there is only one distantly related native *Lycium* species (*Lycium australe* F. Muell.) that has an overlapping distribution with *L. ferocissimum,* across parts of Western Australia, South Australia, Victoria and New South Wales (Adair, 2013). However, some literature suggests that *L. afrum* has also been introduced and is naturalised in Australia. This species overlaps with *L. ferocissimum* in Australia, and may be able to hybridise with *L. ferocissimum* (Venter, 2000; Randall, 2007). However, no signs of hybridisation were detected in Australia.

If further in-field host specificity studies were to be conducted in South Africa, further genetic studies would be needed to confirm whether there is hybridisation occurring in the population as this might produce false positive or false negative results. In addition, it remains important to keep checking the invasive populations in Australia to ensure no morphologically similar species are introduced that may hybridise with the *L. ferocissimum* already there.

6.3 Native range studies

In weed biological control, foreign field surveys aim to go to the country of origin to search for potential biological control agents and reunite them with the invasive weed. Due to financial and time constraints these surveys are often quick and do not cover the entire native distribution of the plant. Some researchers argue that this method is not always successful and does not always select the most effective agent (Olckers *et al.*, 2002). Van Klinken and Raghu (2006) suggested that for the most effective potential biological control agent to be selected and released against the target weed, more time needs to be spent in the native region surveying for potential agents instead of short duration, *ad hoc* surveys that lead to unsuitable agents being imported into quarantine facilities. In this study we have highlighted a number of Van Klinken and Raghu (2006) concerns, showing firstly that it is possible to make taxonomic identification errors in the native range resulting in insects collected from the wrong species (Chapter 3), and that agents vary over the plant's native distribution (Chapters 4 & 5) as well as between seasons (Chapters 4 & 5).

Surveying plants over a period of three years and at various times of the year provided useful insights into the true insect diversity associated with *L. ferocissimum* (Chapter 4). During surveys, the Cape provinces of South Africa were experiencing the worst drought for 100 years, potentially influencing the insects associated with the species. The drought has only recently broken, and whether more insects would be collected if the plants were re-surveyed remains to be determined. This method of continuously collecting insects on the target weed in the native region has proven to be useful and differs from conventional collection methods previously used by researchers, where insects are collected over a limited time period (Olckers *et al.*, 2002). By continuously collecting insects and monitoring we were able to filter out insects that were generalists, visiting, or resting on the plants and those that were associated with the plant, saving time and resources.

Native range surveys can provide important information on the weed and its response to herbivory or any other form of attack in the native region. For example, native range surveys may reveal cryptic species that are often missed in this study two very similar species of *Cleta* were found, one from the Western Cape Province population, *Cleta* sp. 1, and the other from the Eastern Cape Province population. Initially, they were thought to be the same species. Both species are phytophagous lady beetles whose larva and the adults feed on the leaves of *L. ferocissimum*. The larva tend to feed on the bottom side of the leaves whilst the adults feed on the top side (Chapter 4). It was only once cultures were being kept in the laboratory that it was determined that the insects were different. These surveys can also inform multitrophic interactions, for example, Pikart *et al.* (2017) surveyed for potential biological control agents of *Pereskia acuteata* Miller (Cactaceae) in Brazil and collected two new species of parasitoids on the larvae of a potential candidate agent (*Adetus analis* Haldeman (Cerambycidae)). Surveys conducted for this thesis were the first to note *Psylliodes calcaratum* Bryant (Chrysomelidae: Galerucinae) host plant and its host range. Furthermore, the surveys conducted here resulted in rediscovery of a rare flea weevil, *N. serietuberculata* which was previously recorded once on an unidentified *Lycium* species in 1985 (see van Noort & Ranwashe, 2017). *Neoplatygaster serietuberculata* has proven to be a potential biological control candidate against *L. ferocissimum* (Table 6. 1; Chapter 5). Additionally a number of species are currently being identified which may yet turn out to be new species to science (Chapter 4).

Biological control programmes on weeds in the genus *Solanum* are challenging, as this is a large genus with congeners found throughout the world, thus the insects associated with them have to show a very high level of specificity to be considered suitable for release. Insects associated with most of the *Solanum* invasive species have been found to feed on non-target species closely related to the invasive species in laboratory testing (Olckers *et al.*, 2002), with Lefoe *et al.* (2019) indicating that some candidate agents will even feed on distantly related species within the genus. Cripps (2013) showed that the species of *Cassida* would feed on non-target species if confined by a cage, but that during field testing of such species they did not feed or complete their life cycle on such species. This is problematic as laboratory-based testing is often one of the main methods for agent selection. However, it has repeatedly been shown that lab-based studies do not always accurately describe the insect's ecological host range (Hill, 1999). In these cases, in-field host specificity trials become important in confirming the insect's ecological host range. In this study, some of the prioritised candidate agents fed on other *Lycium* species during host specificity testing (Chapter 5). Our results indicated that *C. distinguenda,* if confined in a cage in the laboratory, would feed on species closely related to *L. ferocissimum* but in open field testing it did not feed or complete its life cycle on any of the non-target species (Chapter 5). Several authors (see Briese, 1999, 2005; Briese *et al.*, 2002; Jacob & Briese, 2002) have argued that laboratory results do not always represent field environmental conditions and potential candidate agents that could be helpful in managing the weed in the invaded region might be rejected through laboratory results. Field studies are needed to assess this threat. In this thesis we showed the importance of doing native range surveys and in-field experiments to validate selected potential biological control before they are imported into quarantine facilities. Further in-field testing is required to confirm these observations, but they have suggested that there might be differences between the species fundamental and ecological host ranges of some of the insects. This is an important finding as one would not want to disregard an excellent biocontrol agent purely on laboratory-based trails. McFadyen (1998) called the process of choosing the best available candidate agent as "the holy grail" of biological control research, and although there have been two decades of biological control research since this statement, it remains valid.

6.4 Solanaceae vs Chrysomelidae

The Solanaceae include such globally important species as the potato (*Solanum tuberosum*), the tomato (*Solanum lycopersicum*), the pepper (*Capsicum annuum*) and aubergine or egg plant (*Solanum melongena*). *Nicotiana tabacum*, originally from South America, is now cultivated throughout the world to produce tobacco (Sforza & Jones, 2007). In addition, many Solanaceae are important weeds in various parts of the world, and some species such as *Solanum mauritianum* Scopoli, *Solanum sisymbriifolium* Lamarck, *Solanum viarum* Dunal and *L. ferocissimum* cause serious ecological and economic problems and need to be managed. In a few cases, e. g. *S. elaeagnifolium* (four biological control agents), *S. mauritianum* (two biological control agents), *S. viarum* (one biological control agents), *S. sisymbriifolium* (one biological control agents) biological control has been used to successfully manage the weed species (Winston *et al.*, 2014).

Interestingly, in the biological control of Solanaceae chrysomelid leaf beetles are the most abundant species agents, suggesting a co-evolution between these two groups. For example, two species of chrysomelid beetles (*Leptinotarsa texana* Schaeffer and *Leptinotarsa defecta* Stål) were released on the invasive *Solanum elaeagnifolium* Cavanilles in South Africa (Olckers & Hulley, 1994). The release of the two *Leptinotarsa* species was delayed in South Africa, due to fears that they would cause damage to *Solanum melongena* L. and *Solanum tuberosum* L., important cultivated vegetable crops in the country (Olckers & Hulley, 1994). *Solanum melongena* makes biological control of *Solanum* weeds difficult, as most insects associated with *Solanum* species can feed on it. This has led most biocontrol scientists to suggest that *S. melongena* is a neutral plant with even indigenous *Solanum* insects feeding on it (Olckers & Hulley, 1994; Olckers *et al.*, 1995). *Gratiana spadicea* (Klug) was released on *Solanum sisymbriifolium* Lam. in 1994 (South Africa) and *Gratiana boliviana* Spaeth on *Solanum virirum* Dunal (United States of America) in 2003, and these agents have successfully controlled the respective weeds in these regions (Hill, 1994; Olckers *et al.*, 1995; Winston *et al.*, 2014).

However, chrysomelids don't always have beneficial attributes and may also be regarded as pests. An example is the cottonwood leaf beetle, *Chrysomela scripta* F., which caused significant economic loss by feeding on the hybrid poplar (*Populus nigra* L. x *Populus maximowiczii* A. Henry (Salicaceae)) and some *Salix* spp. cultivated for biomass, pulp, and timber throughout the United States (Tenczar & Krischik, 2006; see also Beenen & Roques, 2010). *Chrysomela scripta* feeding can kill saplings and significantly damage growing trees by reducing the height and diameter. *Leptinotarsa decemlineata* Say (also known as the Colorado potato beetle) causes significant damage to plants in the Solanaceae and is the most devastating chrysomelid so far threatening the food security in North America spreading to France, Russia and other areas of Europe (Bieńkowski & Orlova-Bienkowskaja, 2018). *Leptinotarsa decemlineata* feeds on several species within the Solanaceae such as *Solanum turberosum*, *S. lycorpersicum*, *S. melongena*, *S. laciniatum*, *S. dulcamara*, *Hyoscyamus niger* and *Atropa belladonna*. Lefoe *et al.* (2019) investigated the fundamental host range of *Leptinotarsa texana* Schaeffer (Chrysomelidae) and showed that an insect used for biological control of weeds in one country might not bring the same results if tested in another country where the weed is also problematic. For example, in Australia *L. texana* completed development on twelve non-target Australian indigenous plants in the *Solanum* genus during both no choice and choice testing in the lab. The candidate agent was also found feeding on *Solanum karsense* Symon, listed nationally as a vulnerable species that needs conservation. However, the insect did not lay eggs on the plants during no choice testing in the quarantine facility in Australia (Lefoe *et al.*, 2019). This agent was released in South Africa for the control of *S. elaeagnifolium* in the 1980s and is considered an effective agent, and no non-target effects have been recorded. In this thesis I found additional Chrysomelids feeding on Solanaceae, adding to the growing body of literature on the interaction of Solanaceae and Chrysomelidae beetles.

6.5 Prioritisation of biological control agents

This thesis prioritised the biological control agents *Cassida distinguenda*, *Cleta eckloni* and *Neoplatygaster serietuberculata* based on feeding guild, abundance and ease of rearing in the laboratory. However, several authors have proposed scoring systems for selecting the best possible biological control agents for invasive weeds in the native region (Harris, 1973; Goeden, 1983; Raghu *et al.*, 2006; van Klinken & Raghu, 2006). For example, van Klinken and Raghu (2006) suggested selecting biological control agents based on different classifications: scoring systems, specific rules and modelling of expert systems, as compared to Harris's systems that only look at scoring systems. This classification system considers ecological theory known about an agent, history of control attempts on weed, experimental data and prior experience, for example, natural enemy feeding guilds. Harris (1973) proposed selecting biological control agents based on the insect's biology and success of other agents in the same family as the proposed agent (Table 6. 1). Harris's method of prioritisation is still extensively used (e.g. Hill, 1994; Olckers, 1996, 1999; Olckers *et al.*, 1999; Madire, 2013; Cripps *et al.*, 2019). Goeden (1983), however, suggested that the system proposed by Harris did not work for unknown insect species encountered during native range surveys for the first time, or agents that have not yet been released in other countries. I have applied Harris's (1973) system to *C. eckloni*, *N. serietuberculata and C. distinguenda* (Table 6. 1). I did not have data on indirect damage the three candidate agents inflict on the target weed and other insects occurring within the population. Furthermore, natural enemies that might cause a decline and mortality of the candidate potential agent are not known. The effectiveness score for our proposed candidate biological control agents indicated that amongst the three candidates *N. serietuberculata,* with a score of 30 points from a possible 34 points, is the most promising agent, followed by *C. eckloni* with 28 points out of a possible 34 (Table 6. 1). *Cassida distinguenda* received only 22 points out of 34. In spite of the scores accumulated during the scoring systems, it is still necessary to ensure that potential biological control agents are screened to meet necessary requirements for release in the invaded region. Hill (1994) using the same method of prioritisation found that both candidate biological control agents [*Metriona elator* Klug (Chrysomelidae) and *Gratiana spadicea* Klug (Chrysomelidae)] for *Solanum sisymbriifolium* showed considerable prospect for biological control of the weed in South Africa, giving them a score of 20 and 24 points each. One agent was later rejected as it was not suitably specific, and the second agent *G. spadicea* was released based on its damage on the plant in South Africa is regarded as substantial (Zachariades, 2018). Hill (1994) argues that even with criticisms Harris's (1973) system provides a much-needed framework for assessing the biological control agent's characteristics.

Table 6. 1. Effectiveness of *C. eckloni*, *C. distinguenda*, and *N. serietuberculata* as candidate weed biological control agents for *Lycium ferocissimum* Miers (Solanaceae) based on Harris's scoring system (Harris, 1973)

Criteria	Score of *C. eckloni*	Score of *C. distinguenda*	Score of *N. serietuberculata*	Maximum possible score
1. Host Specificity	3	1	3	3
2. Direct Damage Inflicted	4	3	5	5
3. Indirect Damage Inflicted	?	?	?	3
4. Phenology of Attack	4	4	4	4
5. Number of Generations	3	3	3	4
6. Number of Progeny per Generation	2	1	1	2
7. Extrinsic Mortality Factors	?	?	0	4
8. Feeding Behaviour	2	2	2	2
9. Compatibility	2	2	2	2
10. Distribution	4	2	6	6
11. Effectiveness	4	4	4	6
12. Size	?	?	?	4

Total	**28**	**22**	**30**	**45**

Raghu *et al.* (2006) proposed a filter model, considering plant demography, plant response to herbivory and verifying agent efficacy. In the matrix several steps are taken, the weed's life cycle is determined, what type of damage the weed is most vulnerable to, what type of feeding guild from the insects causes most damage to the weed, and lastly determining the host range of insects (see example in Raghu *et al.*, 2006). Here I was not able to contribute to the entire model as the majority of this model focusses on the weed within its invaded range but I was able to contribute to the knowledge base of insects associated with the species. As such, if the Raghu *et al.* (2006) model is followed and the guild that is most important to the management of the plant is identified then the suitable agent could be easily identified from this work and prioritised (Chapters 4 & 5).

It is also important when prioritising biological control agents to consider climatic tolerance of the insects. In our example the agents were collected from more than one climatic zone in Eastern and Western Cape provinces (Figure S1. 1; Figure S1. 2). Wapshere (1983) found that agents sourced from climatically matched native distribution to their intended invaded range are more likely to flourish and establish upon release and provide control over the weed. McEvoy & Coombs (2000) indicated that due to climatic variation in the native region of potential biological control agents 44% of biocontrol agents released in the invaded region have failed to establish in their newly adopted environment. The basic principle of climatic matching implies that biological control agents should be collected in the native distribution where climatic conditions are similar to those in the invaded distribution and agents are more likely to establish and persist (Robertson *et al.*, 2008). For example, *S. mauritianum* has a broad distribution in South Africa favouring the higher rainfall regions, particularly the Western and Eastern Capes, KwaZulu-Natal, Gauteng, Mpumalanga and Limpopo provinces (Olckers, 2003, 2011). In 2008 a biological control agent *Anthonomus santacruzi* Hustache (Coleoptera: Curculionidae) was released to reduce *S. mauritianum*'s flowering capacity, excessive seed production and dispersal (Klein, 2011; Singh & Olckers, 2017). Unfortunately, despite significant release efforts, the agent only established along a climatically limited area of the southern coastline of KwaZulu-Natal Province (Olckers, 2011; Cowie *et al.*, 2016; Singh & Olckers, 2017). Conducting this climatic matching for the biological control programme against *S. mauritianum* prior to the importation of *A. santacruzi* may have resulted in prioritisation of a better suited agent, or a better suited lineage of *A. santacruzi*. Hence, in this thesis climatic matching of agents was preliminarily considered using the Koppen Geiger climate classification system (Figure S1. 1; Figure S1. 2). These indicated that the invaded climates of Australia were suitable for the establishment of the prioritised agents (*C. eckloni, C. distinguenda* and *N. serietuberculata*) from South Africa. However, it is suggested that further climate matching using specialised modelling programmes such as MaxEnt or Climex be considered to determine the exact area suitable for the insects in Australia (Trethowan *et al.*, 2011).

6.6 Conclusion and recommendations

Lycium ferocissimum is a weed of National Significance in Australia causing considerable damage to Australian flora and fauna. The shrub has been controlled using mechanical and chemical means, but these methods are costly, leaving biological control as another method to be explored to mitigate the problem. This thesis provides valuable information on the target weed and insects associated with reference to biological control.

In addition, the method of conducting repeated, long-term native range surveys across the entire distribution of the target plant's population, showed how valuable it is for selecting the most appropriate agents for the plant. Matching the two populations (invaded vs native) enabled us to collect potential biological control agents of the same haplotype as in the invaded region, further improving our agent selection methods.

One of the limitations of this study is that the genetic methods I employed could not identify the exact origin of the weed in the native region, South Africa. Molecular analysis using DNA structure data is required to pinpoint the origin of the first introduction of the weed. However, we were able to show that there have been multiple introductions of *L. ferocissimum* in Australia, with at least one introduction originating from the Western Cape Province, South Africa, represented by two haplotypes that were found only in Western Cape Province and some part of Australia.

This thesis prioritised four candidate biological control agents: *Cleta eckloni*, *Cassida distinguenda*, *Neoplatygaster serietuberculata* and a rust fungus *Puccinia rapipes*. These candidate agents were further studied, therefore, determining the most efficient agent that should be considered for biological control of the shrub. *Cassida distinguenda* and *P. rapipes* showed some inconsistency during laboratory trials, completing their life cycles and damaging the non-target plant *L. barbarum* which has economic value. It is very possible that if *C. distinguenda* and *P. rapipes* were to be released on *L. ferocissimum* in Australia or any other part of the world where *L. barbarum* occurs, they might extend their host ranges. However, if the two are released in areas where *L. barbarum* do not occur, they could bring about substantial control of *L. ferocissimum*. *Cleta eckloni* and *N. serietuberculata* showed significant promise as biological control agents as they were observed to inflict significant damage on the plant. They were also able to build up high numbers in the field, causing leaf drop. Even though they were shown to be host-specific in the laboratory, further testing is required in any country looking to introduce them as biological control agents.

Biological control is repeatedly shown as the most cost effective, safe and sustainable option for the management of invasive species. With the global movement to more green and sustainable management options, biological control should become an increasingly favoured management option (Lake & Minteer, 2018). As biological control receives more attention it will become increasingly important to ensure the success of programmes and to ensure the continued safety currently associated with the science (Moran *et al.*, 2005; Wheeler *et al.*, 2017; Paynter *et al.*, 2018; Hinz *et al.*, 2019). Not only has this thesis contributed four potential biological control candidates, which might reduce the negative impacts of *L. ferocissimum* in Australia, which in turn could result in a reduction of chemical and mechanical control use in Australia and other countries. It has also contributed to the growing knowledge of conducting native range studies, the complexities of plant taxonomy and the importance of agent prioritisation.

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Supplementary Materials

Table S1. 1. Collection details and molecular identification of assumed *Lycium ferocissimum* Miers (Solanaceae) plants from South African and Australian populations, based on either three concatenated chloroplast regions (trnH-psbA intergenic spacer, maturase K, trnT-trnL intergenic spacer), or the nuclear GBSSI gene). Numbers in parentheses indicate the number of plants sequenced from the site and inverted comma indicated where more than one species was found (McCulloch *et al.,* in press).

Region	Site	Latitude	Longitude	Molecular identification	
				Chloroplast genes	GBSSI
Eastern Cape, South Africa	EC1	-33.3108	26.5169	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	EC2	-33.2023	26.6190	*"Lycium sp. unknown"* (3), *Lycium ferocissimum* (1)	*"Lycium sp. unknown"* (3), *Lycium ferocissimum* (1)
	EC3	-33.1474	26.6415	*"Lycium sp. unknown"* (3), *Lycium ferocissimum* (1)	*"Lycium sp. unknown"* (3), *Lycium ferocissimum* (1)
	EC7	-32.8292	26.8917	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (1)
	EC8	-33.2962	26.1474	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	EC9	-33.3160	26.5207	*"Lycium sp. unknown"* (1)	*"Lycium sp. unknown"* (1)
	EC10	-33.2872	26.5309	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	EC11	-33.2767	26.5417	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	EC12	-33.2668	26.5547	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	EC13	-33.4719	26.4846	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	EC14	-33.6785	26.6772	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	EC15	-33.6817	26.6797	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	EC16	-33.5966	26.8870	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	EC18	-33.3916	26.7075	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	EC19	-33.3118	26.5141	*Lycium ferocissimum* (4)	*Lycium ferocissimum* (2)
	EC20	-33.5746	25.8704	*Lycium ferocissimum* (4)	*Lycium ferocissimum* (3)
	EC21	-33.6945	25.7999	*"Lycium sp. unknown"* (4)	*"Lycium sp. unknown"* (3)
	EC22	-33.7488	25.6886	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	EC23	-33.7194	24.7202	*"Lycium sp. unknown"* (3)	*"Lycium sp. unknown"* (3)
	EC24	-34.1239	24.8038	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	EC25	-34.0569	24.9238	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	EC26	-33.9330	25.0106	*"Lycium sp. unknown"* (3)	*"Lycium sp. unknown"* (3)
	EC27	-33.4571	26.6893	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
Western Cape, South Africa	WC1	-33.6938	19.5959	*"Lycium sp. unknown"* (1), *Lycium ferocissimum* (1)	*"Lycium sp. unknown"* (1), *Lycium ferocissimum* (1)
	WC2	-33.4893	18.7234	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC3	-33.4888	18.7231	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC4	-33.1945	18.7059	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC5	-32.1149	18.8554	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	WC8	-33.3017	18.6862	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC9	-34.2353	18.4749	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC10	-34.2318	18.4744	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	WC11	-34.4180	19.1729	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC12	-34.4707	19.8884	*"Lycium sp. unknown"* (2)	*"Lycium sp. unknown"* (1)
	WC13	-34.2762	19.5351	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)

	WC14	-33.9712	18.3715	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	WC15	-34.0411	18.3595	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC16	-34.1303	18.4490	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	WC18	-33.7237	18.5006	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC19	-33.7262	18.7403	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
	WC20	-33.7289	23.2617	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	WC21	-32.3186	18.4058	*Lycium ferocissimum* (1)	*Lycium ferocissimum* (1)
Australia	AU1	-26.5759	148.7822	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	AU2	-27.1204	151.7731	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	AU3	-27.2306	151.2638	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU4	-27.2478	152.7653	*Lycium ferocissimum* (4)	*Lycium ferocissimum* (3)
	AU5	-27.3065	151.5048	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU6	-27.3532	151.5195	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU7	-27.5450	152.6136	*Lycium ferocissimum* (4)	*Lycium ferocissimum* (3)
	AU8	-27.5648	151.6507	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU9	-28.5276	150.3088	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	AU10	-30.9361	143.4155	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU11	-30.9544	147.5005	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU12	-33.2992	147.8048	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	AU13	-33.6090	150.8993	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU14	-33.8277	144.9163	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU15	-34.2957	144.4307	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU16	-34.7892	149.6808	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU17	-33.2020	144.9656	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	AU18	-35.9256	145.5439	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU19	-36.9940	143.7104	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU20	-37.8371	143.6252	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU21	-37.8884	143.7121	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	AU22	-37.9819	147.0708	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	AU23	-38.0169	143.9653	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU24	-38.0842	145.1644	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU25	-38.1490	144.2990	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU26	-38.8103	146.0565	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU27	-32.5500	138.8040	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU28	-33.7444	135.1820	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU29	-34.4656	138.2883	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU30	-34.8393	139.2130	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU31	-35.1623	139.3781	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU32	-35.3928	138.4034	*Lycium ferocissimum* (2)	*Lycium ferocissimum* (2)
	AU33	-35.5766	137.6298	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU34	-36.4029	139.7703	*Lycium ferocissimum* (4)	*Lycium ferocissimum* (3)
	AU35	-39.8635	143.8670	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU36	-39.8721	143.8615	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
	AU37	-41.0587	146.7904	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU38	-41.1595	146.3007	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
	AU39	-42.0331	147.4947	*Lycium ferocissimum* (4)	*Lycium ferocissimum* (4)

AU40	-28.8029	114.6218	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (2)
AU41	-32.0823	115.7587	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
AU42	-33.5766	120.1187	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)
AU43	-33.7960	121.9111	*Lycium ferocissimum* (3)	*Lycium ferocissimum* (3)

Table S1. 2. Geographic occurrences of herbivorous insect taxa collected on *Lycium ferocissimum* in the Eastern and Western Cape provinces of South Africa. The X denotes that the species was not recorded in the respective province.

Foliage feeders	**Eastern Cape Province**	**Western Cape Province**
Acanthocoris spinosus Spinola	EC1, EC23	x
Aculus sp. *or Aculops* sp.	EC9, EC15, EC16, EC21, EC26	x
Thrips simplex (Morison) */Frankliniella schultzei* Trybom	EC4, EC20	WC4, WC20
Antestiopsis thunbergii	EC1, EC15, EC19, EC23	WC12, WC18
Cassida distinguenda Spaeth	EC1, EC6, EC10, EC12, EC13, EC14, EC15, EC16, EC18, EC19, EC20, EC22, EC23, EC25, EC26	WC13, WC14, WC19
Cassida melanophthalma Boheman*/Cassida reticulipennis* Borowiec & Świętojańska	x	WC3
Cenaeus carnifex	EC18	x
Chnootriba sp	EC1, EC10, EC11, EC13, EC15, EC18, EC19, EC20, EC21, EC22	WC2, WC4, WC14, WC21
Cicadidae sp 1	x	WC13
Cleta eckloni Mulsant	EC1, EC7, EC10, EC12, EC13, EC18, EC19, EC20, EC21, EC22, EC26, EC30	WC21
Clytrini	EC2	x
Cryptocephalus nr liturellus Suffrian	EC7, EC13, EC16, EC19, EC23	x
Curculionidae	EC13, EC17, EC21, EC22	WC1, WC2, WC4, WC13, WC19
Epilachna sp 2 *(Coleptera, Coccinelidae)*	EC12, EC15, EC19, EC26	WC15, WC16
Epilachna sp x	EC2, EC20	x
Epitrix sp.	EC2, EC12, EC23	x
Epitrix sp 2	EC2, EC12, EC23	x
Lamprosomatinae	EC2, EC3, EC11, EC13, EC18, EC20, EC25	WC4, WC5
Lixini sp.	EC22	x
Membracidae	EC9, EC18, EC25, EC30	WC19
Neoplatygaster serietuberculata (Gyllenhal)	EC4, EC21, EC22, EC23, EC26, EC30	WC1, WC14, WC18, WC21
Pseudambonea capeneri Schuh*/Schuhistes lekkersingia* Menard	EC1, EC2, EC3, EC4, EC5, EC6, EC9, EC10, EC11, EC12, EC13, EC14, EC15, EC18, EC19, EC20, EC21, EC23, EC28	WC2, WC13, WC19
Peploptera sp.	EC2, EC5	x
Fruit feeders		
Acanthocoris spinosus Spinola	EC1, EC23	x
Antestiopsis thunbergii	EC1, EC15, EC19, EC23	WC12, WC18
Ceratitis sp.	EC12	WC9, WC10, WC12, WC13, WC15, WC18,

		WC19, WC22
Tephritidae	EC10, EC13	WC3
Seed feeders		
Cenaeus pectoralis Stål	EC18	WC10
Flower feeders		
Thrips simplex (Morison) /*Frankliniella schultzei* Trybom	EC4, EC20	WC4, WC20
Antestiopsis thunbergii	EC1, EC15, EC19, EC23	WC12, WC18
Melyridae	EC2, EC3, EC9, EC14, EC16, EC20	WC5, WC21
Micraspis sp. 1	x	WC2, WC3
Promeces sp .1	EC3, EC4	x
Root feeders		
Epitrix sp. 2	EC2, EC12, EC23	x
Peploptera	EC2, EC5	x
Undetermined		
Amphipsocidae sp. 1	x	WC14, WC15
Apalochrus sp. 1	x	WC13, WC20, WC21
Beaufortiana cornuta Distant	EC8, EC13, EC17, EC18	
Chrysomelidae	EC10, EC11, EC14, EC15, EC18, EC20, EC22, EC23, EC29	WC19
Coccoidea sp. 1	EC8, EC12	WC16
Homoptera	EC17, EC19	WC1, WC4, WC9, WC13
Hymenoptera	EC13, EC15, EC18, EC22, EC25	WC10, WC11, WC12, WC16, WC18, WC19, WC21, WC22
Macetes sp.	EC11, EC23	x
Malachiinae sp.	EC2, EC3, EC4, EC5, EC6, EC14, EC15, EC20, EC23, EC25, EC26, EC28	WC13
Pachycnema sp 1	x	WC4, WC7, WC12, WC13, WC22
Pentatomidae sp.	EC19, EC22	x

Table S1. 3. Prevalence (% of total abundance; prev.) and distribution (number of sites) of herbivorous insects associated with *Lycium ferocissimum* Miers (Solanaceae) in the Eastern Cape (EC) and Western Cape (WC) provinces of South Africa. Diet specificity (monophagous, oligophagous and polyphagous) was assessed based on published literature, consultations with taxonomists and anecdotal evidence. Species encountered only once were excluded from the analysis. The X symbol represents unknown diet specificity. The letters in parenthesis represent foliage feeders that are also flower feeders (fl), fruit feeders (fr) or root feeders (rt).

Taxa			**Diet specificity**	**Eastern Cape**		**Western Cape**	
Order	**Family**	**Species**		**prev.**	**sites**	**prev.**	**sites**
FOLIAGE FEEDERS							
Coleoptera	Chrysomelidae	*Cassida distinguenda* Spaeth	Monophagous	5,7	15	1,1	3
Coleoptera	Chrysomelidae	*Cassida melanophthalma* Boheman/ *Cassida reticulipennis Borowiec & Świętojańska*	Monophagous	0	0	1,1	1
Coleoptera	Chrysomelidae	*Clytrini*	X	0,2	1	0	0
Coleoptera	Chrysomelidae	*Cryptocephalus* nr *liturellus* Suffrian	X	1,1	5	0	0
Coleoptera	Chrysomelidae	*Epitrix* sp. *(rt)*	Oligophagous	0,9	3	0	0
Coleoptera	Chrysomelidae	*Epitrix* sp. *2*	Oligophagous	1	2	0	0
Coleoptera	Chrysomelidae	Lamprosomatinae	X	0,9	7	0	0
Coleoptera	Chrysomelidae	*Macetes* sp.	X	0,2	2	0	0
Coleoptera	Chrysomelidae	*Peploptera* sp. *(rt)*	X	0,5	2	0	0
Coleoptera	Chrysomelidae	Chrysomelidae	X	1,4	9	0,4	1
Coleoptera	Coccinellidae	*Chnootriba* sp.	Polyphagous	10,6	10	5,5	4
Coleoptera	Coccinellidae	*Cleta eckloni* Mulsant	Monophagous	11,4	12	0,4	1
Coleoptera	Coccinellidae	*Epilachna* sp. 1(Coleptera, Coccinelidae)	X	0,4	2	0	0
Coleoptera	Coccinellidae	*Epilachna* sp. 2 (Coleptera, Coccinelidae)	X	0,4	4	1,5	2
Coleoptera	Coreidae	*Acanthocoris spinosus* Spinola	Oligophagous	0,6	2	0	0
Coleoptera	Curculionidae	Lixini sp.	Polyphagous	0,6	1	0	0
Coleoptera	Curculionidae	*Neoplatygaster serietuberculata* Gyllenhal	Monophagous	1,8	6	4,1	4
Coleoptera	Curculionidae	Curculionidae	X	0,5	4	11,4	5
Hemiptera	Cicadellidae	*Cicadidae* sp. 1	X	0	0	0,7	1
Hemiptera	Coccoidea	*Coccoidea* sp. 1	X	1,8	1	0	0

Hemiptera	Membracidae	*Beaufortiana cornuta* Distant	X	0,6	4	0	0
Hemiptera	Membracidae	Membracidae	X	3	4	0,7	1
Hemiptera	Miridae	*Pseudambonea capeneri* Schuh/ *Schuhistes lekkersingia Menard*	Oligophagous	13,7	19	1,8	3
Hemiptera	Pentatomidae	*Antestiopsis thunbergii* Gmelin *(fl)(fr)*	Polyphagous	2,7	4	1,5	2
Hemiptera	Pentatomidae	Pentatomidae sp. 1	X	0,3	2	0	0
Hemiptera	Pyrrhocoridae	*Cenaeus carnifex* Fabricius	Polyphagous	0,2	1	0	0
Homoptera		Homoptera	X	0,4	2	3,3	4
Thysanoptera	Thripidae	*Thrips simplex* Morrison/ *Frankliniella schultzei* Trybom *(fl)*	Polyphagous	0,3	2	1,1	2
Trombidiformes	Eriophyidae	*Aculus/Aculops* sp.	X	32,4	5	0	0
FRUIT FEEDERS							
Diptera	Tephritidae	*Ceratitis* sp.	Polyphagous	0,2	1	6,6	8
Diptera	Tephritidae	Tephritidae	X	0	0	4,1	3
Hemiptera	Coreidae	*Acanthocoris spinosus* Spinola	Polyphagous	0,6	2	0	0
SEED FEEDERS							
Hemiptera	Pyrrhocoridae	*Cenaeus pectoralis* Stål	X	0,2	1	2,6	1
FLOWER FEEDERS							
Coleoptera	Cerambycidae	*Promeces* sp. 1	X	0,2	2	0	0
Coleoptera	Coccinelidae	*Micraspis* sp. 1	X	0	0	4,8	2
Coleoptera	Malachiidae	*Apalochrus* sp. 1	X	0	0	1,5	3
Coleoptera	Melyridae	Malachiinae sp.	X	4,1	12	1,8	1
Coleoptera	Melyridae	Melyridae	X	1	6	1,1	2
UNDETERMINED							
Coleoptera	Scarabaeidae	*Pachycnema* sp. 1	X	0	0	6,6	4
Psocoptera	Amphipsocidae	*Amphipsocidae* sp. 1	X	0	0	3	2

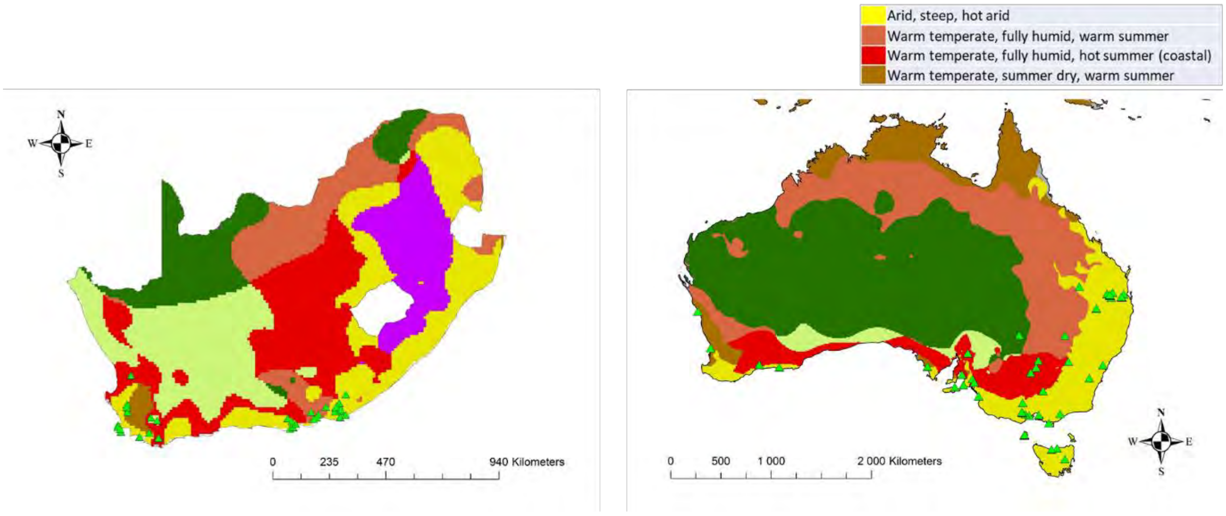

Figure S1. 1. Climate matching of insects localities in the native region vs invaded region using Köppen-Gieger climate classification.

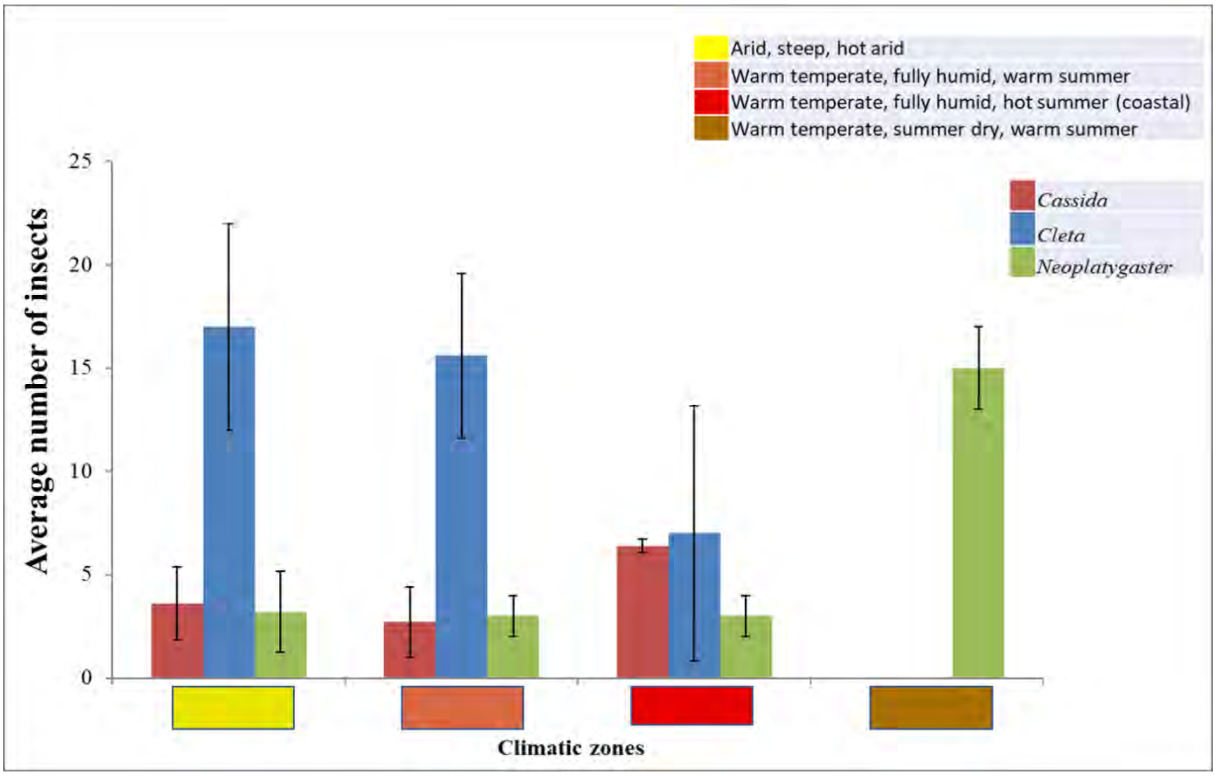

Figure S1. 2. Average number of *Cassida distinguenda*, *Cleta eckloni* and *Neoplatygaster serietuberculata*, found per *Lycium ferocissimum* plant sampled within the four Köppen-Geiger climate classification zones in South Africa.