Biology and ecology of stem-boring insects associated with the invasive weed *Senecio madagascariensis* (Asteraceae) and related species in their native range in KwaZulu-Natal, South Africa

By

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DECLARATION

The research described in this dissertation was carried out in the School of Life Sciences (Pietermaritzburg campus), College of Agriculture, Engineering and Science, University of KwaZulu-Natal, from April 2017 to June 2024 under the supervision of Prof T. Olckers and Dr D. Egli.

The work presented in this thesis represents the original work of the author and has not been otherwise submitted in any other form, for any degree or diploma, to any other University. Where use has been made of the work of others, this has been duly acknowledged in the text.

Signed:

Dineshen Singh (Candidate)

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION OF PLAGIARISM

I, Dineshen Singh, declare that:

1. The research reported in this thesis is, except where otherwise indicated, my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. Their words have been re-written but the general information attributed to them has been referenced.
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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References section.

Signature: Dineshen Singh

Date: 18 June 2024

ABSTRACT

Senecio madagascariensis Poiret (Asteraceae), commonly known as fireweed, is an herbaceous plant that is native to South Africa and Madagascar and has become a major agricultural weed in many countries around the world, most notably Australia where it poisons livestock, hybridizes with native plants and reduces pasture productivity. Consequently, it has been targeted for biological control. This study formed part of the collaboration between the University of KwaZulu-Natal and Commonwealth Scientific and Industrial Research Organisation in Australia to source suitable control agents for fireweed. The aims of the study were to: (1) determine the seasonal abundance of stem-boring insects associated with fireweed; (2) use DNA barcoding to determine the identity of important insect species; (3) determine the field host range of these insects by surveying related native *Senecio* species and comparing the insect taxa found; (4) determine the impact of larvae of the stem-boring weevil, *Gasteroclisus tricostalis* (Thunberg) (Curculionidae), on fireweed plants and; (5) determine the laboratory host range of *G. tricostalis* to determine its suitability for release in Australia.

Stem-boring taxa that were considered to have biocontrol potential included Coleoptera (specifically Curculionidae) and Lepidoptera (specifically Tortricidae and Pterophoridae). Season played a significant role in determining the abundance of insect taxa, with Curculionidae larvae displaying two peaks in abundance (May and January), while Lepidoptera larvae displayed a single peak in April. DNA barcoding of the COI gene region revealed 19 weevil species associated with native *Senecio* species, with *G. tricostalis* being restricted to the *S. madagascariensis* species complex. DNA barcoding also revealed six Lepidoptera species, with two species restricted to the *S. madagascariensis* species complex.

Following these results, the weevil *G. tricostalis* was prioritized as the most promising candidate agent due to its narrow field host range. During impact trials involving varying larval loads, arising from differential oviposition densities, on the growth and reproductive traits of *S. madagascariensis*, larvae of *G. tricostalis* were able to significantly reduce the floral productivity and shoot production of fireweed plants, both of which influence the abundance and spread of fireweed. Following these results, further host-range testing was conducted on *G. tricostalis* in the laboratory. Although the weevil displayed a narrow field host range, these tests revealed that it was capable of surviving on some non-target Australian *Senecio* species. *Gasteroclisus tricostalis* larvae were recovered on seven native Australian *Senecio* species, whilst adults were reared on four species, in numbers that were not significantly different to

those recorded on fireweed. This host range was considered to be unacceptably broad and the weevil was rejected as a potential agent for Australia. Although *S. madagascariensis* is a challenging target for biological control, other invaded countries that have fewer native species within the genus *Senecio* (such as New Zealand and Hawaii, USA), could consider *G. tricostalis*, and other previously discounted candidate agents, for release, due to lower chances of non-target impacts.

KEYWORDS: Agent-impact studies; fireweed; host-specificity testing; invasive plants; native-range surveys; weed biological control

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List of publications

This PhD thesis represents a conversion from my Master of Science dissertation, for which the degree was awarded. Data from my original study were revised (Chapters 1-3) and two additional results-based chapters were added to the thesis (Chapters 4 and 5). All results-based chapters have either been published in, or submitted to, international peer-reviewed journals as follows:

Singh, D., Egli, D., Willows-Munro, S., Gooden, B., & Olckers, T. (2022). Seasonal abundance of stem-boring insects associated with the invasive *Senecio madagascariensis* in its native range (KwaZulu-Natal, South Africa) and their potential for biological control. *Biocontrol Science and Technology*, 32(5), 607–623. **[Chapter 2]**

Singh, D., Egli, D., Willows-Munro, S., Gooden, B., & Olckers, T. (2023a). Host-plant affinities of stem-boring weevils and moths associated with fireweed (*Senecio madagascariensis*) in South Africa: Evaluating native host ranges of candidate biocontrol agents using DNA barcoding. *Biocontrol Science and Technology*, 33(1), 48–60. **[Chapter 3]**

Singh, D., Egli, D., Gooden, B., & Olckers, T. (2023b). Response of the invasive fireweed (*Senecio madagascariensis*) to variable levels of larval stem-boring by *Gasteroclisus tricostalis* (Coleoptera: Curculionidae), a potential biological control agent. *Biological Control*, 187, article 105395. **[Chapter 4]**

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Chapter 1: Introduction and literature review

Invasive alien plants and the process of invasion

Alien invasive plants (AIP) have become a major problem, not only in South Africa but around the world (Van Wilgen et al., 2000; Meyerson and Mooney, 2007; Richardson and Rejmánek, 2011). Since the 1800s, these plants have caused a range of negative impacts on biodiversity, ecosystem functioning, biological processes as well as on the economic output of agricultural land and forestry plantations (Brooks et al., 2004; Theoharides and Dukes, 2007; Holzmueller and Jose, 2009; Kumar and Prasad, 2014). In the United States alone, it is estimated that the costs of damage and control of AIP amount to nearly $30 billion annually (Holzmueller and Jose, 2009). AIPs are plant species that have become naturalized in areas where they have been introduced and, due to the absence of their natural enemies, grow rapidly and uncontrolled, often replacing and reducing indigenous flora and causing changes in the biogeochemical cycles of areas (Brooks et al., 2004; Theoharides and Dukes, 2007; Holzmueller and Jose, 2009; Kumar and Prasad, 2014). These plants are introduced into new geographical ranges, either intentionally as cultivated or ornamental species, or accidentally by humans as they travel, or via the international trade of contaminated goods (Theoharides and Dukes, 2007; Foxcroft et al., 2008; Van Driesche et al., 2008).

AIPs have received a great deal of attention from scientists within a range of fields, such as ecologists, biologists, conservationists and many others due to their extensive negative impacts on the biology, ecology and functioning of various habitats (Sakai et al., 2001; Foxcroft et al., 2008; Kumar and Prasad, 2014). In recent years, AIPs have been recognised as one of the most destructive forces that directly alter the structure, functioning and composition of ecosystems (Holzmueller and Jose, 2009; Kumar and Prasad, 2014). These plants have major economic importance by degrading agriculture, forestry plantations, riverine ecosystems, fisheries and conservation areas (Foxcroft et al., 2008; Kumar and Prasad, 2014; Paini et al., 2016). The invasion of alien plants works hand-in-hand with various other aspects of environmental change, such as an increase in land use change, habitat destruction, climate change and increases in greenhouse gases (such as CO_2 and N) (Theoharides and Dukes, 2007; Tamura and Tharayil, 2014; Liu et al., 2017; Rai and Singh, 2020). The mechanism by which invasion occurs can be separated into three distinct phases which include their introduction, establishment/colonization and expansion (Richardson et al., 2000; Theoharides and Dukes, 2007; Aikio et al., 2010; Kumar and Prasad, 2014).

Introduction phase of invasion

The introduction of an exotic species involves its transport over long distances to new regions which it would not have reached by itself (Theoharides and Dukes, 2007; Kumar and Prasad, 2014). Although plant species have always dispersed via natural means, such as birds (feeding on fruits and dispersing seeds), other animals (e.g. seeds attaching to fur), wind and water, the current dispersal patterns are occurring at accelerated rates, which are aggravated by the much greater distances travelled, mostly due to human movement and global trade (Theoharides and Dukes, 2007; Kumar and Prasad, 2014). Nowadays, there is a skewed pattern of invasion by alien plants, with more AIPs located in Africa and the Americas than other parts of the world (Theoharides and Dukes, 2007). This is mostly due to the past trends in human colonization, agriculture and trade of goods (Theoharides and Dukes, 2007; Kumar and Prasad, 2014).

Although many AIPs were introduced to new areas by accident (via cargo ships, contaminated agricultural produce, livestock or people), others were introduced intentionally and are now cultivated because of beneficial traits, such as producing edible fruit or other products (Henderson, 2001; Theoharides and Dukes, 2007). Examples in South Africa include *Eucalyptus* L'Hér. species (honey), *Morus alba* L. (fruit), *Psidium guajava* L. (fruit) and *Opuntia ficus-indica* L. Mill. (fruit) (Henderson, 2001). Other invasive species that are grown for firewood, forage or timber in South Africa include species of Australian *Acacia* Mill. and *Eucalyptus* and species of European *Pinus* L. (Henderson, 2001). Many species were also grown for their ornamental (e.g. *Lantana camara* L., *Rosa rubiginosa* L.) or medicinal value (e.g. *Hypericum perforatum* L.) in South Africa (Henderson, 2001; Theoharides and Dukes, 2007).

Since the 19th century, many AIPs were deliberately introduced to new areas by human colonists due to their aesthetic or ornamental value (Theoharides and Dukes, 2007). These species are typically more devastating than those that were accidentally introduced, due to higher initial founder populations and repeated introductions (Theoharides and Dukes, 2007).

Colonization and establishment phase

Although AIPs may be introduced into an area, this does not imply an ability to become established (Theoharides and Dukes, 2007). The ability of a species to colonize and then establish in a new area depends on various factors such as the environmental and climatic conditions, and various other biotic and abiotic factors (Theoharides and Dukes, 2007; Van Driesche et al., 2008). One important aspect that strongly affects a species' invasive ability is

referred to as “propagule pressure” (Sakai et al., 2001; Colautti and MacIsaac, 2004; Theoharides and Dukes, 2007; Van Driesche et al., 2008; Lockwood et al., 2009; Simberloff, 2009). Propagule pressure, also known as “introduction effort”, refers to the number of individuals of a plant species that are introduced into an area, the number of introduction events, as well as the composition of the introductions (e.g. genetic demographic structure) (Lockwood et al., 2005; Theoharides and Dukes, 2007). This is similar to a term in conservation biology referred to as “minimum viable population”, which simply states that the higher the number of individuals and/or number of introduction events, the higher the chances that a species will survive stochastic events and overcome Allee effects caused by low conspecific numbers, while greater genetic diversity and variation will allow an alien species to adapt to the conditions of a new area (Sakai et al., 2001; Theoharides and Dukes, 2007; Lockwood et al., 2009). The type of environment is vital in determining the amount of propagule pressure required to facilitate invasion (Colautti and MacIsaac, 2004; Lockwood et al., 2005; Theoharides and Dukes, 2007; Van Driesche et al., 2008). In areas which are climatically favourable and where there is little interspecific competition, low propagule pressure can promote invasion, while areas with harsh environmental conditions and intensive competition would require high propagule pressure for invasion to be successful (Theoharides and Dukes, 2007).

For an alien plant to become established in a newly colonized area, it must develop a self-sustaining population that allows it to expand in size (Richardson et al., 2000; Theoharides and Dukes, 2007; Aikio et al., 2010). During the establishment phase, biotic filters are important factors in reducing/constraining the population size of alien plants (Theoharides and Dukes, 2007; Van Driesche et al., 2008). These biotic filters provide barriers that can reduce invasion and are formed by the presence of living organisms within an area (Theoharides and Dukes, 2007; Van Driesche et al., 2008). Biotic filters include the interactions of resident plant species (such as facilitation and competition) which can influence a new species’ ability to survive, grow and reproduce and hence its ability to establish in an area (Theoharides and Dukes, 2007; Van Driesche et al., 2008; Öster et al., 2009; Hulvey and Aigner, 2014). The biotic community (i.e. the type of plants) in an area is also important in determining whether invasion by a plant species would be successful or not (Richardson et al., 2000; Mitchell et al., 2006). In areas where plant communities are similar between the native and new ranges (i.e. homogeneous), invasion may be less likely due to intense competition, while in dissimilar communities (areas which are heterogeneous in resources and diversity) alien plants may have a higher chance of

surviving and establishing due to higher resource availability and lower competition (Liu et al., 2006; Theoharides and Dukes, 2007; Westphal et al., 2008).

There are various properties and traits that alien plants exhibit which increase their ability to establish within an area. These include, but are not limited to, having secondary compounds which reduce herbivory by natural enemies (e.g. *Schinus terebinthifolius* Raddi which produces the secondary compound schinol) and having allelopathic properties which are highly toxic to local plants (e.g. *Centaurea maculosa* Lam. which produces catechin that reduces the growth of plants around its roots) (Bais et al., 2003; Cappuccino and Arnason, 2006). Other traits which are common to invasive plants include rapid growth rates and short life cycles as well as prolific flowering and high seed production (Kumar and Prasad, 2014). Examples of invasive plants which display most of these traits include *Solanum mauritianum* Scop. (see Olckers, 2011) and *Senecio madagascariensis* Poir. (see Sindel, 2009; Egli and Olckers, 2015; 2017).

The establishment phase is often followed by a lag phase, where there is little growth in the population of the alien species (Mack et al., 2000; Theoharides and Dukes, 2007; Aikio et al., 2010). During this lag phase, small populations of established alien plants adapt to their new area, particularly the local habitats and environmental conditions, and undergo genetic filtering/adaptation, which leads to the formation of new genotypes and allows the population to overcome Allee effects and increase its genetic diversity (Theoharides and Dukes, 2007; Aikio et al., 2010). This is exemplified by the invasive plant *Ageratina adenophora* (Spreng.) King and H. Rob. (Crofton weed) in China (Wang and Wang, 2006). Crofton weed is native to Mexico and was introduced to Britain in 1826, after which it spread to other parts of the world including China (Wang and Wang, 2006). After a lag phase of 20 years (1940-1960), it spread rapidly at an average rate of 20 km per year and is now considered to be one of the worst alien plant species in China (Wang and Wang, 2006).

Expansion phase

After the lag phase, there is a period of expansion when the invading species increases its population size rapidly which is sometimes referred to as landscape spread (Theoharides and Dukes, 2007; Aikio et al., 2010; Kumar and Prasad, 2014). Expansion is affected by a range of factors such as the life history of the invader (i.e. its growth rate, history of invasion, and ability to disperse), the local plant community and the structure of the landscape (i.e. the size and distribution of habitats, distance between areas and connectivity of areas) (Wangen and

Webster, 2006; Theoharides and Dukes, 2007). According to Pyšek and Hulme (2009), the average rate of spread of an invasive species can vary between 2 m and 370 m per year.

The ability of an AIP to spread within an area is mainly affected by the pattern of the landscape, notably the barriers that need to be overcome (With, 2002; Theoharides and Dukes, 2007), but also pressure from native organisms, particularly herbivorous insects and pathogens (see below). The pattern of the landscape is determined by three main factors, including the geology, biology and disturbance regime of the area (With, 2002; Theoharides and Dukes, 2007). In more recent years, anthropogenic processes have greatly enhanced the spread of AIPs worldwide (With, 2002; Theoharides and Dukes, 2007). Humans often alter the natural disturbance regimes within ecosystems (e.g. animal impacts, extreme weather events etc.) which can severely change the landscape pattern and promote the invasion of alien plants (With, 2002; Theoharides and Dukes, 2007).

Control of AIPs

There are many characteristics of invasive weeds which allow them to become established and gain an advantage over native floral communities (Williamson and Fitter, 1996; Allendorf and Lundquist, 2003; Culliney, 2005; Pyšek and Richardson, 2007; Van Kleunen et al., 2016). These include, but are not limited to: rapid growth rates (seedling growth and early/fast reproduction); being able to grow in a range of environmental conditions; having a range of pollinators and/or being able to self-pollinate; seeds being spread by a range of mechanisms; being able to reproduce sexually and asexually; being able to produce a range of secondary plant compounds which reduces herbivory and local plant growth (allelopathy); an ability to produce a large number of viable seeds; an ability to grow rapidly after a disturbance and; an ability to adapt to areas and control measures (Williamson and Fitter, 1996; Culliney, 2005; Pyšek and Richardson, 2007; Holzmueller and Jose, 2009; Kumar and Prasad, 2014; Van Kleunen et al., 2016).

Due to the extensive ecological and economic damage caused by AIP species, three control options have been used to manage their populations, notably mechanical, chemical and biological control (Culliney, 2005; Clewley et al., 2012; Van Wilgen et al., 2013). Mechanical control is labour intensive and involves the direct removal of AIPs by hand-pulling, mowing, cutting, bulldozing, hoeing or tillage (Culliney, 2005). Chemical control uses a range of approved herbicides and application techniques (see XACT information (2005) for South Africa) to kill individual plants and reduce population spread (Culliney, 2005). Both chemical

and mechanical control are effective within small areas, where invasion by the targeted plant is limited (Culliney, 2005). However, although these control options are widely practiced, they are extremely costly (albeit not in comparison to the cost of AIPs) since they incur labour and logistic costs and require continuous follow-up applications (Culliney, 2005). In addition, both methods have other more serious impacts such as mechanical control causing environmental damage (e.g. disturbing habitats and wildlife as well as contributing to soil compaction and erosion) and chemical control negatively affecting non-target species, the ecosystem and human health (e.g. causing immune and neurological problems and, in some cases, cancer) (Culliney, 2005).

In the past century, biological control has become one of the most desirable solutions for invasive weeds (Culliney, 2005; Clewley et al., 2012; Van Wilgen et al., 2013; Lake and Minteer, 2018). Biological weed control involves the importation of herbivorous natural enemies (notably insects or plant pathogens) from the plant's native region and their release in the invaded areas (Culliney, 2005; Van Driesche et al., 2008; Clewley et al., 2012), in order to reduce the weed's abundance to levels found in its native range (Culliney, 2005; Clewley et al., 2012). Biological control is often used when an invasive species has become widely established and where mechanical and chemical control are not feasible due to them being ineffective, too costly or having adverse effects on the environment (Van Wilgen et al., 2013). Biological control agents can either damage their host plants directly (by feeding on various plant parts, resulting in reduced reproduction or plant mortality) or indirectly (by causing stress on their host plants which reduces their growth and reproduction) (Culliney, 2005).

Biological weed control

Biological control programs typically involve a range of procedures before a biocontrol agent can be released (Culliney, 2005; Moran et al., 2013). The first step is to conduct extensive research on the target weed to determine its ecology, phylogeny (e.g. different varieties, hybrids and genotypes), environmental and economic impact and whether there are any conflicts of interest (Culliney, 2005; Van Driesche et al., 2008; Moran et al., 2013). The next step is to survey the weed in its country of origin to locate potentially suitable control agents and import them into quarantine in the invaded country for stringent testing (Culliney, 2005; Van Driesche et al., 2008; Moran et al., 2013). Each potential control agent undergoes a range of host-specificity tests in quarantine to demonstrate that it is host specific and thus unable to attack non-target plant species (Colpetzer et al., 2004; Culliney, 2005; Van Driesche et al., 2008; Moran et al., 2013). Non-target plant species, especially those that are closely related to the

target weed (e.g. in the same genus or family) and are economically important or native to the area, are routinely tested as part of the process (Van Driesche et al., 2008; Moran et al., 2013).

Once host-specificity testing is completed and the agent is shown to be host specific, applications for its release in the invaded country are submitted to the relevant regulatory authorities (Van Driesche et al., 2008; Moran et al., 2013). Once approved for release, the agent is mass reared to provide a viable population size for release and released at specific sites that would enable the population to survive and spread to new locations (Culliney, 2005; Moran et al., 2013). Thereafter, post-release studies are conducted to confirm the agent's establishment and distribution and eventually to determine its impact on the target weed (Culliney, 2005; Moran et al., 2013). Although, in recent years, biological control has achieved much progress and support in the control of invasive weeds, it has not always been successful. Since the mid-19th century, some 949 biological control agents were released for the control of 133 invasive plants worldwide, with about 25% of these projects having been completely successful and 50-70% of projects resulting in slight to variable control of their target plants (Culliney, 2005; Van Wilgen et al., 2013; Hinz et al., 2020). Besides incompatibility of the agents with the climate of the new range and the recruitment of natural enemies, reasons for the lack of success include biological control being used as a last resort when other weed control options are no longer feasible (Culliney, 2005).

Despite these obstacles, there are several aspects which make biological control a favourable strategy (Culliney, 2005; Van Driesche et al., 2008; Van Wilgen et al., 2013; Hinz et al., 2020). Once established, agents are often self-sustaining and able to spread to isolated locations without human intervention (Culliney, 2005; Van Driesche et al., 2008). When successful, the control of the weed is permanent, resulting in lower costs than mechanical and chemical control programs which require re-application (Culliney, 2005; Van Driesche et al., 2008). Finally, unlike mechanical and chemical control, biological control agents cause little disturbance to the environment, besides their impact on the target weed and changes in the flow of energy, and are non-toxic and biodegradable (Culliney, 2005). However, some of the obstacles to implementation are that agent performance in the new range is unpredictable and that the initial investment into research may be high. Most of the costs relate to the number of scientist-years invested in quarantine host-specificity testing, particularly when agents are rejected due to unacceptably broad host ranges (Van Driesche et al., 2008).

One solution to reduce costs and provide an early assessment of the potential for success is to conduct research in the weed's country of origin where studies can be conducted under natural field conditions (Van Driesche et al., 2008). In this regard, a collaborative research project involving the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia and the University of KwaZulu-Natal (UKZN) was established to source suitable biocontrol agents for the South African plant *Senecio madagascariensis* Poiret, which is invasive in several countries across the world (Sheppard et al., 2013).

Senecio madagascariensis (fireweed) – the target plant

Senecio madagascariensis (more commonly referred to as fireweed) belongs to the family Asteraceae, which is one of the largest families of flowering plants and includes more than 1600 genera and 23 600 species of herbs, shrubs and trees around the world (Jansen and Palmer, 1987; Gao et al., 2010; Wijayabandara et al., 2022; EBI, 2024). Many species in the Asteraceae family are extremely important for their medicinal, ornamental and economic value (Jansen and Palmer, 1987; Gao et al., 2010). There are also numerous species that are important food crops, including *Lactuca sativa* L. (lettuce), *Cynara cardunculus* L. (artichoke), *Cynara scolymus* L. (globe artichoke) and *Helianthus tuberosus* L. (Jerusalem artichoke) (Panero, 2008; Gao et al., 2010). Other agricultural species include *Helianthus annuus* L. (sunflowers) and *Carthamus tinctorius* L. (safflower), which are used in the production of oil (Panero, 2008; Gao et al., 2010). Several Asteraceae species have ornamental value such as species of *Chrysanthemum* L., *Dahlia* Cav. and *Tagetes* L. (Panero, 2008; Gao et al., 2010). Species with medicinal value include *Artemisia annua* L., which is used for the treatment of malaria, *Saussurea involucrata* Matsum and Koidz., which is used as an anti-inflammatory, and various *Echinacea* Mornch species, which are used to reduce inflammation, speed up recovery and increase immunity in response to bacteria and viruses (Panero, 2008; Gao et al., 2010). Weed species that are closely related to economically important plants are typically difficult targets for biological control, due to the rigours of host-range testing (Moran et al., 2013).

Fireweed belongs to the tribe Senecioneae (Vincent and Getliffe, 1992), in which the genus *Senecio* is one of the largest genera of flowering plants, with at least 1250 species that make up about 42% of the 3000 species within the tribe (Frodin, 2004; Pelser et al., 2007). The genus *Senecio* is cosmopolitan with some species found in various parts of the world (Gómiz and Llamas, 2005; Pelser et al., 2007), such as *Senecio vulgaris* L. which occurs around the world (CABI, 2024a) and *Senecio angulatus* L.f. which occurs in South Africa, Australia, New Zealand and around Europe (CABI, 2024b). The genus *Senecio* has had a complicated history

in terms of the placement of its species (Vincent and Getliffe, 1992; Pelser et al., 2007). In the past, and even more recently, there has been much deliberation and uncertainty around the morphology, phenology and species composition of *Senecio* and other closely related genera, due to the close relatedness of species within the tribe Senecioneae (Pelser et al., 2007). One of the most notable aspects of the genus *Senecio* is that the majority of the species are highly toxic, containing high levels of secondary compounds, most commonly pyrrolizidine alkaloids (Pelser et al., 2005). These toxins are especially problematic for many mammalian herbivores as ingestion causes liver damage and mortality in severe cases; consequently, the value and quality of animal products such as meat and milk is reduced (Lambers et al., 1998; Gardner et al., 2006). Although not as frequent, there have been some cases where humans have accidentally ingested these plants and been poisoned (Gardner et al., 2006).

Due to the vast amount of genetic and morphological variation within *S. madagascariensis* (and the genus *Senecio*), there has been much confusion around the taxonomy of the species (Le Roux et al., 2006; CABI, 2024c). Many species within the genus *Senecio* are often quite closely related, which thus poses a major inconvenience for plant taxonomists and biocontrol practitioners (Le Roux et al., 2006). For example, fireweed and *Senecio inaequidens* DC. are often mistaken in the field and display little genetic differences in their DNA sequences (see Le Roux et al., 2006). *Senecio madagascariensis* has five synonyms which include *S. bakeri* Scott-Elliot, *S. burchelli* Cabrera, *S. incognitus* Cabrera, *S. junodianus* O. Hoffm. and *S. ruderalis* Harv. (TPL, 2010; Wijayabandara et al., 2022; CABI, 2024c).

Description of fireweed

Fireweed is a short-lived perennial herb (surviving for more than two years) or in some cases an annual or biennial woody shrubby plant (Parsons and Cuthbertson, 2001; Starr et al., 2003; Csurhes and Navie, 2010). Fireweed can range in height from 7-50 cm, but in some cases where soil and nutrients are favourable the plant may reach 70 cm in height (Parsons and Cuthbertson, 2001; Motooka et al., 2003; Csurhes and Navie, 2010). The growth form of fireweed is variable with some plants having a single main stem and others up to several stems forming the central crown at the base of the plant (Parsons and Cuthbertson, 2001; Csurhes and Navie, 2010; Wijayabandara et al., 2022). The stems have multiple branches towards the top of the plant, all of which produce many capitula (Csurhes and Navie, 2010; Figure 1). The stems arise from a shallow branched tap root system which grows from 10-20 cm deep (Parsons and Cuthbertson, 2001; Starr et al., 2003; Wijayabandara et al., 2022).

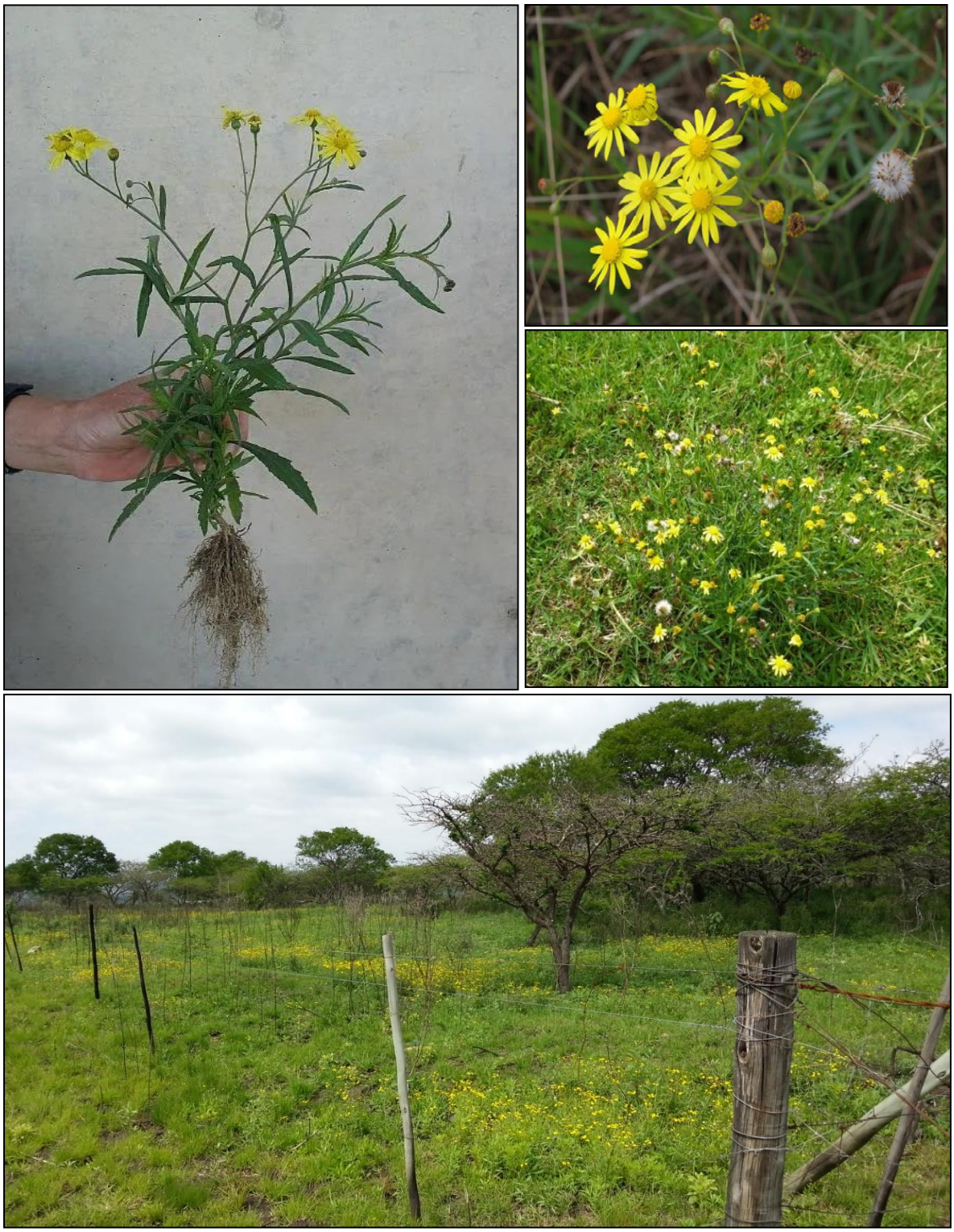


Figure 1. *Senecio madagascariensis* displaying various floral stages and abundance in a field in KwaZulu-Natal, South Africa.

The leaves of fireweed are bright green, simple and are arranged alternately on the stems (Parsons and Cuthbertson, 2001; Csurhes and Navie, 2010). The shape of the leaves does vary

and can be either serrated, entire or have toothed margins, but are typically narrow and elongated (Motooka et al., 2003; Csurhes and Navie, 2010). The leaves are usually clasped around the stem of the plant and can be from 2-12 cm long and 3-5 cm wide, depending on the plant's developmental stage and the amount of nutrients available (Starr et al., 2003; Csurhes and Navie, 2010).

A single plant can produce variable numbers of heterogamous capitula, ranging from two in smaller plants up to 200 or more in larger more developed plants (Starr et al., 2003; Csurhes and Navie, 2010; Figure 1). The capitula are small and daisy-like, bright yellow in colour and are usually 1-2 cm in diameter and found at the tops of branches (Starr et al., 2003; Csurhes and Navie, 2010). The capitula usually consist of 13 rays (ligulate ray florets) that surround ca. 120 small tubular disk florets and are between 6-14 mm long and surrounded by 9-21 involucral bracts, but numbers of ray florets can vary from 12-15 (Csurhes and Navie, 2010).

When capitula mature, they form white, thistle, ball-like structures (Figure 1) which are easily blown by the wind (Motooka et al., 2003; Starr et al., 2003). These structures are comprised of numerous small seeds, which are between 1-3 mm long and less than 0.5 mm wide, each bearing a pappus of short silky white hairs (Starr et al., 2003; Csurhes and Navie, 2010). Single capitula can produce up to 150 seeds and a single plant can produce up to 30 000 seeds within a single growing season (Parsons and Cuthbertson, 2001; Motooka et al., 2003). Viable seedbanks can persist for 3-5 years, possibly for up to 10 years (Wijayabandara et al., 2022).

Seeds of fireweed are light, slender and highly viable, with up to 90% viability in seeds produced in a growing season (Starr et al., 2003). Germination of seeds is triggered by rainfall or moist soil conditions and mild to warm temperatures (Starr et al., 2003; Csurhes and Navie, 2010). Germination can occur over a range of temperatures (15-27°C), but optimal temperatures for germination are from 20-25°C (Starr et al., 2003; Csurhes and Navie, 2010). Seeds can germinate throughout the year (depending on weather conditions), but mostly germinate from autumn to mid-summer (Starr et al., 2003; Csurhes and Navie, 2010). Seedlings can produce capitula after a reasonably short time (6-10 weeks after emergence) and the seeds remain viable for 7-10 years (Starr et al., 2003; Csurhes and Navie, 2010).

Seeds of fireweed are dispersed primarily via wind, which enables them to disperse over great distances (Starr et al., 2003; Csurhes and Navie, 2010). However, seeds can also be

dispersed accidentally via contaminated hay or grain products, by humans via adherence to clothes or vehicles, or by birds and other animals (Starr et al., 2003; Csurhes and Navie, 2010).

Origin and secondary distribution

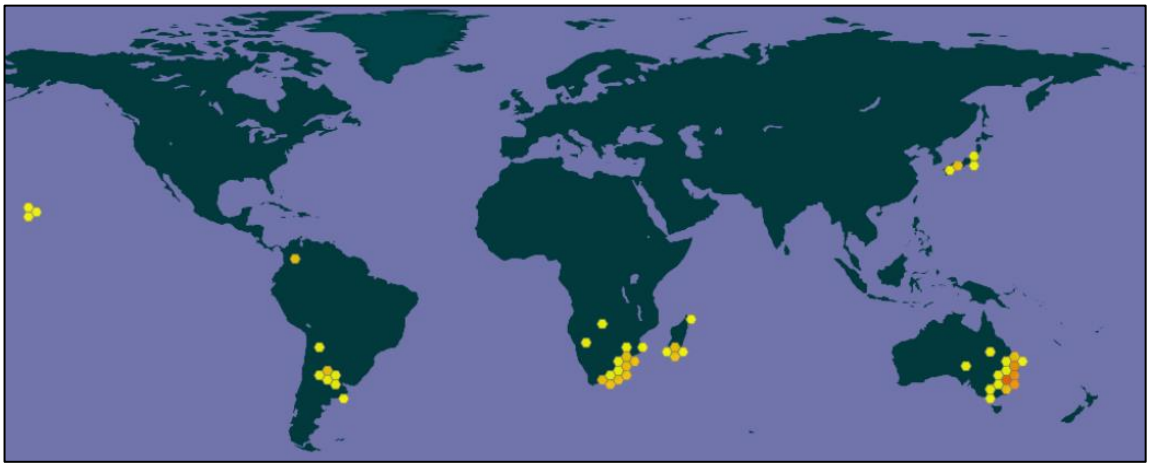
Fireweed is native to Madagascar and southern Africa and is particularly abundant in KwaZulu-Natal Province in South Africa (Scott et al., 1998; Motooka et al., 2003; Starr et al., 2003; CABI, 2024c). However, the plant has spread to several subtropical and warm temperate regions around the world, where it invades a range of ecosystems such as pastures, woodlands and grasslands as well as disturbed areas such as roadsides, parks and degraded areas (Parsons and Cuthbertson, 2001; Sindel, 2009; Figure 2). Fireweed was introduced to other countries on the African continent such as Kenya (Sindel and Michael, 1992), Mauritius and Mozambique (CABI, 2024c). The weed is particularly problematic in Australia, where it is considered to be one of the worst invaders of coastal pastures in south-eastern Queensland and New South Wales (Sindel, 2009; McFadyen and Morin, 2012; Figures 3 and 4). It was introduced from Australia to Hawaii, where it is now a major weed of pastures (Le Roux et al., 2006). It was also introduced to various South American countries such as Argentina (Lopez et al., 2008), Brazil (Cruz et al., 2010) and Colombia (Le Roux et al., 2010), where it invades agricultural land and grasslands. The plant was first reported in Japan in 1976 and since 2006 has been declared as an AIP (Tsutsumi, 2011).

Fireweed has become a major problem in most of its introduced countries, notably in Australia, the USA (Hawaii), Japan and Argentina (Sindel, 2009). The populations in Hawaii are closely matched (both genetically and taxonomically) to those found in Australia, which implies that the plant was introduced to Hawaii from Australia (Sindel, 2009). In more recent years, the populations of fireweed from both Hawaii and Australia have been matched via DNA barcoding to populations from KwaZulu-Natal, South Africa (Scott et al., 1998; Le Roux et al., 2006). Although only recently introduced to Hawaii (since the late 1980s), the plant has become widespread across the island and is one of its most problematic weeds (Le Roux et al., 2006; Sindel, 2009).

In Australia, fireweed was first recorded in 1918, presumably introduced via the ballasts of ships, which traded goods between Europe, Australia and South Africa (Parsons and Cuthbertson, 2001; Sindel, 2009, McFadyen and Morin, 2012). It was then spread along the eastern coast of Australia via contaminated seed crops during the 1940s and by the 1960s was classified as a major invader (Sindel, 2009). By the 1980s, fireweed had spread dramatically,

both south and north of the coastal areas (New South Wales and southern Queensland) into areas that were climatically similar to southern Africa (Sindel, 2009; McFadyen and Morin, 2012). Fireweed has continued to spread in Australia forming large populations in areas where it was previously only found in small patches (Sindel, 2009; McFadyen and Morin, 2012).

(A)



(B)

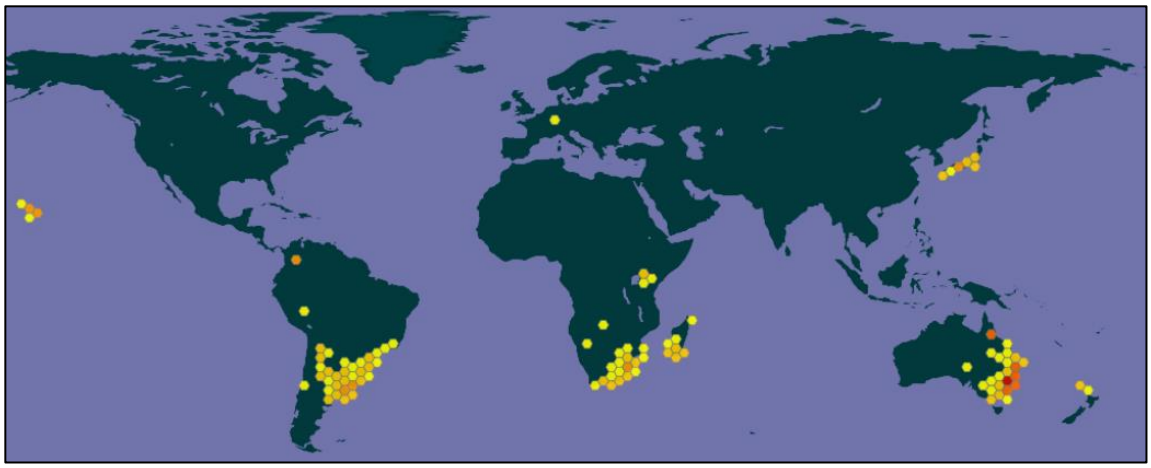


Figure 2. Distribution of *Senecio madagascariensis* in (A) 1999 and (B) 2019, showing its expansion in range within 20 years. Source: https://www.gbif.org/species/3107722

As with many invasive species, fireweed is an extremely aggressive invader which can dominate a variety of habitats in a relatively short period of time (Sindel, 2009; McFadyen and Morin, 2012). However, the distribution of fireweed is mostly restricted to humid and subtropical regions of the world (Sindel, 2009; Wijayabandara et al., 2022). Fireweed can grow in a range of environmental conditions such as altitudes ranging from 0-1500 m a.s.l., but can be found at higher altitudes, such as in Kenya and Colombia where it occurs at 2600 m a.s.l. and 2800 m a.s.l., respectively (Sindel, 2009; Wijayabandara et al., 2022; CABI, 2024c). It grows in areas receiving annual rainfall ranging from 500-1500 mm, but also occurs in higher

rainfall regions such as in Japan where annual rainfall is between 1000-1700 mm (Sindel, 2009; CABI, 2024c). Fireweed can tolerate mean annual temperatures of between 10-20°C and can survive in a range of soil conditions, but prefers soils that are well drained, not compacted and have high nutrient levels (Sindel, 2009; CABI, 2024c). However, it can survive in soils with low nutrient levels, rendering it especially problematic (Sindel, 2009; CABI, 2024c).

Figure 3. *Senecio madagascariensis* infestation in a pasture in Australia. Source: A.W. Sheppard, CSIRO.

Figure 4. *Senecio madagascariensis* infestation in a pasture in the Bega Valley, New South Wales, Australia. Source: B. Gooden, CSIRO.

Impacts of fireweed

As with most of the species within the tribe Senecioneae, fireweed is characterized by its production of secondary metabolites, namely pyrrolizidine alkaloids, which are extremely toxic (Pelser et al., 2005; Sindel, 2009; Hooda and Chauhan, 2024). These secondary metabolites deter herbivory (by both vertebrates and invertebrates) and are toxic to most herbivores (Pelser et al., 2005; Hooda and Chauhan, 2024). Consequently, fireweed has many negative impacts when consumed by domestic livestock (Le Roux et al., 2006). Fireweed is especially toxic to cattle and horses, causing a range of symptoms such as poor growth rates, weakness, loss of condition, jaundice, blindness, irritability, twitching, irreversible liver damage and, in severe cases, death (Starr et al., 2003; Csurhes and Navie, 2010). The plant is toxic during all growth stages and in both green and dry foliage (Starr et al., 2003).

Although sheep and goats are not immune to the plant's toxic effects, they are less affected than other livestock (Starr et al., 2003; Csurhes and Navie, 2010). Although most animals tend to avoid the plant, it is accidentally consumed when pastures are not managed correctly, no other food is available, when the plants are young, when hay is contaminated or when livestock are exposed to it for the first time (Starr et al., 2003; Csurhes and Navie, 2010). In rare cases, humans may also be affected via the consumption of contaminated wheat or from other animal products such as honey, dairy products or eggs that were derived from infested pastures (Sindel, 2009; Sheppard et al., 2013; CABI, 2024c).

There are vast economic costs associated with fireweed infestations. In Australia, it was estimated that fireweed reduces pasture productivity and profits by 15-50% and that chemical control of the weed would cost on average A$9000 per farm or up to A$18 million annually (Sheppard et al., 2013; Sindel and Coleman, 2012). There are a range of additional costs, such as a loss in production due to poisoned livestock, loss in the quality of produce, reduction in pasture carrying capacity, an increase in production costs due to chemical and physical control and less productivity through ineffective paddock rotations (i.e. using sheep or goats rather than cattle) (Sheppard et al., 2013; Sindel and Coleman, 2012). Other negative implications include a reduction in land value and legislation forcing landowners to control and remove the weed (Sheppard et al., 2013).

Fireweed is an extremely aggressive invader that can change and dominate invaded areas (Figures 3 and 4), being able to outcompete other plant species for light, nutrients (namely phosphorus and nitrogen) and moisture (Sindel, 2009). One of the most invasive traits of

fireweed is its ability to colonize a range of habitats that include pastures, woodlands, grasslands, bushlands, roadsides, parks and unmaintained waste areas (Csurhes and Navie, 2010; Wijayabandara et al., 2022; Hooda and Chauhan, 2024). Another invasive property of the plant is its ability to grow and reproduce throughout the year (Sindel, 2009; Csurhes and Navie, 2010; Sindel and Coleman, 2012). During winter, when other pasture species are dormant and less productive, fireweed has a competitive advantage and grows rapidly, forming dense infestations within pastures (Sindel, 2009; Sindel and Coleman, 2012).

In Australia, a major environmental concern is the weed's hybridization with native *Senecio* species growing in close proximity to infestations (Prentis et al., 2007; Sindel, 2009; Sheppard et al., 2013). This could have a range of consequences including an increase in the weediness of hybrid progeny, the creation of new hybrid species and lineages, and a decrease in biodiversity due to the decline or possible extinction of hybridizing species (Prentis et al., 2007; Sindel, 2009). In particular, the progeny of hybrids between *Senecio pinnatifolius* A. Rich. (Coast groundsel) and fireweed are largely sterile and have very low viability (Prentis et al., 2007; Sindel, 2009). In addition, fireweed has a hybridization advantage over *S. pinnatifolius* and, in areas where fireweed is more common, *S. pinnatifolius* could be under threat of extinction (Prentis et al., 2007; Sindel, 2009).

Control of *Senecio madagascariensis*

Attempts to control fireweed in Australia have involved conventional methods such as physical and chemical control, but since the 1980s biological control has been viewed as a more practical and long-term solution (Sindel, 2009; McFadyen and Morin, 2012).

Chemical control

A range of herbicides have been used for the control of fireweed in Australia, with glyphosate and bromoxynil considered the most effective (Sindel, 2009; Wijayabandara et al., 2022). Bromoxynil is most effective on young plants when applied at a rate of 280g ha^{-1}, but less than 55% of mature plants are killed at this rate (Sindel, 2009; Wijayabandara et al., 2022). In mature plants where buds have formed, a rate of 500- 560g ha^{-1} is required for effective control (Sindel, 2009). Both glyphosate and bromoxynil are especially effective in reducing the germination of fireweed when applied on plants during their late flowering stage, with glyphosate proving more effective (Sindel, 2009). However, glyphosate is non-selective and will kill desirable pasture plants, whereas bromoxynil is selective and thus less damaging to pastures (Sindel, 2009). Other herbicides used against fireweed in Australia and Hawaii include 2,4-

dichlorohenoxyacetic acid, dicamba, MCPA, metsulfuron and triclopyr (Parsons and Cuthbertson, 2001; Motooka et al., 2003; Starr et al., 2003). Since fireweed can produce seeds throughout the year, once-yearly herbicide applications to pastures are insufficient to control the weed (Sindel, 2009).

Physical control

Physical control entails a range of processes such as pasture management, slashing/ mulching of infestations and hand pulling of plants (Starr et al., 2003; Sindel, 2009). Pasture management forms the basis for any control program against fireweed (Starr et al., 2003; Sindel, 2009). Any process that allows vacant land to be exposed, such as overgrazing, drought, areas left barren after trampling, and uncompetitive pasture species promote the establishment of fireweed (Sindel, 2009). Best pasture practices, such as planting/ sowing of competitive pasture species, maintaining proper grazing regimes and adding fertilizer at the right times, reduces the onset of fireweed (Starr et al., 2003). In Australia, good competitive winter species include Phalaris (*Phalaris aquatica* L.), ryegrass (*Lolium* L. species), fescue (*Festuca arundinacea* Schreb.), subterranean clover (*Trifolium subterraneum* L.) and oats (*Avena* L. species), while good competitive summer species include Kikuyu (*Pennisetum clandestinum* Hochst. ex Chiov.), *Paspalum* L. species, Setaria (*Setaria sphacelata* (Schumach.) Stapf and C.E. Hubb.) and Rhodes grass (*Chloris gayana* Kunth.) (Sindel, 2009). In particular, Kikuyu has a longer period of growth than most grass species and, even under heavy grazing, the dominance exhibited by Kikuyu can reduce the growth of fireweed (Sindel, 2009).

The proper application of fertilizer to pastures is vital when trying to prevent fireweed outbreaks (Starr et al., 2003; Sindel, 2009). Fertilizers should be added to pastures during the growth phase of competitive grass species, to maximize their growth and yield whilst decreasing the density of fireweed (Sindel, 2009). However, if fertilizers are applied outside the growth phase of these grass species, the lack of competition and high soil nutrients will promote fireweed growth (Sindel, 2009).

Proper grazing management and rotation are very important when managing fireweed (Sindel, 2009). Heavy grazing by cattle can cause open patches to form, allowing fireweed seedlings to grow above pasture canopy and cause outbreaks in pastures (Sindel, 2009). It is especially important that stocking rates are kept low during the peak emergence of fireweed (e.g. during autumn) to allow pastures to exert more competition and maximize control (Sindel, 2009). Grazing by sheep and goats can partially control fireweed in pastures since they are 10-

20 times more tolerant to the weed's toxins than cattle and horses (Starr et al., 2003; Sindel, 2009). However, this only provides short-term relief since sheep and goats are not invulnerable to poisoning (Starr et al., 2003; Sindel, 2009).

Hand pulling of plants can be used in areas where they are isolated, provided that this occurs before seed set (Starr et al., 2003). However, hand pulling is ineffective for large populations where mowing may be preferable (Sindel, 2009). Although mowing does not kill individual plants it reduces their biomass, slows their growth and delays flowering (Sindel, 2009). As fireweed seeds do not germinate in the absence of light, burying seeds via mulching or tillage can contribute towards control (Starr et al., 2003; Sindel, 2009).

Biological control

Although Australia and Hawaii considered the possibility of biological control of fireweed in the 1980s, actual initiatives were undertaken during the 1990s (McFadyen and Morin, 2012; Wijayabandara et al., 2022). In Australia, host-range testing of the flower-feeding moth *Phycitodes* sp. nov. (Pyralidae) and the stem-boring moth *Lobesia* sp. nov. (Tortricidae) were initiated in 1990, following their importation from Madagascar (Sindel, 2009; McFadyen and Morin, 2012). Although both species caused substantial damage to fireweed, they attacked several native Australian *Senecio* species during cage trials and were thus rejected as biocontrol agents (Sindel, 2009; McFadyen and Morin, 2012). Subsequently, in 1992, permits were obtained for the importation and testing of two other promising agents, a flower-feeding moth *Homoeosoma stenotea* Hampson (Pyralidae) and a stem-boring fly *Melanagromyza* sp. (Agromyzidae), from South Africa (McFadyen and Morin, 2012). However, the project was suspended in 1995 after the insects were unable to be collected and funding for the project was terminated (McFadyen and Morin, 2012). The last candidate agent to be tested in Australia was the rust fungus *Puccinia lagenophorae* Cooke, which was imported from South Africa. However, a strain of the fungus already occurs naturally in Australia and inflicted the same levels of damage as the introduced strain, resulting in the rejection of the latter strain (Sindel, 2009; McFadyen and Morin, 2012).

Biocontrol efforts against fireweed in Hawaii started in 1999 with the importation from Madagascar of 11 insect species and the rust fungus *P. lagenophorae*, which were successfully cultured in quarantine (Culliney et al., 2003; Ramadan et al., 2011; McFadyen and Morin, 2012). However, by 2011 most of these potential agents were discarded due to a lack of host specificity or insufficient impact on the weed (Culliney et al., 2003; Ramadan et al., 2011). Of

the 11 insect species, only two were considered promising, namely the leaf-feeding moth *Secusia extensa* Butler (Erebidae) and the flowerhead-feeding fly *Sphenella* sp. (Tephritidae) (Culliney et al., 2003).

Host-specificity testing revealed that *S. extensa* had a suitably narrow host range that was limited to species within the tribe Senecioneae, with three species, *S. madagascariensis*, *S. vulgaris* and *Delairea odorata* Lemaire accepted as hosts; none of which are native to Hawaii (Ramadan et al., 2011; Krushelnycky et al., 2018). Although *S. extensa* was released in Hawaii in 2013 (HDOA, 2013; Krushelnycky et al., 2018), it cannot be released in countries like Australia that have a high diversity of native *Senecio* species (McFadyen and Morin, 2012). The rust fungus, despite good infections of fireweed, proved unsuitable for release as it was able to infect non-target species that are endemic to Hawaii (Ramadan et al., 2011). So far, *S. extensa* is the only agent to have been deployed against fireweed anywhere in the world.

Recent biological control initiatives in South Africa

Due to the high costs of physical and chemical control and the increasing status of fireweed as one of the worst invasive species of coastal pastures in Australia, interest in finding suitable biocontrol agents was rekindled (Sheppard et al., 2013). Since earlier genetic barcoding suggested that Australian populations of fireweed originated from KwaZulu-Natal Province, South Africa, a collaboration between the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the University of KwaZulu-Natal (UKZN) was established in 2011 to source candidate agents in the province (Sheppard et al., 2013). During initial surveys conducted by Egli and Olckers (2015, 2017, 2020), several species of root-feeding, flower-feeding and stem-boring taxa were identified as potential biocontrol agents.

Despite the possibility of numerous candidate agents for fireweed, subsequent studies (see Egli et al., 2020; Zuma et al., 2021; Mkhize et al., 2023) revealed that many of the these were unsuitable, as they were found on numerous non-target *Senecio* species during field surveys. Consequently, priority was given to stem-boring taxa that included species of Coleoptera, Diptera and Lepidoptera (Singh et al., 2022; 2023a; see Chapters 2 and 3). Furthermore, more detailed studies focused on the stem-boring weevil, *Gasteroclisus tricostalis* (Thunberg) (Coleoptera: Curculionidae), which was shown to have a relatively narrow host range in the field (Singh et al., 2023a; see Chapter 3).

Gasteroclisus tricostalis is a small to medium-sized weevil (Figure 5A), which has a variable life span of between 8-12 months (sometimes up to 24 months). Adults feed on leaves

and shoot tips of *S. madagascariensis* plants, with females ovipositing on the stems and branches (Figure 5B). Hatching larvae burrow in the stems, moving into the main stem and down towards the roots as late instars (Figure 5C). The time taken from oviposition to adult emergence is highly variable and can take between 9 and 17 weeks, depending on the conditions under which larval-infested plants are maintained.

While biological control is considered a cost effective and long-lasting solution to the management of alien weeds (McConnachie et al., 2003; Culliney, 2005; Messing and Wright, 2006; Suckling and Sforza, 2014; Harms et al., 2021), in recent years it has received much scrutiny regarding the impacts of ineffective agents on the environment (Messing and Wright, 2006; Hinz et al., 2019). Often ineffective agents can have both direct and indirect non-target impacts in the area in which they are released (Cory and Myers, 2000; Carvalheiro et al., 2008). Direct environmental impacts occur when introduced biocontrol agents feed on, oviposit and develop on native and other species that were not the intended targets (Taylor et al., 2007; Van Driesche et al., 2008; DePrenger-Levin et al., 2010). Indirect environmental impacts are often much harder to determine and occur when agents have unintended effects in the new range, such as influencing food web interactions and causing compensatory growth in the target plants (Carvalheiro et al., 2008; Van Driesche et al., 2008; Hinz et al., 2020; Todd et al., 2021).

Thus, determining the impact and potential effectiveness of a biocontrol agent before it is released is an important aspect of weed biocontrol programmes (McClay and Balciunas, 2005; Gerber et al., 2008; Morin et al., 2009; Wang et al., 2011; 2012). Such pre-release studies can reduce the risk of non-target impacts, prevent the wastage of time and funds on unsuitable agents, and reduce the risk of releasing agents that have little effect on the target plant (McClay and Balciunas, 2005; Van Driesche et al., 2008; Wang et al., 2011). Consequently, the impact of varying larval loads of *G. tricostalis* on the growth and reproduction of *S. madagascariensis* plants was assessed prior to laboratory host-range studies (Singh et al. 2023b; see Chapter 4).

Figure 5. *Gasteroclisus tricostalis*, A) adult mating pair, B) oviposition scars on fireweed, C) late-instar larva, and D) sleeved mating pairs in the laboratory culture.

Host-specificity testing represents the final stage of pre-release studies to determine whether an agent is suitable for release in the invaded range (Schaffner, 2001; Haye et al., 2005; Sheppard et al., 2005; Van Driesche et al., 2008). These tests are conducted to ensure that the direct risks of non-target effects associated with the release of an agent are acceptably low (Van Klinken, 1999; Müller-Schärer and Schaffner, 2008). Several considerations are taken into

account when conducting host-range tests and include the compilation of a suitable list of test-plant species, as well as the selection of appropriate test designs (Kuhlman et al., 2006; Van Driesche et al., 2008).

A suitable test-plant list should include a variety of plant species that is sufficient to confirm that the biocontrol agent is host specific, with a low probability of non-target damage (Sheppard et al., 2005; Kuhlman et al., 2006). The list should include plant species that are taxonomically or phylogenetically related to, or ecologically similar to, the target plant, but also more distantly related plant species that are useful, rare or endangered, or economically important (see Sheppard et al., 2005; Kuhlman et al., 2006).

Host-specificity testing typically involves both no-choice and choice tests (Schaffner, 2001; Sheppard et al., 2005). No-choice tests are conducted by exposing a single test-plant species to the candidate agent to determine if the plant can support feeding, oviposition and development of the agent (Schaffner, 2001; Sheppard et al., 2005). Choice tests include paired-choice tests, in which the agent is exposed to the target plant and a single non-target test species, and multiple-choice tests, in which the agent is exposed to the target plant and several non-target test species (Schaffner, 2001; Sheppard et al., 2005). The range of plant species that support the development of a candidate agent under laboratory conditions is referred to as its "fundamental host range", whereas the range of plant species utilized by the agent under field conditions is referred to as its "ecological host range" (Haye et al., 2005; Sheppard et al., 2005). Typically, an agent's fundamental host range is broader than its ecological host range (Haye et al., 2005). The fundamental host range of *G. tricostalis* was thus determined by host-specificity testing (Chapter 5) for comparison with its ecological host range (Chapter 3).

Objectives of the project

This study formed an integral part of the research collaboration between the CSIRO and the UKZN, with the aim of determining the suitability of stem-boring insects for the biological control of fireweed in Australia. The main objectives were as follows:

(1) Determine the seasonal abundance of stem-boring insects associated with *S. madagascariensis* populations in the field in KwaZulu-Natal (Chapter 2).
(2) Using genetic barcoding, differentiate the different stem-boring species associated with *S. madagascariensis* and match the endophagous immature stages within the stems to the adults (Chapters 2 and 3).

(3) Using genetic barcoding, determine the ecological host range of these stem-boring species by surveying related *Senecio* species in the field in KwaZulu-Natal and comparing the associated taxa to those associated with *S. madagascariensis* (Chapter 3).

(4) Determine the growth and reproductive responses of *S. madagascariensis* plants to varying levels of attack by the stem-boring weevil *G. tricostalis*, to assess its potential efficacy as a candidate biocontrol agent (Chapter 4).

(5) Determine the fundamental host range of *G. tricostalis* by testing closely related non-target plants from Australia and South Africa in the laboratory, to assess its suitability for release in Australia (Chapter 5).

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Chapter 2: Seasonal abundance of stem-boring insects associated with the invasive *Senecio madagascariensis* in its native range (KwaZulu-Natal, South Africa) and their potential for biological control

ABSTRACT

Fireweed, *Senecio madagascariensis* Poiret (Asteraceae), is a target for biological control in Australia and Hawaii. Candidate agents recorded in KwaZulu-Natal Province, South Africa, the region of origin of the invasive populations, include stem-boring insects. The seasonal abundance of stem borers associated with *S. madagascariensis* populations was studied at four field sites in the KwaZulu-Natal Midlands region, during 2017/18. DNA barcoding was used to differentiate and link insect taxa with their endophagous larvae. Monthly sampling revealed that stem-boring larvae comprised largely Diptera (42% of specimens) and Curculionidae (38.9%), with Lepidoptera (19.8%) the least abundant. Differences in larval herbivore loads between sites were statistically significant, with the warmer and drier sites supporting 59% higher larval numbers. DNA barcoding focused on Curculionidae and Lepidoptera, which took precedence due to restricted host ranges demonstrated in an earlier study. Of the four lineages of weevil larvae recorded, 95% of sequenced specimens comprised two maternal lineages of *Gasteroclisus tricostalis* (Thunberg). Collectively, *G. tricostalis* was detected across nine months of the year, with peaks in abundance in autumn and summer. Similarly, of the three lineages of lepidopteran larvae recorded, 83% of sequenced specimens comprised *Metamesia elegans* (Walsingham) (Tortricidae). *Metamesia elegans* was collected across six months of the year, with an autumn peak. These results support the prioritization of *G. tricostalis* and *M. elegans* as the most promising stem-boring agents for *S. madagascariensis*. The higher seasonal abundance of *G. tricostalis*, together with the good track record of weevils in weed biocontrol, suggest that it should take precedence in subsequent laboratory host-range assessments.

KEYWORDS: Cytochrome oxidase I gene sequencing, fireweed, *Gasteroclisus tricostalis*, *Metamesia elegans*, stem borers, weed biocontrol

Introduction

Senecio madagascariensis Poiret (Asteraceae), commonly referred to as fireweed or Madagascar ragwort, is a short-lived biennial (sometimes annual), woody, herbaceous plant that has invaded several countries (Lopez et al., 2008; Tsutsumi, 2011; Bartle et al., 2013;

Sheppard et al., 2013). Native to southern Africa and Madagascar, the plant continues to reduce pastoral productivity, poison livestock, and contaminate animal products across invaded landscapes, notably in Australia (Sindel and Michael, 1992; Sindel, 2009; McFadyen and Morin, 2012) and Hawaii (Le Roux et al., 2006). The extent of invasion in Australia and Hawaii has resulted in efforts to source biological control agents from its regions of origin. Although candidate agents were first imported from Madagascar (Ramadan et al., 2011; McFadyen and Morin, 2012), later genetic studies revealed that *S. madagascariensis* populations in both Australia and Hawaii were likely derived from native populations in KwaZulu-Natal Province, South Africa, rather than Madagascar (Scott et al., 1998; Le Roux et al., 2006; Sindel, 2009).

With the intention of sourcing new biocontrol candidates for Australia, natural enemy surveys were conducted in KwaZulu-Natal Province. Consequently, several insect taxa comprising root-feeding, capitulum-boring, and stem-boring species were prioritized as potential agents for *S. madagascariensis* (Egli and Olckers, 2015; 2020). Although the root-feeding flea beetle *Longitarsus basutoensis* Bechyné (Chrysomelidae: Galerucinae: Alticini) was initially ranked as the most promising candidate (Egli and Olckers, 2017), subsequent field surveys and laboratory trials revealed an unacceptably broad host range for countries like Australia that host a diverse native *Senecio* flora (Zuma et al., 2021). Consequently, the focus of surveys shifted to stem-boring insects, with *Gasteroclisus tricostalis* (Thunberg) (Coleoptera: Curculionidae) and *Metamesia elegans* (Walsingham) (Lepidoptera: Tortricidae) considered as priority candidates (Egli and Olckers, 2020). Indeed, endophagous insect species like stem borers, which spend much of their life cycle developing within plant tissues (Almeida et al., 2006), typically display a higher degree of host specificity than ectophagous species, due to their more intimate host-plant relationships (Hattendorf et al., 2006; Harvey et al., 2015). Endophagous agents have thus been widely used in weed biocontrol programmes (Winston et al., 2014), including those targeting invasive Asteraceae (Egli and Olckers, 2017). Besides their host specificity, stem-boring insects have often been favoured as biocontrol agents due to the high levels of damage inflicted on their target weeds (Klein, 2011; Winston et al., 2014).

Data on candidate biocontrol agent population abundance and phenology across the native range are important in predicting their potential efficacy for successful biocontrol of target weeds (Van Driesche et al., 2008; Forrest and Miller-Rushing, 2010). Effective insect agents are typically compatible with the climate of the new range, display a close synchrony with the target weed's phenology (Culliney, 2005) and achieve high population densities that can reduce host plant performance (Strobel, 1991; Gassmann, 1996). Many insect species display

predictable changes in their population dynamics, which are largely seasonal (Wolda, 1978, 1988; Denlinger, 1980; Ramya et al., 2017). Since high agent densities in the native range can improve the chances of biocontrol success (Gassmann, 1996, Müller-Schärer and Schaffner, 2008; Winston et al., 2014), seasonal abundance studies on candidate agents in their native range can indicate their potential in their new range (Strobel, 1991; Syrett et al., 2000). Seasonal abundance studies can also identify peaks in agent abundance, which is useful for predicting impact and facilitating collections (Bellows and Fisher, 1999; Arun and Vijayan, 2004).

In this study, we monitored the seasonal abundance of the major stem-boring insects associated with *S. madagascariensis* in KwaZulu-Natal Province, South Africa. Sampling of *S. madagascariensis* populations was conducted monthly over 12 consecutive months in 2017/18 to determine the relative abundance and population peaks of these taxa across seasons. Taxa displaying the highest numbers of stem-boring larvae across the most seasons represent priorities for biocontrol research, since *S. madagascariensis* reproduces throughout the year in both its native and invaded ranges (McFadyen and Morin, 2012). We adopted a DNA barcoding approach using the Cytochrome Oxidase I gene region, described by Egli et al. (2020), to identify the immature stages of stem-boring taxa and link them with named adult specimens.

Materials and methods

Study sites

Field surveys were conducted monthly between April 2017 and March 2018 for stem-boring insect taxa associated with *S. madagascariensis* populations, at four sites in the KwaZulu-Natal Midlands (Table 1). These sites were selected as they supported dense patches of *S. madagascariensis* (ca 10-15 mature plants/m^2) with sufficient numbers of plants available for sampling throughout the year. Sites at the Ukulinga Research Farm (Ukulinga) and African Bird of Prey Sanctuary (Raptor Centre) were situated at lower altitudes than those at the Cedara College of Agriculture (Cedara) and Groundcover Leather Company (Groundcover). The lower altitude sites are typically subject to lower mean annual rainfall and higher mean daily temperatures than the higher altitude sites (Table 1).

Sampling procedure

At each field site, five S. *madagascariensis* plants that were roughly 40-60 cm in height were sampled every month by cutting them at the base of the stem at the soil surface and placing the

material into brown paper bags. Although the size of the sampled plants was kept as constant as possible, this was dependant on plant availability. In some months, smaller plants were sampled due to plant mortality and previous sampling efforts. The samples were preserved in a freezer in the insectary of the School of Life Sciences, University of KwaZulu-Natal (UKZN), Pietermaritzburg campus (29°37'35" S, 30°24'10" E) to preserve the stems for later inspection. The stems of each sampled plant were dissected under a dissecting microscope and all insect larvae were recorded and placed into 1.5 ml Eppendorf tubes containing 100% ethanol to preserve them for later genetic analyses. Consequently, a total of 240 plants (5 plants x 4 sites x 12 months) were inspected for stem-boring larvae over the duration of the study.

Table 1. Description of sites in the KwaZulu-Natal Midlands, South Africa where *Senecio madagascariensis* populations were sampled monthly during 2017/18 for stem-boring insect taxa.

Site name	Co-ordinates (S, E)	Altitude (m amsl)	Habitat	Mean annual rain (mm)*	Mean daily temp (°C)*
Ukulinga (Mkhondeni)	29°39'44.291" 30°24'18.215"	759	Grassland/ paddock	738	18.4
Raptor Centre (Ashburton)	29°40'33.254" 30°30'42.987"	787	Dry savannah/ roadside	738	18.4
Cedara (Hilton)	29°32'22.128" 30°16'05.732"	1068	Grassland/ paddock	885	16.3
Groundcover (Curry's Post)	29°23'19.046" 30°10'31.065"	1280	Grassland/ paddock	985	15.8

* Data provided by F.J. Mitchell and R.D. Chapman (Department of Natural Resources, KwaZulu-Natal Department of Agriculture and Rural Development).

Three additional plants were uprooted each month at each of the four field sites. Following the removal of all floral material, these plants were placed into containers of water and maintained for two months in rearing cages in the laboratory. All adult herbivores that emerged from the stems were recorded and either pinned to create a reference collection or preserved in 100% ethanol for genetic sequencing. Voucher specimens are lodged with the KwaZulu-Natal Museum in Pietermaritzburg (Coleoptera and Diptera) and the Durban Natural Science Museum (Lepidoptera). Where possible, sequenced adult specimens, notably Coleoptera

(Curculionidae) and Lepidoptera, were matched to larval specimens for species separation. Specimens of Diptera were not considered further due to their lower priority as candidate agents for Australia (see Egli et al., 2020).

On each sampling occasion, sweep netting was also conducted for the collection of adult specimens that could be matched with larvae recorded in the stems. Ten sweep net (42 cm diameter) samples were collected at each site every month. A single sweep net sample involved sweeping 10 plants, with three sweeps per plant, ensuring that a total of 100 plants were swept at each site every month. The contents of each sweep net sample were placed into plastic Ziploc^{TM} bags, which were then frozen for later inspection. The sweep net samples were then inspected for adult insects under a dissecting microscope. All adults of Curculionidae and Lepidoptera were recorded and placed into 1.5 ml Eppendorf tubes containing 100% ethanol for genetic sequencing and matching with stem-boring larvae.

DNA barcoding

Photographs of all larval and adult specimens to be sequenced were taken at the Microscopy and Microanalysis Unit (MMU) at UKZN. The DNA from the lepidopteran and Curculionidae samples was extracted using the Zymo Tissue and Insect DNA miniprep extraction kit (Zymo Research, Irvine, CA, USA) following the manual; except for the final step where 60 µl DNA elution buffer was added to the ZymoSpinTM IIC column instead of 100 µl. Thereafter, the column was centrifuged at 10 000 g for 30 seconds. The 60 µl DNA from the 1.5 ml microcentrifuge tube was then pipetted back into the ZymoSpinTM IIC column and centrifuged again. This was done to maximize DNA yield.

For lepidopteran samples, the Cytochrome Oxidase I (COI) gene region was amplified using the primers LepF (5′-ATTCAAATCATAAAGATAT-3′) and LepR (5′-TAAACTTCTGGATGTCCAAAAA-3′). Each 13.5 µl Polymerase Chain Reaction (PCR) consisted of 1.75 µl sterilized distilled water, 1 µl $MgCl_2$, 1 µl BSA, 0.25 µl of each primer, 6.25 µl OneTaq 2x master mix with standard buffer (New England Biolabs, Ipswich. Massachusetts, USA) and 3 µl of sample DNA. For PCR amplification the following conditions were used: initial denaturation for 1 minute at 94°C, 15 cycles of [denaturation for 1 minute at 94°C, annealing for 1 minute 30 seconds at 48°C, extension for 1 minute 15 seconds at 68°C], 36 cycles of [denaturation for 1 minute at 94°C, annealing for 1 minute 30 seconds at 53°C, extension for 1 minute 15 seconds at 68°C] and final extension for 5 minutes at 68°C.

For curculionid samples, the COI gene region was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). Each 12.5 µl PCR consisted of 1.75 µl sterilized distilled water, 1 µl $MgCl_2$, 1 µl BSA, 0.25 µl of each primer, 6.25 µl OneTaq Quick load master mix and 2 µl of sample DNA. For PCR amplification the following conditions were used: initial denaturation for 3 minutes at 94°C, 10 cycles of [denaturation for 30 seconds at 94°C, annealing for 45 seconds at 46°C, extension for 1 minute 25 seconds at 68°C], 25 cycles of [denaturation for 30 seconds at 94°C, annealing for 45 seconds at 59°C, extension for 1 minute 25 seconds at 68°C] and final extension for 10 minutes at 68°C. No-template negative controls were included to check for contamination of reagents.

PCR amplicons were sent to the Central Analytical Facility at Stellenbosch University, South Africa for Sanger sequencing using the BigDye Terminator V3.1 sequencing kit and ABI3730xl machine (Applied Biosystems). All electropherograms were checked using BioEdit 7.0.5 (Hall, 2005). Poor quality end and primer binding sites were trimmed. Sequences were converted to amino acids to check for the presence of stop codons. All sequences were BLASTed against NCBI GenBank (www.ncbi.nlm.nih.gov/genbank) and the Barcode of Life Database (BOLD, www.barcodeoflife.org). Specimens were allocated to putative species if they had sequence similarity values between 95% and 100% (Govender and Willows-Munro, 2019). COI sequences for the weevil (accession numbers: MT259813-MT259827; MT259829-MT259832; MT259854; MT259876-MT259903; MT259905-MT259919) and lepidopteran specimens (accession numbers: MT277493-MT277516; MT 277525-MT277527; MT277529-MT277533; MT277535-MT277540; MT277542-MT277575) were uploaded onto GenBank. The sequence data were aligned in BioEdit 7.0.5 (Hall, 2005). Specimens were also assigned to species-level operational taxonomic units (OTUs) using a phylogenetic approach as specimens belonging to the same species were expected to cluster together in the phylogenetic trees (Hebert et al. 2003). The use of molecular OTUs, which represent genetically close clusters, can facilitate native range surveys of biocontrol agents where morphological identifications are lacking (see Nawaz et al., 2021). The Akaike Information Criterion (AIC) implemented in jModelTest 2 (Darriba et al., 2012) was used to determine the best-fit nucleotide substitution model for each alignment. For both the Lepidoptera and Curculionidae datasets, the best-fit model was the General Time Reversible model (GTR) with gamma distribution (G) and proportion of invariable sites (I).

Maximum likelihood trees were estimated using RAxML 8.0 (Stamatakis, 2014). Branch support was assessed using 1000 bootstrap replicates. A consensus tree was inferred from the bootstrap replicates using the CONSENSE module of Phylip 3.69 (Felsenstein, 2005). Bayesian inference was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Two analyses were run simultaneously, each consisting of four Markov chains run for 20 million generations. Trees were sampled every 500 generations. Convergence was determined in Tracer 1.5 (Rambaut et al., 2018) when Effective Sampling Size (ESS) values were above 200 for all parameters (Drummond et al. 2006). The first 20% of trees were removed as burn-in and 50% majority rule consensus trees were created in Phylip. All trees were midpoint rooted in Figtree 1.3.1 (Rambaut, 2009).

Statistical analysis

Variation in the abundance of stem-boring larvae across months and between sites was analysed using IBM SPSS version 27.0. Since the high prevalence of zero counts precluded the use of either parametric tests or generalized linear modelling, non-parametric Kruskal-Wallis tests, followed by Mann-Whitney U-tests for pairwise comparisons (Zar, 1999), were used as a last resort.

Results

Distribution of stem-boring larvae across sites

Stem-boring larvae associated with *S. madagascariensis* (n = 132) were comprised of Coleoptera (Curculionidae), Diptera, and Lepidoptera. Summed across all sites, 41.7% comprised Diptera (n = 55), while 39.4% comprised Curculionidae (n = 52) and 18.9% comprised Lepidoptera (n = 25). The larval load in the stems varied significantly between the four sites ($H = 14.849$; $df = 3$; $P = 0.002$), with plants at the Raptor Centre site supporting 61.4% of the larvae (Figure 1). A total of 81 larval specimens (36 Curculionidae, 26 Diptera, and 19 Lepidoptera) were collected at the Raptor Centre site throughout the year (Figure 1b). The Ukulinga and Cedara sites supported a similar herbivore load as one another, with 24 larvae recorded at each site. However, the composition of taxa varied between Ukulinga (15 Curculionidae and nine Diptera; Figure 1a) and Cedara (18 Diptera, five Lepidoptera and one Curculionidae; Figure 1c). The Groundcover site displayed the lowest herbivore load with only three larvae (two Diptera and one Lepidoptera) recorded throughout the year (Figure 1d).

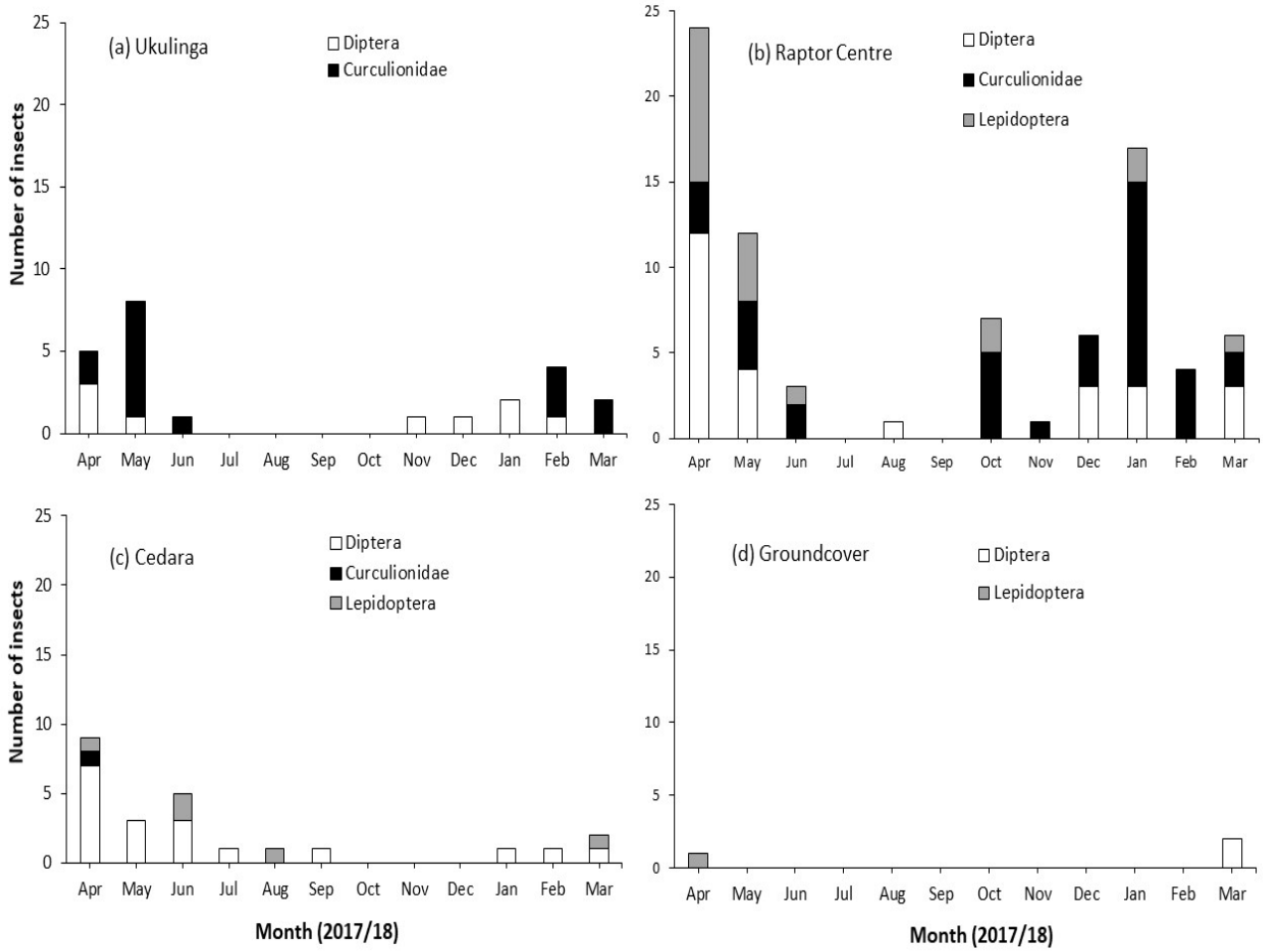

Figure 1. Total numbers of insect larvae recorded monthly in the stems of five *Senecio madagascariensis* plants during 2017/18 at lower altitude (a-b) and higher altitude (c-d) sites in the KwaZulu-Natal Midlands, South Africa.

Species delimitation with DNA COI barcoding

The COI gene region was successfully sequenced from 63 curculionid specimens (44 larvae and 19 adults) and 72 lepidopteran specimens (24 larvae and 48 adults). All sequences were barcode-compliant (>500 bp of length, with no stop codons, misidentifications, or contamination). The final alignment for Curculionidae and Lepidoptera was 618 bp (367 variable characters) and 626 bp (306 variable characters) in length, respectively. Maximum likelihood and Bayesian phylogenies were similar with no incidents of conflict. Bootstrap values and posterior probability values were annotated onto the most likely phylogeny produced by RAxML for the Curculionidae (Figure 2) and Lepidoptera (Figure 3).

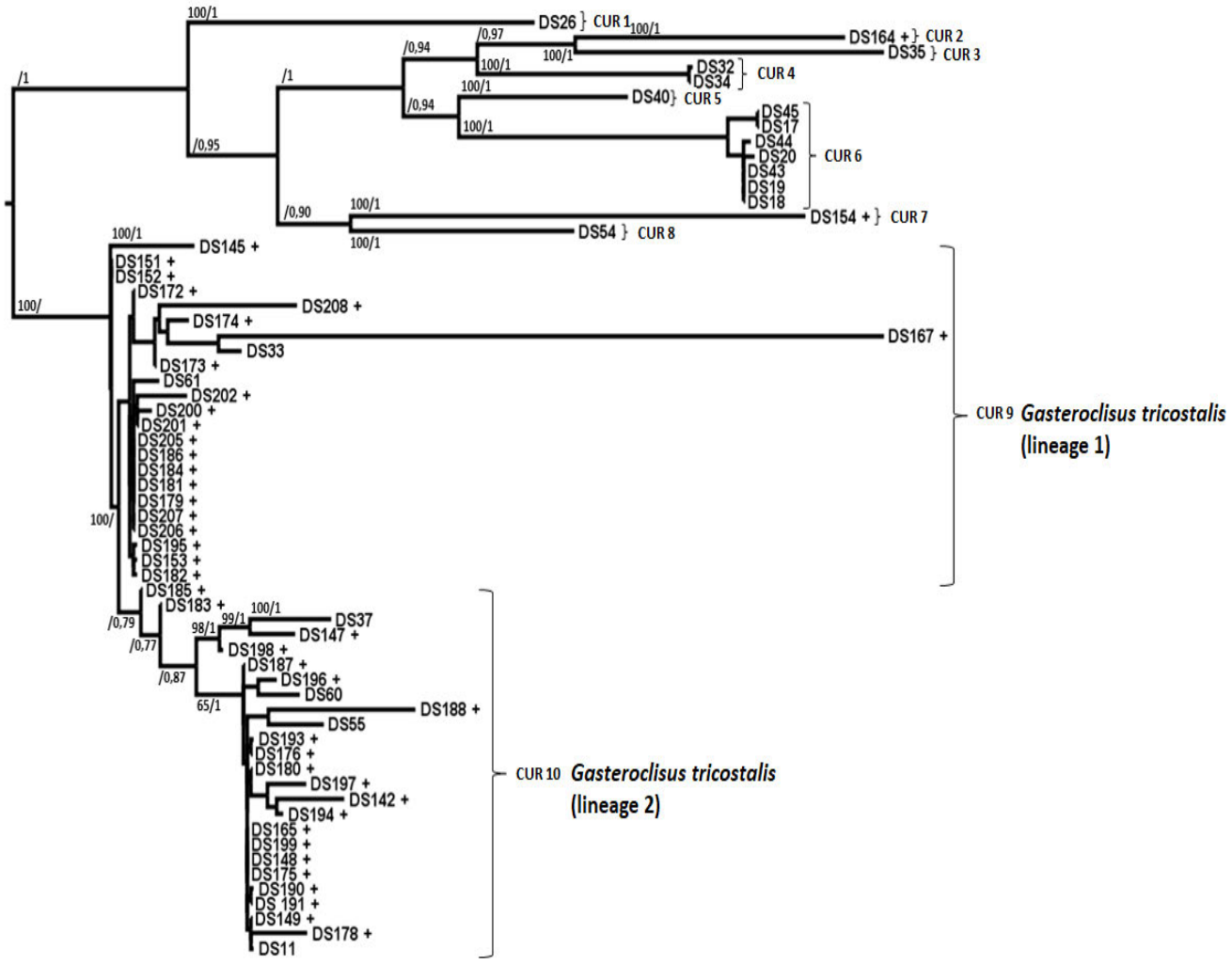

Figure 2. Mid-point rooted maximum likelihood COI phylogeny for Curculionidae associated with *Senecio madagascariensis* (+ denotes larval specimens). Values on branches indicate bootstrap values and posterior probability values. Only bootstrap values ≥ 65% and posterior probability ≥ 0.65 are shown. Different operational taxonomic units (CUR) are indicated.

Adult weevils and stem-boring weevil larvae recorded on *S. madagascariensis* (Figure 2) comprised 10 OTUs, with six represented by adults only (CUR 1, 3, 4, 5, 6, 8), two by larvae only (CUR 2, 7) and two by both adults and larvae (CUR 9, 10). Most of the COI sequences were assigned to two maternal lineages of *G. tricostalis* (CUR 9, 10), while the remaining OTUs remained unidentified.

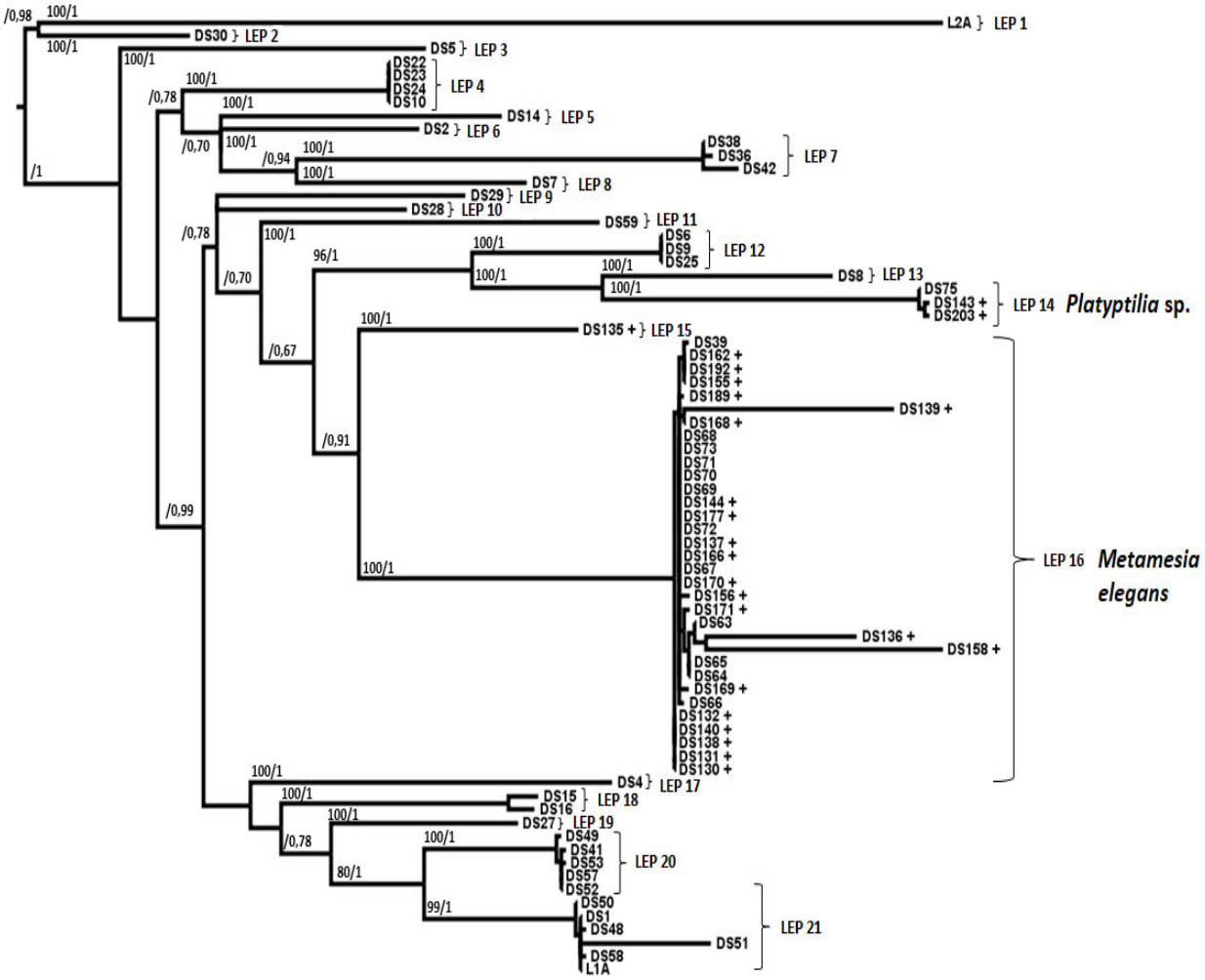

Figure 3. Mid-point rooted maximum likelihood COI phylogeny for Lepidoptera associated with *Senecio madagascariensis* (+ denotes larval specimens). Values on branches indicate bootstrap values and posterior probability values. Only bootstrap values ≥ 65% and posterior probability ≥ 0.65 are shown. Different operational taxonomic units (LEP) are indicated.

Adult lepidopterans and stem-boring lepidopteran larvae recorded on *S. madagascariensis* (Figure 3) comprised 21 OTUs, with 18 represented by adults only, one by larvae only (LEP 15) and two by both adults and larvae (LEP 14, 16). While the presence of adult lepidopterans was deemed of lesser importance, since these may well comprise casual associations, the presence of larvae was indicative of host utilization. Twenty one larval COI sequences matched to *M. elegans* (LEP 16), with two sequences belonging to an unidentified species of *Platyptilia* Hübner (Pterophoridae; LEP 14) and one sequence to an unknown species of Lepidoptera (LEP 15).

Adult stem borers reared across sites

Twelve adult Curculionidae, which matched to *G. tricostalis*, were reared from the stems of *S. madagascariensis* plants that were collected at two sites, namely Raptor Centre (10) and Ukulinga (two) (Figure 4). Twenty-six adult Lepidoptera comprising largely Tortricidae (22 specimens) but also Pterophoridae (three) and Crambidae (one) were reared from plants collected at three sites, namely Raptor Centre (17 specimens), Cedara (five) and Groundcover (four).

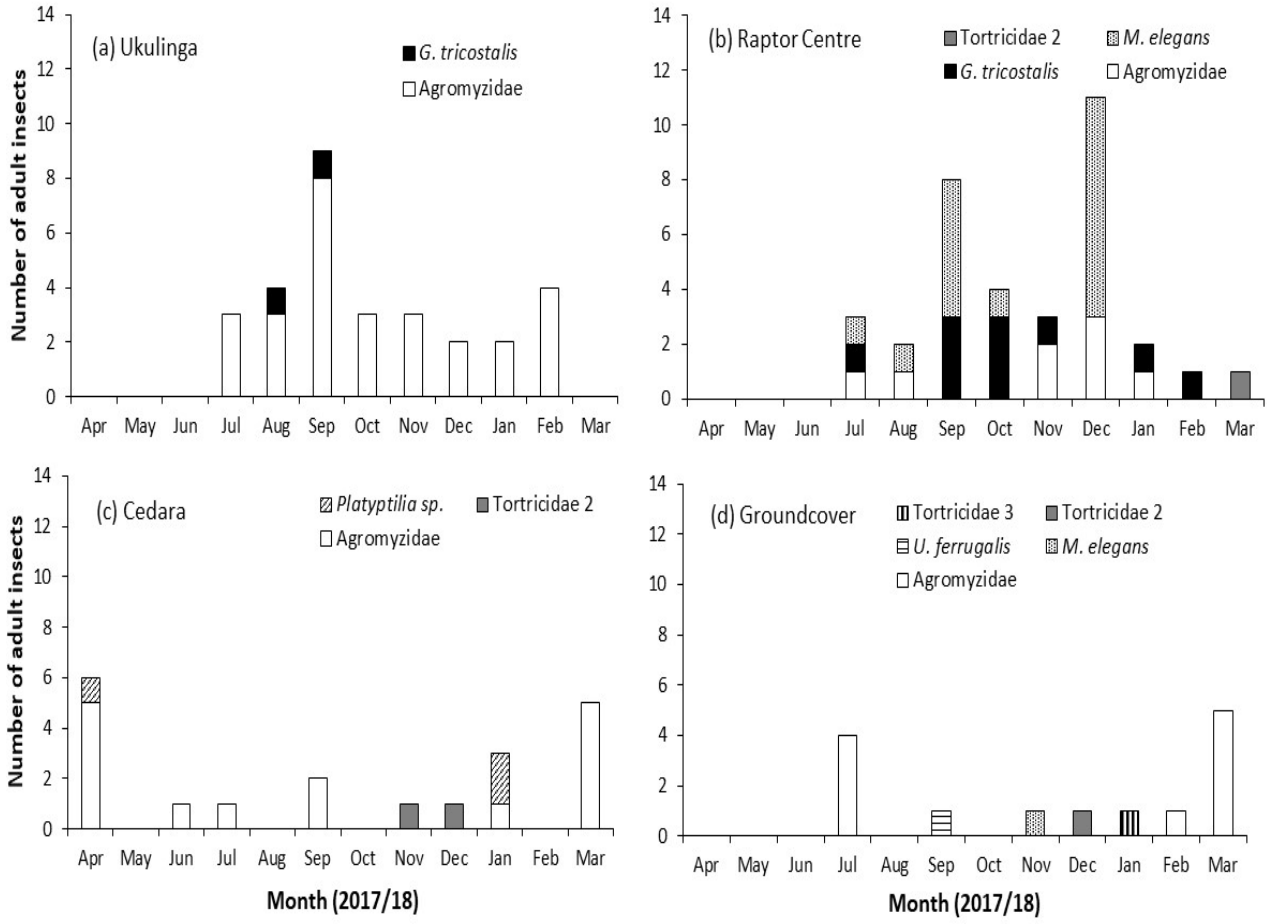

Figure 4. Total numbers of adult insects reared monthly from the stems of three *Senecio madagascariensis* plants during 2017/18 at lower altitude (a-b) and higher altitude (c-d) sites in the KwaZulu-Natal Midlands, South Africa.

Of the three tortricid species reared, most were ascribed to *M. elegans*, with 17 specimens from two sites (Raptor Centre and Groundcover). Four tortricid specimens from three sites (Raptor Centre, Cedara and Groundcover) were ascribed to Tortricidae species 2. A single specimen each of Tortricidae species 3 and *Udea ferrugalis* (Hübner) (Crambidae: Spilomelinae) was recovered from one site only (Groundcover) (Figure 4). The pterophorid

specimens, which conformed to the unidentified species of *Platyptilia*, were recovered from a single site (Cedara).

Sixty-one adult Diptera, comprising unidentified Agromyzidae were reared from plants collected at all four sites (Figure 4). The agromyzids comprised 93% of the adults reared from the Ukulinga material, compared to 75% from Cedara, 71% from Groundcover and 23% from Raptor Centre (Figure 4). However, since none of the dipterans recorded in this study were considered as candidate agents for Australia, due to broad host ranges (see below), they were not considered in further analyses.

Abundance of Curculionidae across sites and months

There were significant differences in the numbers of weevil larvae recorded in *S. madagascariensis* stems between sites ($H = 20.469$; $df = 3$; $P < 0.0001$) but not months ($H = 10.563$; $df = 11$; $P = 0.481$). Across all four sites, a total of 51 weevil larvae were recorded throughout the year with peaks in May 2017 (19.6% of specimens) and January 2018 (23.5%) (Figure 5a). No weevil larvae were recorded during July to September 2017 at any of the sites (Figure 5a). During the remainder of the months sampled, larval numbers remained low.

Of the 51 larvae collected, 44 (86.3%) were successfully sequenced and comprised four lineages (CUR 2, 7, 9 and 10) with the remaining seven larvae not successfully sequenced and treated as unknown species. CUR 9 and 10, which conformed to two maternal lineages of *G. tricostalis*, were the dominant lineages (42 specimens) and were matched with adults that were reared from stems and collected by sweep netting (Figure 2). The two *G. tricostalis* lineages were each represented by 21 specimens that together were collected during eight of the 12 months sampled (Figure 5a). CUR 2 and 7 were each represented by single specimens collected in June and May 2017, respectively (Figure 5a) and were not matched with adult weevils that were collected by sweep netting (Figure 2). The seven specimens that could not be sequenced were collected in April and May 2017.

Thirty-five weevil larvae were collected at the Raptor Centre (68.6% of specimens) followed by Ukulinga (15 larvae; 29.4%), with only one larva recorded at Cedara (2.0%) and none at Groundcover (Figure 5c). Consequently, there were significantly higher numbers of weevil larvae at Raptor Centre than at Cedara ($P < 0.0001$) and Groundcover ($P < 0.0001$), but not Ukulinga ($P = 0.102$), while Ukulinga supported significantly higher numbers than Groundcover ($P = 0.012$). Larvae of *G. tricostalis* lineage 1 were recovered at three sites, with those of lineage 2 recovered at two sites (Figure 5a). Larvae of CUR 2 and 7 were recovered

at only one site each, while larvae that could not be sequenced originated from two sites (Figure 5c).

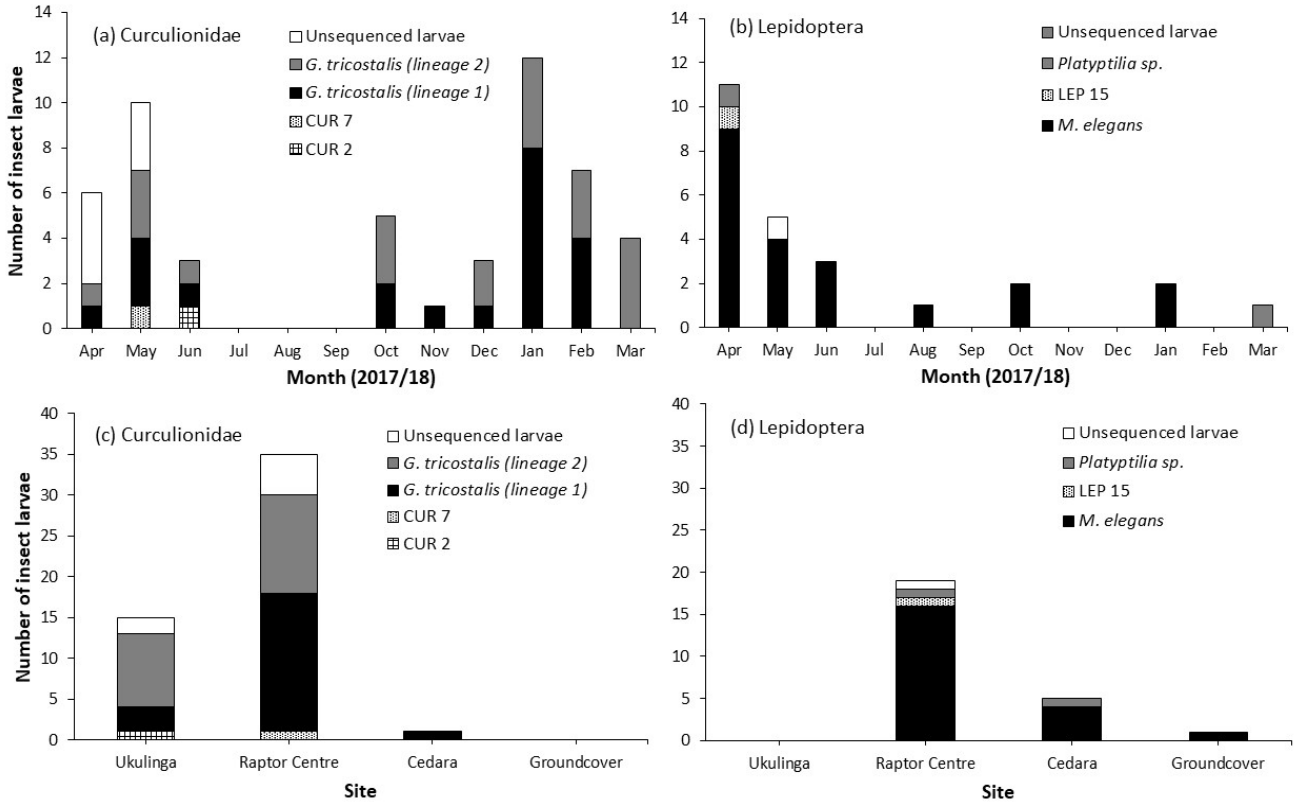

Figure 5. Total numbers of larvae of species of Curculionidae (a, c) and Lepidoptera (b, d) in the stems of *Senecio madagascariensis* sampled at sites in the KwaZulu-Natal Midlands, South Africa during 2017/18. Abundance is represented by month across all sites (a, b) and by site across all months (c, d).

The sweep net samples recovered a total of 25 adult weevils on the foliage of *S. madagascariensis* plants, of which 19 were successfully sequenced and comprised eight different lineages. Six adult specimens were matched with stem-boring larvae (Figure 2) and conformed to the two lineages of *G. tricostalis*. The remaining six lineages are considered to be casual associates, particularly since four lineages were represented by single specimens (Figure 2). Single adult specimens of *G. tricostalis* lineage 1 were collected in April and November 2017 and February and March 2018, while single specimens of *G. tricostalis* lineage 2 were collected in November 2017 and March 2018. Adults of *G. tricostalis* lineage 1 were collected at three sites (Raptor Centre, Ukulinga and Cedara) while those of lineage 2 were all collected at Raptor Centre.

Abundance of Lepidoptera across sites and months

There were significant differences in the numbers of lepidopteran larvae recorded in *S. madagascariensis* stems between sites (H = 11.285; df = 3; P = 0.010) but not months (H = 14.360; df = 11; P = 0.214). Across all four sites, a total of 25 lepidopteran larvae were recorded throughout the year, with a single peak in April 2017 (11 specimens; 44%) followed by fewer recoveries in May 2017 (five specimens; 20%) (Figure 5b). During the remainder of the months sampled, larval numbers remained low with none recorded in five months.

Of the 25 lepidopteran larvae collected, 24 were successfully sequenced and comprised three lineages (LEP 14, 15 and 16) with the remaining larva that could not be sequenced belonging to an unknown species. LEP 16, which matched to *M. elegans*, was clearly the dominant lineage (20 specimens) and was linked with adults that were reared from stems and collected by sweep netting (Figure 3). Larvae of *M. elegans* were recorded across six months of the year (Figure 5b). In contrast, LEP 15 was only recorded once (April 2017), while LEP 14, which conformed to the unidentified species of *Platyptilia*, was recorded twice (April 2017 and March 2018). The unknown specimen was collected in May 2017.

Nineteen lepidopteran larvae were collected at the Raptor Centre (76% of specimens) followed by Cedara (five specimens; 20%), with only one specimen recorded at Groundcover (4.0%) and none at Ukulinga (Figure 5b). There were significantly higher numbers of lepidopteran larvae at Raptor Centre than at Ukulinga (P = 0.003) and Groundcover (P = 0.010), but not Cedara (P = 0.222). Larvae of *M. elegans* were recovered at three of the four sites, while those of LEP 15 and *Platyptilia* sp. were recovered at one and two sites, respectively (Figure 5d).

The sweep net samples recovered a total of 39 adult lepidopterans on the foliage of *S. madagascariensis* plants, of which 36 (92.3%) were successfully sequenced and comprised 21 different lineages. Of these, only one adult specimen was matched with stem-boring larvae and BLASTed as *M. elegans*. The remaining 20 lineages were considered as casual associates, particularly since adult Lepidoptera are not typically herbivorous. The single adult specimen of *M. elegans* was collected in December 2017 at Raptor Centre.

Discussion

Senecio madagascariensis is a highly aggressive invader in several countries and a major problem in the rangelands of south-eastern Australia. Since earlier testing of candidate insect

agents from Madagascar revealed none that were suitable for release in Australia (McFadyen and Morin, 2012), the sourcing of new agents has shifted to the plant's native range in South Africa. Previous surveys in KwaZulu-Natal Province (Egli and Olckers, 2015, 2020) revealed several stem-boring taxa of interest. The results of our current 12-month survey confirmed that the assemblage of stem-boring taxa hosted by *S. madagascariensis* populations in the KwaZulu-Natal Midlands region comprised coleopteran, dipteran and lepidopteran larvae. Dipteran larvae were most frequently encountered, followed by curculionid larvae and lastly lepidopteran larvae. The overall herbivore load within *S. madagascariensis* stems (i.e., all taxa combined) varied significantly between sites. Plants at the Raptor Centre site displayed the highest herbivore load, with insect larvae recovered in most months of the year, while those at the Groundcover site were largely devoid of larvae.

Weed biocontrol agents can be highly effective when they are abundant throughout the year, thereby inflicting sustained damage on their host plants (Bellows and Fisher, 1999; Van Driesche et al., 2008). However, insect abundance is typically seasonal (Wolda, 1978, 1988; Pinheiro et al., 2002), since the climatic parameters (e.g. temperature, rainfall and humidity) that influence the developmental biology of insects and their host plants (Gilbert and Raworth, 1996; Jaworski and Hilszczański, 2013) are affected by changing seasons (Stine and Huybers, 2012; Du Plessis and Schloms, 2017). Although monthly differences were not significant, we detected a seasonal pattern in stem-boring insect activity, with peaks in the austral summer (January) and autumn (April-May) and very little/no activity in winter (July-August) and early spring (September). In particular, both the weevil *G. tricostalis* and tortricid moth *M. elagans* exhibited seasonal differences in abundance, albeit non-significant. The lack of significant differences was likely an effect of low/no monthly larval counts at the four sites, which is typical of herbivorous insects in their native range (e.g. Zuma et al., 2021). Also, the severe 2015/16 drought experienced in KwaZulu-Natal, which continued into 2017/18, may well have reduced stem borer numbers, since drought disrupts the growth, phenology and food quality of plants (Mattson and Haack, 1987; Rouault et al., 2006; Barnabas et al., 2008; Farooq et al., 2009).

Adult insects reared from the stems of *S. madagascariensis* comprised seven distinct taxa, with *G. tricostalis* and *M. elegans* the most abundant. Other lepidopteran taxa included two unidentified species of Tortricidae, *U. ferrugalis* (Crambidae) and an unidentified species of *Platyptilia* (Pterophoridae). *Platyptilia* sp. has also been reared from the plant’s capitula (Egli and Olckers, 2020). Diptera comprised an unidentified species of Agromyzidae, but was

excluded from DNA barcoding because of low biocontrol potential for Australia. In particular, the Agromyzidae were previously recovered from the stems of several non-target *Senecio* species in the field, rendering them unsuitable for countries with a diverse native *Senecio* flora (Egli et al., 2020). Sequencing of the weevil and lepidopteran larvae that were collected across the 12 months revealed that *G. tricostalis* and *M. elagans* were the most abundant species and confirmed their status as the top stem-boring candidates (Egli and Olckers, 2020). Other lineages of stem-boring weevils or lepidopterans that were either recovered as larvae, or reared from the stems, were collected infrequently and may indicate species that utilize *S. madagascariensis* as an occasional host.

Collectively, the two COI lineages of *G. tricostalis* were present across nine of the 12 months sampled and displayed two peaks in larval abundance, notably in May (autumn) and January (mid-summer). Although no larvae were recovered from July to September (late winter to early spring), adults were reared from plants collected during these months, suggesting larval presence, but very low abundance, during winter. In particular, the frozen plants that were dissected for larvae may have contained weevil eggs, resulting in an underestimation of larval abundance. The presence and abundance of *G. tricostalis* varied significantly between sites, with most larvae recovered at the two lower altitude sites (Raptor Centre and Ukulinga), where conditions are typically hotter and drier (Table 1).

The tortricid moth *M. elegans* was detected across six of the 12 months and displayed a single peak in larval abundance in April (mid-autumn), followed by a winter decline and low numbers during the rest of the year. Adults of *M. elegans* were also reared from plants that were collected during months when no larvae were recovered, similarly suggesting higher actual numbers (see above). These discrepancies indicate that seasonal abundance studies such as this can be bolstered by a combination of data from larval dissections and adult emergence, since the latter can indicate false-negative detections that arise through direct dissection only. The defoliating moth *Secusio extensa* (Butler) (Erebidae) that was released against *S. madagascariensis* in Hawaii also displayed a single peak in abundance, albeit in early spring (March in Hawaii), with a decline in abundance during the rest of the year (Krushelnycky et al., 2018). The numbers of *M. elegans* larvae recorded were also significantly influenced by site location. Lepidopteran larvae, which were dominated by *M. elegans*, were more abundant at the Raptor Centre site, from which 76% of the specimens were detected. No lepidopteran larvae were recovered, and no adults were reared, from material collected at Ukulinga, despite

recoveries of stem-boring Lepidoptera at this site in 2011 (Egli and Olckers, 2015). This may also have been a consequence of the drought conditions experienced in the current study.

In conclusion, the results of this study revealed strong seasonal and spatial variation in the abundance of the most promising stem-boring candidates, with two distinct peaks in larval activity (autumn and mid-summer) for *G. tricostalis* and one for *M. elagans* (autumn). Our study also revealed a higher abundance of these candidate agents at the warmer and drier sites sampled, despite prevailing drought conditions. This was in stark contrast to the situation with the root-feeding *L. basutoensis*, where the Cedara and Groundcover sites, with cooler temperatures and higher rainfall, had higher population abundances (Zuma et al. 2021). Our study also highlights the invaluable role of DNA COI barcoding in identifying the larvae of candidate biocontrol taxa and linking them with the adult stages that were either reared from plant material or collected in the sweep net samples (see Gaskin et al., 2011). The higher seasonal abundance of *G. tricostalis*, together with the good track record of weevils in weed biocontrol (Herrick and Kok, 2010; Winston et al., 2014), suggest that it should take precedence in subsequent laboratory assessments of host specificity. In addition, the impact of these candidate agents on the growth and reproduction of *S. madagascariensis* needs to be quantified. Indeed, earlier field observations in South Africa suggested that both species may have limited biocontrol potential, since they feed on the pith inside the stems and appear to inflict limited damage (Marohasy, 1991). However, our observations concur with those of Hawaiian scientists, which suggested that damage by *G. tricostalis* is substantially higher, with signs of stem breakage and general weakening of the plants, albeit with no evidence of plant mortality (M. Ramadan, Hawaii Department of Agriculture, pers. comm., 2020).

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Chapter 3: Host-plant affinities of stem-boring weevils and moths associated with fireweed (*Senecio madagascariensis*) in South Africa: evaluating native host ranges of candidate biocontrol agents using DNA barcoding

ABSTRACT

Native to South Africa and Madagascar, the invasive fireweed, *Senecio madagascariensis* Poiret (Asteraceae), is a target for biological control in Australia and Hawaii, where substantial infestations persist. Earlier studies in the weed's region of origin, namely KwaZulu-Natal Province in South Africa, assessed the seasonal abundance of stem-boring insect herbivores in *S. madagascariensis* populations. The weevil *Gasteroclisus tricostalis* (Thunberg) (Curculionidae) and moth *Metamesia elegans* (Walsingham) (Tortricidae) were prioritized as candidate agents for Australia due to their persistence across seasons. In this study, the host ranges of these two candidate agents were assessed by comparing the stem-boring curculionids and lepidopterans that are associated with *S. madagascariensis* with those associated with non-target *Senecio* species in the KwaZulu-Natal Midlands region. DNA barcoding of the adults and endophagous larvae recovered during field sampling of *S. madagascariensis* and 17 non-target *Senecio* species in 2017/18, as well as those from earlier field surveys, was used to differentiate the insect species and elucidate their host-plant affinities. Although weevil larvae were recovered from the stems of seven non-target *Senecio* species, *G. tricostalis* was associated only with *Senecio inaequidens* DC. and *Senecio skirrhodon* DC., both in the *S. madagascariensis* species complex. Lepidopteran larvae were associated with three non-target *Senecio* species, all in the *S. madagascariensis* species complex, with *M. elegans* associated only with *Senecio harveianus* MacOwan and *S. inaequidens*. These results suggest that *G. tricostalis* and *M. elegans* have restricted host ranges and that further host-specificity studies in quarantine are warranted to demonstrate their suitability for release.

KEYWORDS: Agent host range, cytochrome oxidase I gene sequencing, stem borers, weed biocontrol

Introduction

Besides standard host-specificity tests, studies in the target plant's native range that include surveys of congeneric plants can provide considerable insight into a candidate agent's host range and thus facilitate the prioritization of promising species for more intensive testing, while eliminating polyphagous species at an early stage (Van Driesche et al., 2008; Sutton et al.,

2021). Also, laboratory host-range tests are typically conservative and may overestimate an insect's host range, while field-based host-range assessments often provide a more realistic indication of its ecologically realised host range (e.g. McConnachie and McKay, 2015). For example, *Bagous hydrillae* O'Brien (Coleoptera: Curculionidae), a stem-boring weevil deployed against *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae) in the USA, displayed a broad host range during laboratory trials, but a considerably narrower one in its native Australian range (Balciunas et al., 1996). Besides native-range surveys, DNA barcoding has become an increasingly valuable tool in weed biocontrol (Gaskin et al., 2011), by identifying unknown or cryptic species when morphological characteristics are limited (Hebert et al., 2003, 2004; Hebert and Gregory, 2005; Hajibabaei et al., 2006; Valentini et al., 2009; Krishnamurthy and Francis, 2012), matching adults with immature stages (Ball and Armstrong, 2006; Alessandrini et al., 2008) and determining the phylogenetic relationships between species (Hebert et al., 2003; Van Driesche et al., 2008; Sutton et al., 2021).

The invasive fireweed *Senecio madagascariensis* Poiret (Asteraceae), considered native to South Africa and Madagascar, is currently a target for biological control in Australia and Hawaii, where severe invasions continue to degrade pastures, poison animals and contaminate agricultural products (Ramadan et al., 2011; McFadyen and Morin, 2012; Olckers et al., 2021; Wijayabandara et al., 2022). Earlier investigations have shown that the plants in both countries likely originated from KwaZulu-Natal Province in South Africa and not Madagascar as originally thought (Scott et al., 1998; Le Roux et al., 2006; Sheppard et al., 2013). Hence, investigations towards the sourcing of candidate agents shifted from Madagascar to South Africa (Olckers et al., 2021). Studies in KwaZulu-Natal revealed several potential insect agents that included root feeders, capitulum borers, and stem borers (Egli and Olckers, 2015; 2020). The flea beetle *Longitarsus basutoensis* Bechyné (Chrysomelidae) was deemed to be the most promising of these. However, field assessments of its host-plant associations and laboratory host-specificity tests revealed that its host range was unacceptably broad (Zuma et al., 2021) and the flea beetle was thus rejected for importation into Australia. This country hosts a diverse native *Senecio* flora and thus requires insects with very narrow host ranges for them to be considered as potential agents (Sheppard et al., 2013). Stem-boring insects (Egli and Olckers, 2020) were subsequently prioritized for further investigation.

Investigations into the seasonal abundance of stem-boring insects confirmed that *Gasteroclisus tricostalis* (Thunberg) (Coleoptera: Curculionidae) and *Metamesia elegans* (Walsingham) (Lepidoptera: Tortricidae) were the most commonly encountered, and hence

most promising, candidates for the biocontrol of *S. madagascariensis* (Singh et al., 2022; Chapter 2). Endophagous insect species are more likely to be host specific than their ectophagous counterparts (Hattendorf et al., 2006; Harvey et al., 2015) and are thus often favoured as biocontrol agents worldwide (Winston et al., 2014). In particular, several stem-boring agents have been deployed against invasive Asteraceae (Egli and Olckers, 2017).

The aim of this study was to provide field-based evidence of the likely host specificity of potential agents to justify their importation into Australia, but also other invaded countries that intend to implement biocontrol, for further evaluation. Following field surveys, we used DNA barcoding to assess the host-plant affinities of stem-boring Curculionidae and Lepidoptera across non-target *Senecio* species that are native to the KwaZulu-Natal Midlands. We compared the endophagous larvae recovered from these non-target species with those commonly associated with *S. madagascariensis* (see Chapter 2). Our analysis included data sets (i.e. DNA sequences) that were compiled across multiple years, seasons and sites (see Egli et al., 2020; Singh et al., 2022) to improve the accuracy and robustness of the host-range assessments of these insect taxa, and also resolve uncertainties around taxonomic circumscription.

Materials and methods

Field surveys

Sampling was carried out during December 2017 to April 2018, mostly during summer (December – March), when many native *Senecio* species were flowering and could more easily be identified. Thirty collections involving 17 non-target *Senecio* species were carried out at various locations around KwaZulu-Natal that supported plant populations with sufficient individuals to permit sampling (Supplementary Table 1). *Senecio harveianus* MacOwan, *Senecio inaequidens* DC. and *Senecio skirrhodon* DC. are the closest relatives to *S. madagascariensis*, as they belong to the *S. madagascariensis* species complex (see Hilliard, 1977). The plants were identified using the taxonomic key of Hilliard (1977) and herbarium specimens in the University of KwaZulu-Natal John Bews Herbarium (NU). Voucher specimens were lodged in the John Bews Herbarium.

Sampling procedure

During each of the 30 collections that targeted a single non-target species (Supplementary Table 1), five whole plants were sampled from the respective *Senecio* population. The stems

were cut at soil level and the above-ground material placed in paper bags. The samples were then transferred to the laboratory and frozen at -8 °C for preservation until later analyses. All stem-boring larvae were dissected from the stems and individually preserved in 100% ethanol in 1.5 ml Eppendorf tubes to enable DNA barcoding.

Sweep-net samples were also taken during each collection to collect adult specimens for matching with the endophagous larvae. When sufficient plants were available at each site, up to 10 samples were taken for each *Senecio* species, with one sample comprising three sweeps for each of 10 plants. The insects collected during each sample were transferred into plastic Ziploc^{TM} bags and later preserved in a freezer at -8 °C. The samples were later processed and all curculionid and lepidopteran adults were preserved for DNA barcoding as previously described.

DNA barcoding

Images of adults and larvae of Curculionidae and Lepidoptera were recorded at the Microscopy and Microanalysis Unit at the University of KwaZulu-Natal, as these are a requirement for the uploading of sequences on the relevant databases. DNA extraction was conducted using the Zymo Tissue and Insect DNA miniprep extraction kit (Zymo Research, Irvine, CA, USA). DNA extraction adopted the manufacturer's protocol until the final stage, when 60 µl DNA elution buffer (instead of 100 µl) was incorporated into the ZymoSpinTM IIC column. In order to maximize yield, after centrifuging at 10 000 g for 30 seconds, the 60 µl DNA in the 1.5 ml microcentrifuge tube was pipetted back into the column and centrifuged again.

Following the procedure outlined in Singh et al. (2022), the Cytochrome Oxidase I (COI) gene region of the curculionid and lepidopteran samples was amplified, using the same primers (i.e. LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) for the Curculionidae and LepF (5′-ATTCAAATCATAAAGATAT-3′) and LepR (5′-TAAACTTCTGGATGTCCAAAAA-3′) for the Lepidoptera). The absence of reagent contamination was verified by the inclusion of no-template negative controls. Sanger sequencing of the PCR amplicons was performed by the Central Analytical Facility (Stellenbosch University, South Africa), using the BigDye Terminator V3.1 sequencing kit and ABI3730xl machine (Applied Biosystems).

Together with sequences obtained from two earlier studies (Egli et al., 2020; Singh et al., 2022), curculionid and lepidopteran sequences obtained from the 2017-2018 samples were BLASTed against the Barcode of Life Database (BOLD) and NCBI GenBank for species

delimitation. Specimens with sequence similarity values of 95-100% were allocated to the same species (Govender and Willows-Munro, 2019). The COI sequences derived for the Curculionidae (accession numbers: MT259813-MT259945) and Lepidoptera (accession numbers: MT277493-MT277575) were uploaded onto GenBank to complement those uploaded previously. BioEdit 7.0.5 was used to align the sequence data (Hall, 2005) and a phylogenetic approach was used to assign specimens into species clusters (SCs), following the assumption that sequences from the same species will cluster together in the phylogenies (Hebert et al. 2003). The Akaike Information Criterion implemented in jModelTest 2 was used to determine the best-fit nucleotide substitution model for each alignment (Darriba et al., 2012). The best-fit model for both the curculionid and lepidopteran datasets was provided by the General Time Reversible model (GTR) with gamma distribution (G) and proportion of invariable sites (I).

RAxML 8.0 was used to estimate maximum likelihood phylogenetic trees (Stamatakis, 2014), with 1000 bootstrap replicates used to assess branch support. From these replicates, a consensus tree was inferred by using the CONSENSE module of Phylip 3.69 (Felsenstein, 2005). Bayesian inference was also conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012). Two analyses were implemented for 20 million generations, with each comprising four Markov chains and sampling of the phylogenetic trees occurring at 500-generation intervals. Convergence was determined using Tracer 1.5 (Rambaut et al., 2018), when Effective Sampling Size values exceeded 200 for all parameters (Drummond et al., 2006). Fifty percent majority-rule consensus trees were created in Phylip 3.69, after removal of the first 25% of trees as burn-in. Figtree 1.3.1 was used to midpoint-root all phylogenetic trees (Rambaut, 2009).

Results

Stem-boring Curculionidae

Of the 17 non-target *Senecio* species sampled during this survey, weevil larvae (33 specimens) were dissected from the stems of only five species, namely *S. glaberrimus* (12), *S. inaequidens* (17), *S. panduriformis* (1), *S. serratuloides* (1) and *S. skirrhodon* (2) (Table 1). Sweep net sampling recovered 43 adult weevils from the foliage of 11 non-target *Senecio* species, with most collected on *S. adnatus* (16 specimens), *S. coronatus* (8), *S. glaberrimus* (4) and *S.* sp. nr *conrathii* (4) (Table 1).

CO1 sequences were obtained from 78.8% of the larval specimens and all of the adult specimens collected on the non-target *Senecio* species (Table 1). The weevil sequences from the non-target *Senecio* species were compared to 44 larval and 20 adult sequences that arose from monthly sampling of *S. madagascariensis* populations, over a one-year period in 2017/18 (Singh et al., 2022). In addition, 57 larval and two adult sequences arising from an earlier survey (Egli et al. 2020), comprising 25 from *S. madagascariensis* and 34 from five non-target *Senecio* species, were included in the analyses (Table 1).

The topologies recovered from the maximum likelihood and Bayesian analyses were the same and the bootstrap values and posterior probabilities were incorporated into the most likely phylogeny (Figure 1). A comparison of the COI sequences of weevils associated with *S. madagascariensis* and the non-target *Senecio* species revealed 19 distinct weevil lineages or species clusters (SCs) (Figure 1). Thirteen SCs were based on adult specimens only, suggesting casual host-plant associations, with the remaining six SCs based also on larval specimens, indicating host utilization. Of the six SCs that included larval sequences, two were associated with *S. madagascariensis* (SC 15 and 19). SC 15 represents an unidentified weevil for which adults were also recorded on seven non-target *Senecio* species, and larvae mostly recorded on *S. glaberrimus*, suggesting that *S. madagascariensis* is an occasional host. In contrast, SC 19, which matched with identified adult specimens of *G. tricostalis*, was largely recovered from *S. madagascariensis*, with fewer specimens from the closely related *S. inaequidens* and *S. skirrhodon* (Figure 1).

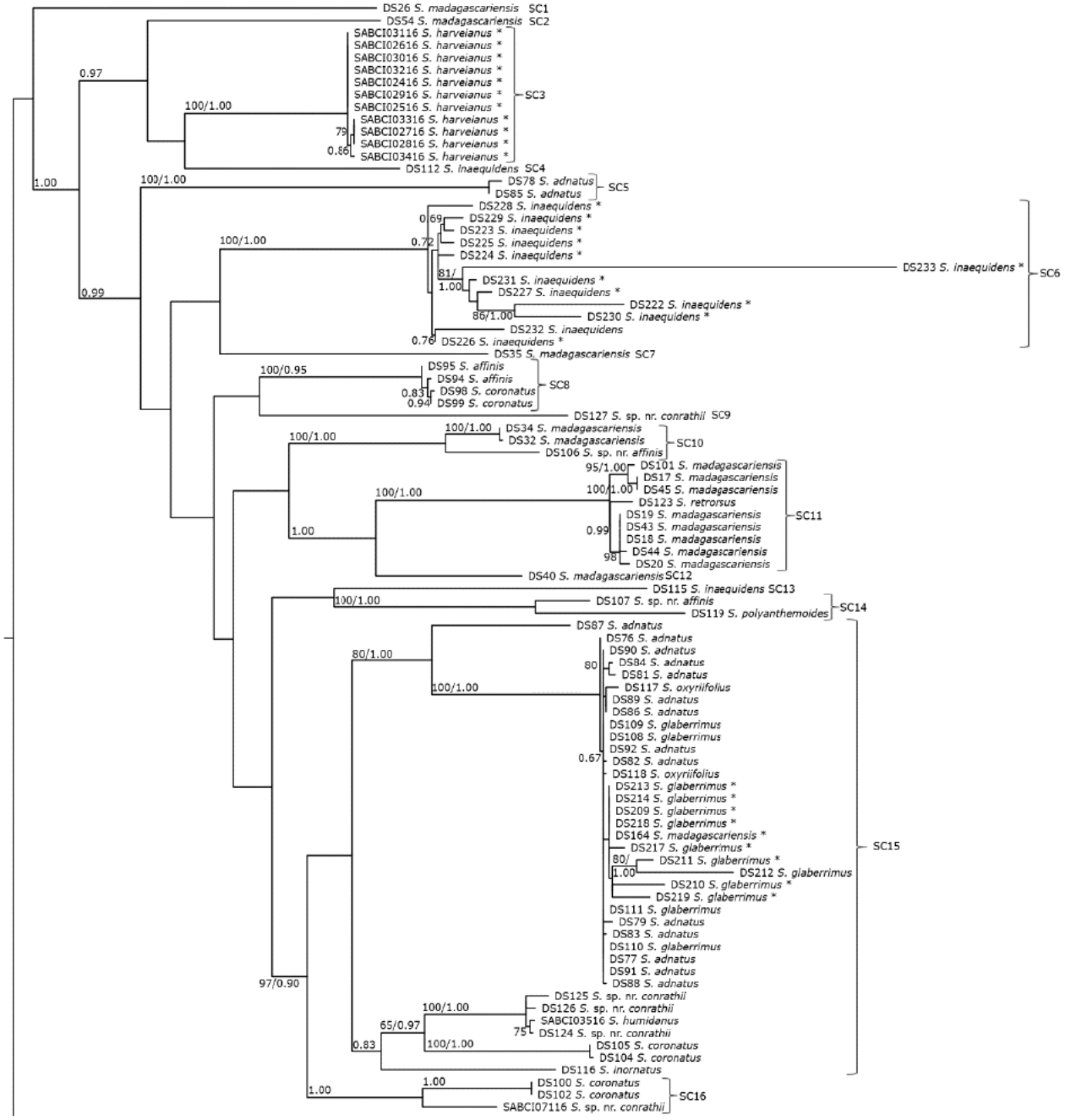
The remaining four SCs were based on larval sequences only and could not be identified. However, these specimens indicated host utilization (Figure 1), with SC 3 associated with *S. harveianus* only, SC 6 with *S. inaequidens* only, SC 17 with *S. humidanus* only, and SC 18 with *S. serratuloides* only.

Table 1. Total number of larvae and adults of stem-boring Curculionidae and Lepidoptera that were collected (Col) on *Senecio madagascariensis* and non-target *Senecio* species during 2017/18, together with subsequent DNA extractions (Ext) and COI sequences obtained (Seq). Numbers in brackets denote additional collections, extractions and sequences from an earlier survey (Egli et al., 2020) that were included in the analyses.

Senecio species	Curculionidae						Lepidoptera					
	Larvae			Adults			Larvae			Adults		
	Col	Ext	Seq	Col	Ext	Seq	Col	Ext	Seq	Col	Ext	Seq
S. adnatus				16	16	16				2	2	2
S. affinis				2	2	2						
S. bupleuroides										2	2	2
S. coronatus				8	8	8						
S. glaberrimus	12	11	9	4	4	4						
S. harveianus	(21)	(14)	(12)				(28)	(15)	(12)			
S. humidanus	(6)	(3)	(2)	(1)	(1)	(1)						
S. inaequidens	17 (11)	17 (4)	14 (2)	2	2	2	(7)	(5)	(4)	2	2	2
S. inornatus				1	1	1				1	0	0
*S. madagascariensis**	51 (58)	51 (28)	44 (25)	26	26	20	25 (44)	25 (39)	24 (20)	51	51	48
S. oxyriifolius				2	2	2						
S. panduriformis	1	1	0									
S. polyanthemoides				1	1	1				1	1	0
S. retrorsus				1	1	1				2	2	2
S. serratuloides	1	1	1									

S. skirrhodon	2 (39)	2 (17)	2 (16)				(11)	(9)	(5)			
S. sp. nr *affinis*				2	2	2						
S. sp. nr *conrathii*	(1)	(1)	(0)	4(1)	4(1)	4(1)				1	0	0
S. sp. nr *serratuloides*										3	2	2
Total	84 (136)	83 (67)	70 (57)	69 (2)	69 (2)	63 (2)	25 (90)	25 (68)	24 (41)	65	62	58

* Specimens collected monthly across 12 months in 2017/18 (Singh et al., 2022; Chapter 2).



Continued on page 67

Figure 1. Mid-point rooted maximum likelihood COI phylogeny for stem-boring Curculionidae associated with *Senecio madagascariensis* and non-target *Senecio* species (* denotes larval specimens). Values on branches indicate bootstrap values and posterior probability values. Only bootstrap values ≥ 65% and posterior probability ≥ 0.65 are shown. Senecio species are labelled at the end of the branches. Different operational taxonomic units or species clusters (SCs) indicate different species; those not followed by species names represent unidentified species.

Continued from page 66

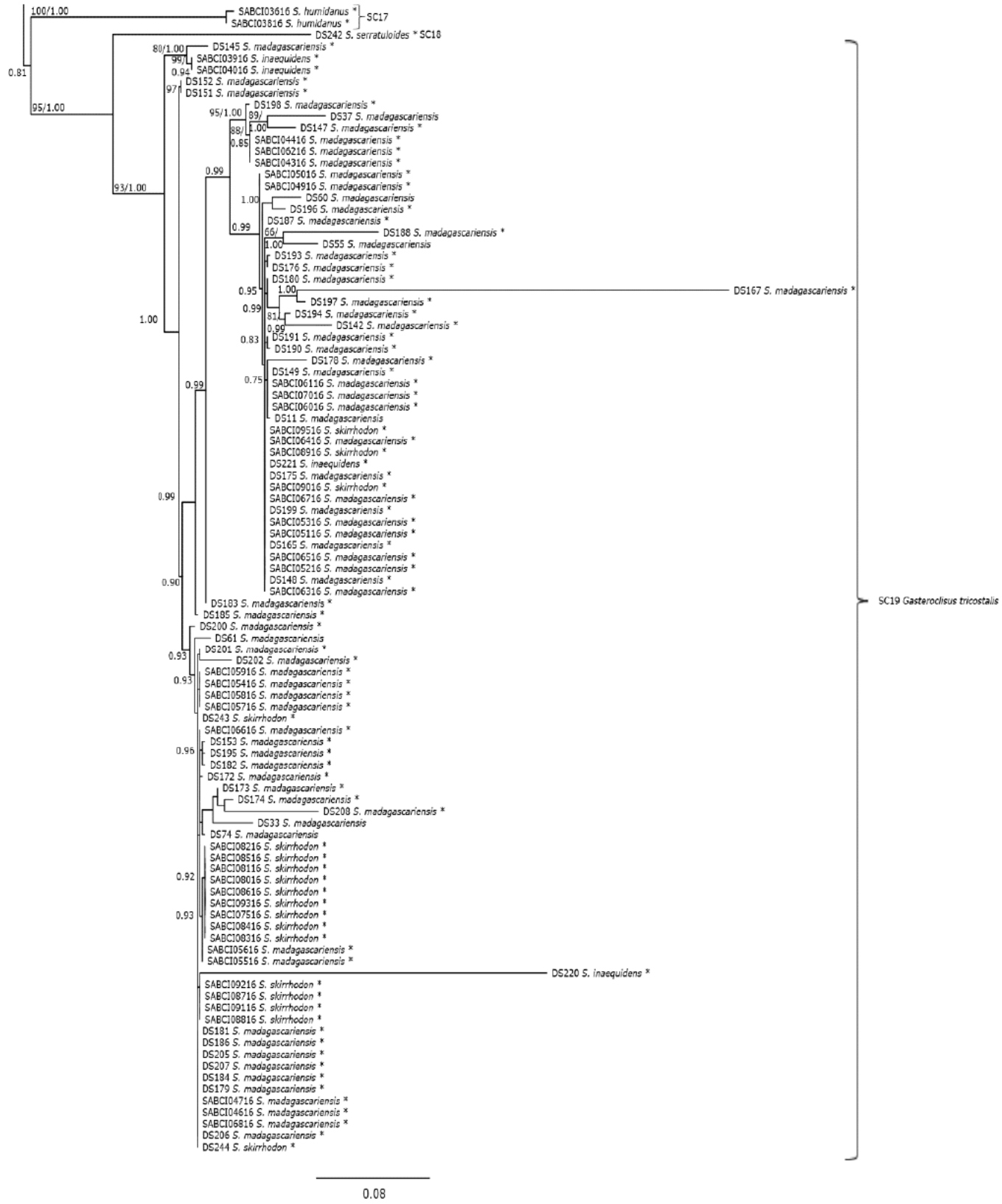


Figure 1: Continued

Stem-boring Lepidoptera

Lepidopteran larvae were not recovered from any of the 17 non-target *Senecio* species sampled during this survey. In contrast, 14 lepidopteran adults were collected from the foliage of eight non-target *Senecio* species during sweep netting (Table 1) but, since these are not typically herbivorous, were considered only for the purposes of matching with larval specimens that were previously collected (Egli et al., 2020; Singh et al., 2022; Chapter 2). CO1 sequences were obtained from all of the adult specimens collected on the non-target *Senecio* species. As before, the adult sequences from the non-target *Senecio* species were compared to 24 larval and 48 adult sequences that arose from monthly sampling of *S. madagascariensis* populations (Singh et al., 2022) and to 41 larval sequences arising from an earlier survey (Egli et al. 2020), comprising 20 sequences from *S. madagascariensis* and 21 from three non-target *Senecio* species.

A comparison of the COI sequences of lepidopteran larvae and adults associated with *S. madagascariensis* and the non-target *Senecio* species revealed eight distinct moth lineages (SCs) (Figure 2). Six SCs were based on few larval sequences that were not matched with adult specimens and thus remained unidentified. Of these, three were associated with *S. harveianus* only (SC 1, 2 and 3), two with *S. skirrhodon* only (SC 6 and 7) and one with *S. madagascariensis* only (SC 4). Most larval sequences matched with identified adult specimens of *M. elegans* (SC 8), which were mostly recovered from *S. madagascariensis*, with a few specimens recovered from *S. inaequidens* and *S. harveianus* (Figure 2). Similarly, sequences that were matched with an unidentified species of *Platyptilia* Hübner (Pterophoridae) (SC 5) were mostly recovered from *S. madagascariensis*, with one sequence from *S. harveianus* (Figure 2).

Figure 2. Mid-point rooted maximum likelihood COI phylogeny for stem-boring Lepidoptera associated with *Senecio madagascariensis* and non-target *Senecio* species (* denotes larval specimens). Values on branches indicate bootstrap values and posterior probability values. Only bootstrap values ≥ 65% and posterior probability ≥ 0.65 are shown. *Senecio* species are labelled at the end of the branches. Different operational taxonomic units or species clusters (SCs) indicate different species; those not followed by species names represent unidentified species.

Discussion

Field-based studies on the host-plant relationships of weed biocontrol agents in their native range can provide evidence of host specificity or support motivations for further studies (Sutton et al., 2021). Such assessments can greatly reduce the time and costs associated with the importation and host-specificity testing of unsuitable agents in quarantine (Legner and Bellows, 1999; Van Driesche et al., 2008). Indeed, Egli et al. (2020) revealed that stem-boring Agromyzidae, which were originally highlighted as promising agents for *S. madagascariensis* (Marohasy, 1991), were associated with several non-target *Senecio* species in South Africa and were thus unsuitable for importation into Australia. Consequently, this study focused on stem-boring Curculionidae and Lepidoptera, which were considered to have narrower host ranges (Egli et al., 2020).

Genetic analyses based on the COI gene region suggest that the weevil *G. tricostalis* is restricted to the *S. madagascariensis* species complex (Hilliard, 1977), with larvae also recovered from *S. inaequidens* and *S. skirrhodon*. The additional sequences arising from this study also helped to resolve the taxonomic uncertainly around *G. tricostalis* (i.e. the possibility of cryptic species). The initial phylogeny of Egli et al. (2020) indicated three closely related species clusters that were deemed to be different maternal lineages of *G. tricostalis*, rather than cryptic species (Olckers et al., 2021). This was reduced to two species clusters following a one-year study on the weevil's seasonal abundance (Singh et al., 2022; Chapter 2). The phylogeny presented in this paper, which incorporated sequences generated from all three studies, has revealed a single species cluster which, given the absence of any morphological differences, was consistent with the contention that a single species is involved (Olckers et al., 2021).

Since *S. inaequidens* and *S. skirrhodon* are taxonomically closer to *S. madagascariensis* than any Australian native *Senecio* species (see Schmidt-Lebuhn et al., 2020; 2022), the narrow host range of *G. tricostalis* has positive implications for Australia. Indeed, Curculionidae have an impressive track record in weed biological control (e.g., Herrick and Kok, 2010; Clewley et al., 2012; Winston et al., 2014; Egli and Olckers, 2017). Examples of stem-boring weevils that were effective against invasive Asteraceae in Australia, include *Lixus cardui* Olivier against *Onopordum* species (Briese, 2012) and *Listronotus setosipennis* (Hustache) against *Parthenium hysterophorus* L. (Dhileepan and McFadyen, 2012). The recovery of *G. tricostalis* on host plants across a broad range of altitudes, including *S. skirrhodon* on the KwaZulu-Natal South Coast (8 m a.s.l) and *S. inaequidens* in the Drakensberg range (1711 m a.s.l.), suggest that this weevil species can persist under diverse environmental conditions.

Stem-boring lepidopteran larvae were not collected on any of the non-target *Senecio* species sampled during 2017-2018. However, the available evidence suggests that the tortricid moth *M. elegans* is similarly confined to plants in the *S. madagascariensis* species complex, with host records only from *S. madagascariensis*, *S. harveianus* and *S. inaequidens*. The pterophorid moth *Platyptilia* sp. was also collected on *S. harveianus*, which although regarded as a valid species in South Africa (Foden and Potter, 2005), has been reported as synonymous with *S. inaequidens* (GBIF Secretariat, 2021). Of the four stem-boring lepidopterans deployed against invasive Asteraceae in Australia, two inflicted extensive damage on their targets, namely *Cochylis atricapitana* Stephens (Tortricidae) and *Platyptilia isodactyla* (Zeller) (Pterophoridae) on *Jacobaea vulgaris* Gaertn (= *Senecio jacobaea* L.) (Ireson and McLaren, 2012; Winston et al., 2014). Similarly, the stem-galling *Epiblema strenuana* Walker (Tortricidae) inflicted extensive damage on *Parthenium hysterophorus* L. and *Ambrosia artemisiifolia* L. in Australia (Dhileepan and McFadyen, 2012; Winston et al., 2014). These examples also support the use of stem-boring lepidopterans against *S. madagascariensis*. Of these, *M. elegans* should take precedence in further assessments, given its considerably higher abundance relative to *Platyptilia* sp. in the field (Singh et al., 2022).

Senecio madagascariensis is a major invasive plant globally, with the potential to invade several countries where it is currently absent (Wijayabandara et al., 2022), but is especially problematic in Australia and Hawaii, where it causes substantial economic and ecological damage. Releases of biocontrol agents targeting *S. madagascariensis* have been restricted to Hawaii, with only one agent, the defoliating moth *Secusia extensa* (Butler) (Erebidae), released so far (Ramadan et al., 2011), but with limited impact on weed populations (Krushelnycky et al., 2018). Biological control initiatives in Australia have been hampered by host-specificity concerns (McFadyen and Morin, 2012; Sheppard et al., 2013), given the diversity of native *Senecio* species. This study supports our contention that the field host ranges of *G. tricostalis* and *M. elegans* are sufficiently narrow to justify their consideration as candidate agents for Australia and has supported the decision to import *G. tricostalis* into Australia for additional studies in quarantine. The weevil was also opportunistically imported into Hawaii in 2014 and successfully cultured from a very small founder population, with host-specificity testing still in progress (M. Ramadan, Hawaii Department of Agriculture, pers. comm., 2020).

However, more intensive laboratory host-range testing, including native Australian Senecioneae as test species, will be required to advocate the release of these stem-boring candidates in Australia. Indeed, both no-choice and choice tests involving the weevil *G.*

tricostalis are currently in progress in South Africa and are scheduled to commence in Australia. Although *S. madagascariensis* is considered to be a "long-shot target" for Australia (Sheppard et al., 2013), biocontrol prospects for Hawaii are considerably more promising, given that the tribe Senecioneae is not represented in the Hawaiian native or endemic flora (Ramadan et al., 2011).

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Chapter 4: Response of the invasive fireweed (*Senecio madagascariensis*) to variable levels of larval stem-boring by *Gasteroclisus tricostalis* (Coleoptera: Curculionidae), a potential biological control agent

ABSTRACT

The stem-boring weevil *Gasteroclisus tricostalis* (Thunberg) (Curculionidae) is under investigation as a biological control agent for *Senecio madagascariensis* Poiret (Asteraceae) in Australia and Hawaii. Besides mandatory host-specificity testing, pre-release studies on the weevil's impact can be a useful predictor of its efficacy when released in the invaded range. In this study, we investigated the response (i.e., growth, capitulum production, and biomass accumulation) of mature flowering *S. madagascariensis* plants to varying densities of *G. tricostalis* larvae in their stems. Although there were weak negative relationships between plant response variables and larval densities, these were only significant in the production of side branches (shoots) and floral components (capitula). Regression analyses showed that six and 12 larvae per mature plant could prevent the production of new capitula and new shoots, respectively. A significant negative relationship between the percentage of eggs that resulted in larval recoveries and the number of eggs deposited per plant may suggest aggressive interactions between the larvae. Given evidence from other biocontrol programmes against asteraceous weeds, *G. tricostalis* may exhibit higher impacts on young pre-flowering plants than on mature plants, a possibility that requires confirmation. Because *G. tricostalis* is present in the field throughout most of the year, additional testing involving multiple generations of the weevil can assess the ultimate impact of sustained damage on plants. Since high floral production is a major driver of invasions by *S. madagascariensis*, substantial reductions in flowering by *G. tricostalis* would likely make a significant contribution to the weed's management.

KEYWORDS: Agent impact studies, floral reduction, stem-boring weevils, weed biocontrol

Introduction

The invasive herb *Senecio madagascariensis* (Poiret) (Asteraceae), commonly known as fireweed, is a target for biological control in Australia (McFadyen and Morin, 2012; Sheppard et al., 2013) and Hawaii (Ramadan et al., 2011; Krushelnycky et al., 2018), due to severe negative impacts on pastoral agriculture, native flora and animal health (Wijayabandara et al., 2022). Although biocontrol efforts commenced in the late 1980s (McFadyen and Morin, 2012;

Olckers et al., 2021), no agents were deemed suitable for release in Australia, while a single agent, the foliage-feeding moth *Secusio extensa* (Butler) (Lepidoptera: Erebidae: Arctiinae) was released in Hawaii in 2013, but with limited impact so far (Ramadan et al., 2011; Krushelnycky et al., 2018).

Although the invasive populations of *S. madagascariensis* in Australia and Hawaii were presumed to originate from Madagascar, later genetic studies confirmed a southern African origin, most likely the KwaZulu-Natal Province of South Africa (Scott et al., 1998; Radford et al., 2000; Le Roux et al., 2006; 2010). Following field surveys in South Africa (Egli and Olckers, 2015; 2020), several insect species were prioritized as candidate agents, but with some later disqualified from further consideration by Australia because of unacceptably broad field host ranges (see Egli et al., 2020; Zuma et al., 2021; Mkhize et al., 2023). However, the stem-boring weevil *Gasteroclisus tricostalis* (Thunberg) (Coleoptera: Curculionidae) has displayed relatively stable abundance across seasons (Singh et al., 2022; Chapter 2) and sufficient host-range restriction in the field in South Africa (Singh et al., 2023; Chapter 3) to warrant further consideration.

Gasteroclisus tricostalis is a medium-sized (8-13 mm in length) weevil that can survive for lengthy periods (*ca* 8-10 months) in culture. Adults feed on leaves at the shoot tips, causing only minor damage. Females deposit single eggs in holes excavated in the stems of plants and concealed by a frass plug. Newly emerged larvae tunnel into the stem, feeding on the pith and boring down towards the roots, eventually pupating in the stems. The weevil's development period from oviposition to adult emergence is highly variable and presumably influenced by host-plant quality. Under quarantine laboratory conditions in Hawaii, this took *ca* 7-17 weeks at 19-23°C, 65-72% RH and a 12L: 12D regime (M. Ramadan, Hawaii Department of Agriculture, pers. comm., 2020), compared to 11 weeks at 22°C in Australia. The weevil was imported into quarantine in Hawaii in 2011/12 and a culture was sustained from a very low founder population. Preliminary studies on its biology and host specificity during 2012-2015 suggest a narrow host range, although testing was not completed (M. Ramadan, Hawaii Department of Agriculture, pers. comm., 2023). Laboratory host-specificity testing was initiated in South Africa in 2022, with field-collected weevils shipped to Australia to establish a quarantine culture for additional testing. In the meantime, an assessment of the impact of larval stem boring on the fitness of *S. madagascariensis* plants was undertaken at the University of KwaZulu-Natal (UKZN) in South Africa.

Pre-release studies on the impact of biocontrol agents on their target weeds are a useful predictor of their efficacy when released in the invaded range (McFadyen, 1998; McClay and Balciunas, 2005; Balciunas and Smith, 2006; Huffaker and Messenger, 2012) and can preclude the release of ineffective agents (Conrad and Dhileepan, 2007; Van Driesche et al., 2008; Morin et al., 2009). Releases of ineffective biocontrol agents have been implicated in negative ecological consequences (Louda and Stiling, 2004; Balciunas and Smith, 2006; Van Driesche et al., 2008). For example, agents that reach high population densities but with limited efficacy can elicit indirect non-target impacts by recruiting and bolstering populations of native parasitoids, stimulating compensatory growth in their target plants, and altering food-web interactions (see Pearson and Callaway, 2003; 2005; Willis and Memmott, 2005). Although such indirect non-target impacts are typically difficult to predict, they emphasize the importance of demonstrating agent efficacy prior to release (Balciunas and Smith, 2006; Kotula et al., 2021; Todd et al., 2021).

Pre-release impact studies can be conducted in the laboratory (e.g. Kluge and Zachariades, 2006; Goolsby et al., 2009; Weed and Casagrande, 2010; Reddy and Mehelis, 2015; Bitume et al., 2019) or in the field in the native range (e.g. Dhileepan et al., 2022). Typically, these determine the target weed's response to herbivory inflicted by a pre-determined insect density, or an ambient field density, relative to insect-free controls. Furthermore, trials that include varying insect densities enable a more accurate determination of their effects on plant growth attributes (Gerber et al., 2008) and can determine agent threshold levels that are required to control the weed. The aim of this study was to determine the response of *S. madagascariensis* plants, as measured by their growth, floral production, and biomass accumulation, to varying numbers of *G. tricostalis* larvae in the stems.

Materials and methods

Insect culture

Gasteroclisus tricostalis was cultured from adults collected between 2019 and 2022 at Ukulinga Research Farm (29°39'44." S, 30° 24'18" E) and the African Birds of Prey Sanctuary (Ashburton) (29°40'33" S 30° 30'43" E), as these sites have supported a high abundance of weevils (see Singh et al., 2022). Weevils were collected by the sweep netting or beating of fireweed plants and placed into plastic screw-top vials (6 x 4 cm). Collected adults were brought to the insectary at the UKZN, Pietermaritzburg campus (29°37'35" S, 30°24'10" E), placed into a glass Petri dish containing fireweed leaves, and observed for mating. Mating pairs

of adults were then placed onto individual potted fireweed plants (17.5 cm pots), which were confined by cylindrical ventilated plastic sleeves (65 cm height x 18 cm diameter) that fitted into the pots. Honey placed on the sides of the sleeves served as a nutrient supplement to increase egg production. The insectary temperature was maintained at 24°C for the duration of the weevils' exposure to the plants.

After one week, the plants were inspected for oviposition and transferred to a shade house in the UKZN Botanical Garden to facilitate larval development. After seven weeks, plants were returned to the insectary and placed into BugDorm-2® insect cages (60 x 60 x 60 cm; MegaView Science) to monitor adult emergence. After a further four weeks, plants were dissected under a dissecting microscope to obtain any larvae, pupae or pre-emergence adults. All reared adults were placed individually into Petri dishes with fireweed leaves for later use in the trials, or to augment the culture. Late-instar larvae were transferred into freshly cut stems that were sealed with Parafilm®, to allow them to complete their development, while pupae were sealed within the original stem. However, larval transfers had varying levels of success, with high levels of mortality. Dead specimens were placed into 1.5 ml Eppendorf tubes containing 100% ethanol and placed into a freezer, in the event that later genetic analyses were required.

Larval impact trials

The larval impact trials were conducted between February and May 2022 (late summer-autumn), when weevil larvae are in high abundance in the field (Singh et al., 2022). Fireweed plants were grown in 17.5 cm pots and maintained in a shade house in the UKZN Botanical Garden, until they had reached a suitable size (i.e., > 35 cm in height and > 3 mm in stem diameter) for exposure to the weevils. A series of 10 trials that included 10 larval-free control plants and 50 larval-infested test plants were conducted in the insectary (24°C). For each trial series, comprising one control and 4-7 test plants of equivalent height, similar-sized plants (mean ± S.D. = 47.8 ± 5.3 cm tall) were used across the treatments.

Prior to their exposure to the weevils, several variables that included height, stem diameter, and the number of shoots, leaves, and capitula were recorded for each individual potted plant. Eight pairs of reproductively-active weevils were used in the trials, with each test plant exposed to one weevil pair for either two, five, or seven days to obtain a range of egg-laying densities. Plastic sleeves (see above) were placed over each plant to confine the weevils. Following adult exposure, the number of eggs was recorded, and the plants were returned to the shade house to

facilitate plant growth and larval development under more natural conditions. Since the rates of egg and larval mortality were unknown, a decision was taken to retain all deposited eggs rather than squash excess eggs to obtain the desired larval numbers in the stems.

After eight weeks, all plants were returned to the insectary and the plant variables were measured again to calculate the increments in growth and reproduction. Dead versus living tissues were recorded in the counts of leaves, shoot tips, and capitula. The capitula were removed from each plant and placed into paper bags. Each plant was cut at the base of the stem and the roots were washed to remove the soil and placed into paper bags. The stems were then dissected, and the late-instar larvae were recorded and preserved in 1.5 ml Eppendorf tubes with 100% ethanol. The dissected stems and leaf material were then also placed into paper bags. The plant tissues (i.e., roots, capitula, and stems/leaves) were oven-dried at 60°C for 24 hours and then weighed to determine the below-ground and above-ground biomass.

Statistical analysis

All statistical analyses were conducted using IBM SPSS version 28.0. Since the datasets met the assumptions of normality and equality of variances, parametric tests were used in the analyses. One-way ANOVA was used to determine whether initial plant size (i.e., height) varied between the recorded larval densities (i.e., number of weevil larvae in the stems). Linear regression was used to determine the relationships between the different response variables and the larval densities. Response variables included the increments in plant height, stem diameter and living tissues (shoots, leaves and capitula) and the final plant biomass (above-ground and below-ground). The relationship between the proportion of eggs that produced late-instar larvae and the number of eggs deposited per plant was also determined by linear regression.

Generalized linear models, corrected for over-dispersion, were used to determine whether the proportion of eggs that produced late-instar larvae varied between females, exposure times or both factors in combination and whether the proportion of dead versus living plant tissues varied between larval densities. These models included a binomial distribution and logit link function, with significance ($P < 0.05$) determined by Wald chi-square statistics. The model used to determine whether mean larval counts differed between the exposure times incorporated a Poisson distribution and log link function. Where significant differences were demonstrated, Least Significant Difference (LSD) tests were used for pairwise comparisons of the means.

Results

Weevil fecundity and larval densities

Daily fecundity was similar between the eight females and ranged from 1-5 eggs per day of exposure on the 50 experimental plants, with a mean (± SE) of 2.0 ± 0.1 eggs per day. Over the varying periods of exposure (2-7 days), the numbers of eggs recovered on the test plants ranged from 1-19. However, there was considerable variation in oviposition success per female, with larval recoveries at the end of the trials ranging from 5.6 % to 73.7% of the eggs deposited. Consequently, there were significant differences between females in the proportions of eggs that produced late-instar larvae (χ^2 = 22.403; df = 7; P = 0.002). While female age may determine oviposition success, plant exposure times might also be influential due to lower late-instar larval recoveries at higher egg loads (see below). Larval densities ranged from 1-6 larvae per experimental plant, with single larvae in 12 plants, two and three larvae in eight plants each and four, five and six larvae in nine, four and three plants, respectively. Mean (± SE) late-instar larval counts accruing from the 5-day adult exposure period (3.45 ± 0.37) were significantly higher (χ^2 = 8.785; df = 2; P = 0.012) relative to the 2-day (1.88 ± 0.30) and 7-day (1.75 ± 1.11) exposure periods. Although proportionally fewer eggs produced larvae after the 7-day adult exposure period (mean ± SE = 15.18 ± 10.30%; n = 4) relative to the 5-day (41.77 ± 5.77%; n = 20) and 2-day (46.11 ± 7.07%; n = 26) exposure periods, the differences bordered on significance (χ^2 = 5.224; df = 2; P = 0.073), presumably due to few samples in the 7-day period. There was no significant interaction between female and exposure time (χ^2 = 8.460; df = 10; P = 0.584) in determining oviposition success.

There were no significant differences in initial plant height between the different larval densities (F = 0.277, df = 6; P = 0.945), confirming that similar-sized plants were used across the larval treatments. However, there was a significant negative relationship (y = -4.567x + 81.979; r^2 = 0.428; P < 0.001) between the percentage of eggs that resulted in larval recoveries and the number of eggs deposited per plant (Figure 1).

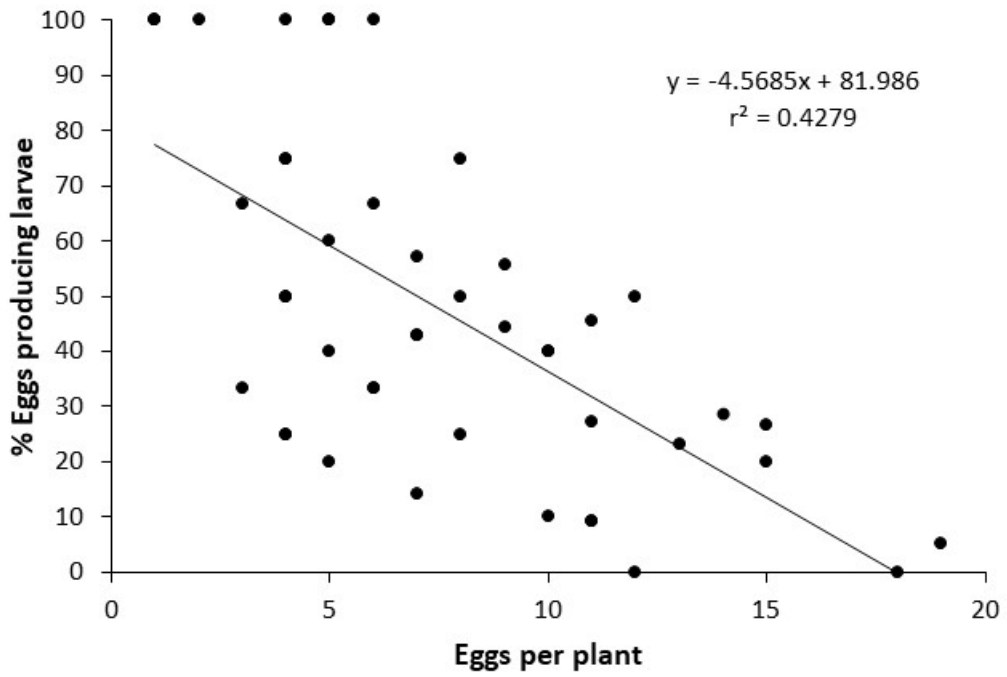

Figure 1. Percentage of eggs that produced late-instar larvae in relation to the number of eggs deposited by individual females of *Gasteroclisus tricostalis* on *Senecio madagascariensis* plants.

Plant responses

At the termination of the trials, 24% of the weevil-exposed plants (12) displayed clear signs of diminished health, with a single dead plant and 11 plants displaying severe stem breakage. These plants typically contained three or more larvae. While the relationships between both new growth (i.e., increased plant height) and new leaf production and larval densities were not significant (Table 1; Figure 2a, b), these were significant in the production of side branches (shoots) and floral components (capitula) (Figure 2c, d). The regression equations predicted that six larvae in the stems could prevent the production of new capitula by a mature plant, while 12 larvae could prevent the production of new shoots (Table 1). The relationships between both root biomass and above-ground biomass and larval densities were not significant (Table 1; Figure 3a, b).

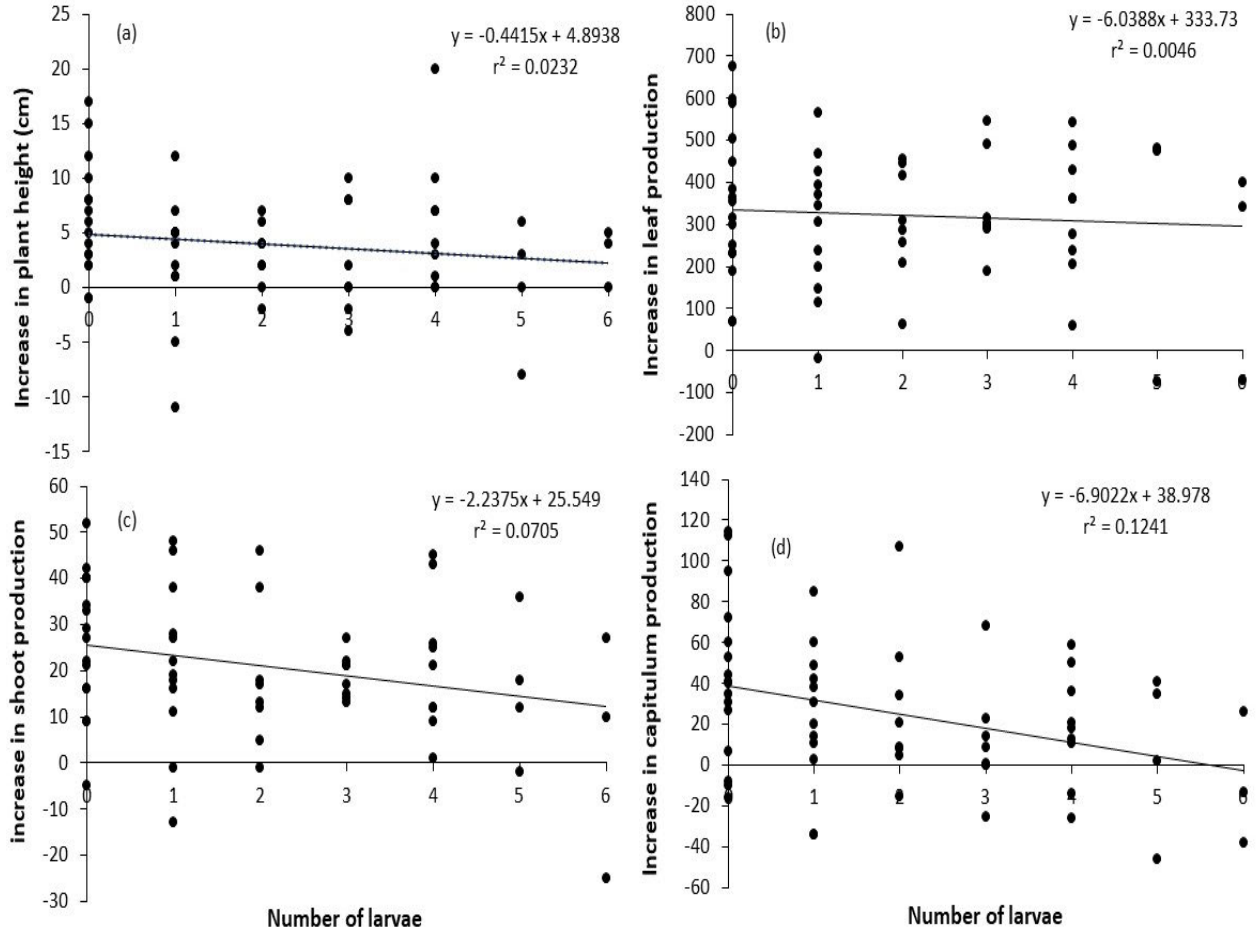

Figure 2. Increase in (a) plant height, (b) leaf production, (c) shoot production and (d) capitulum production by *Senecio madagascariensis* plants in relation to increasing numbers of *Gasteroclisus tricostalis* larvae.

Table 1: Response of *Senecio madagascariensis* growth and reproductive variables (y) to stem boring by increasing numbers of *Gasteroclisus tricostalis* larvae (x).

Response variable	Regression equation	r^2 value	P value	Larvae*
Plant height	y = -0.441x + 4.894	0.023	0.246	-
Stem diameter	y = -0.002x + 0.113	0.001	0.802	-
Shoot production	y = -2.238x + 25.549	0.071	0.040	12
Leaf production	y = -6.039x + 333.732	0.005	0.606	-
Capitulum production	y = -6.902x + 38.978	0.124	0.006	6
Above-ground biomass	y = -0.046x + 5.223	0.004	0.636	-

Below-ground biomass	y = -0.035x + 1.111	0.023	0.250	-

* Estimated number needed to curtail increases in the response variable; excluded for non-significant equations (P > 0.05).

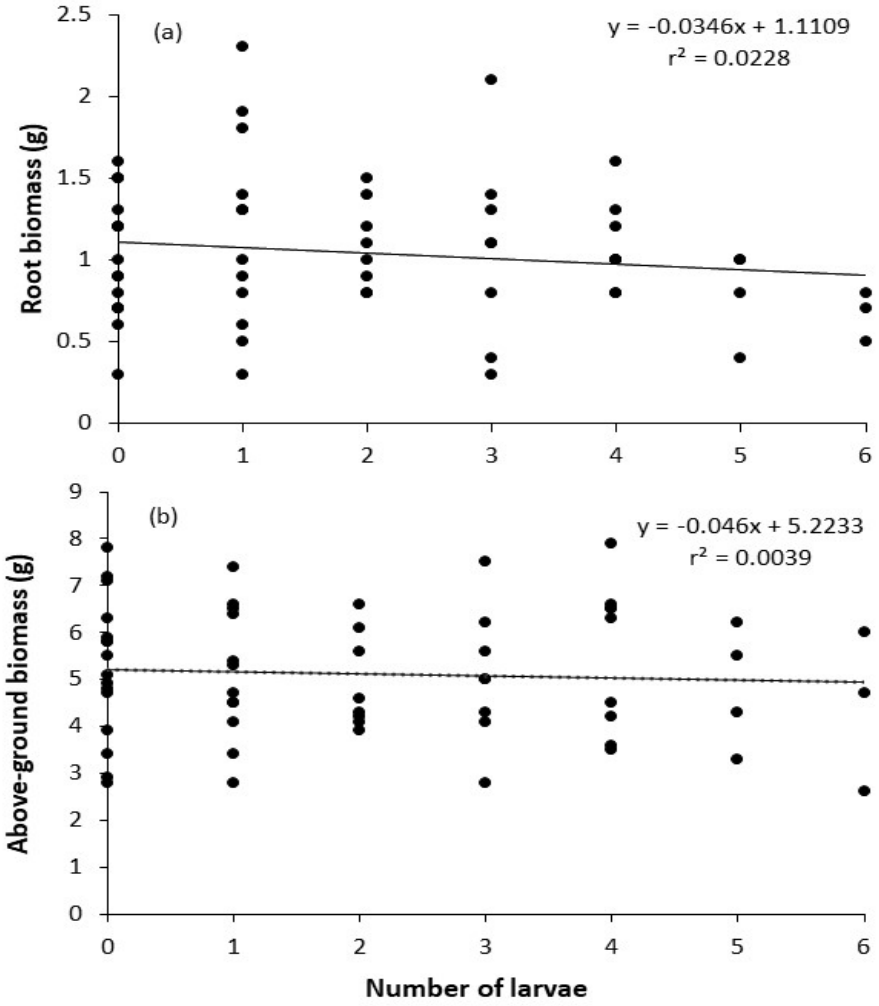

Figure 3. Final biomass of the (a) roots and (b) above-ground material of *Senecio madagascariensis* plants in relation to increasing numbers of *Gasteroclisus tricostalis* larvae.

Despite the significant negative relationship between new shoot production and larval densities, there was no significant effect of larval numbers on shoot mortality (χ^2 = 3.996; df = 3; P = 0.677) (Figure 4a). In contrast, there were significant differences in capitulum mortality between larval densities (χ^2 = 16.175; df = 6; P = 0.013), with treatments of one, three and six larvae causing significantly higher mortality than in the control plants (Figure 4b).

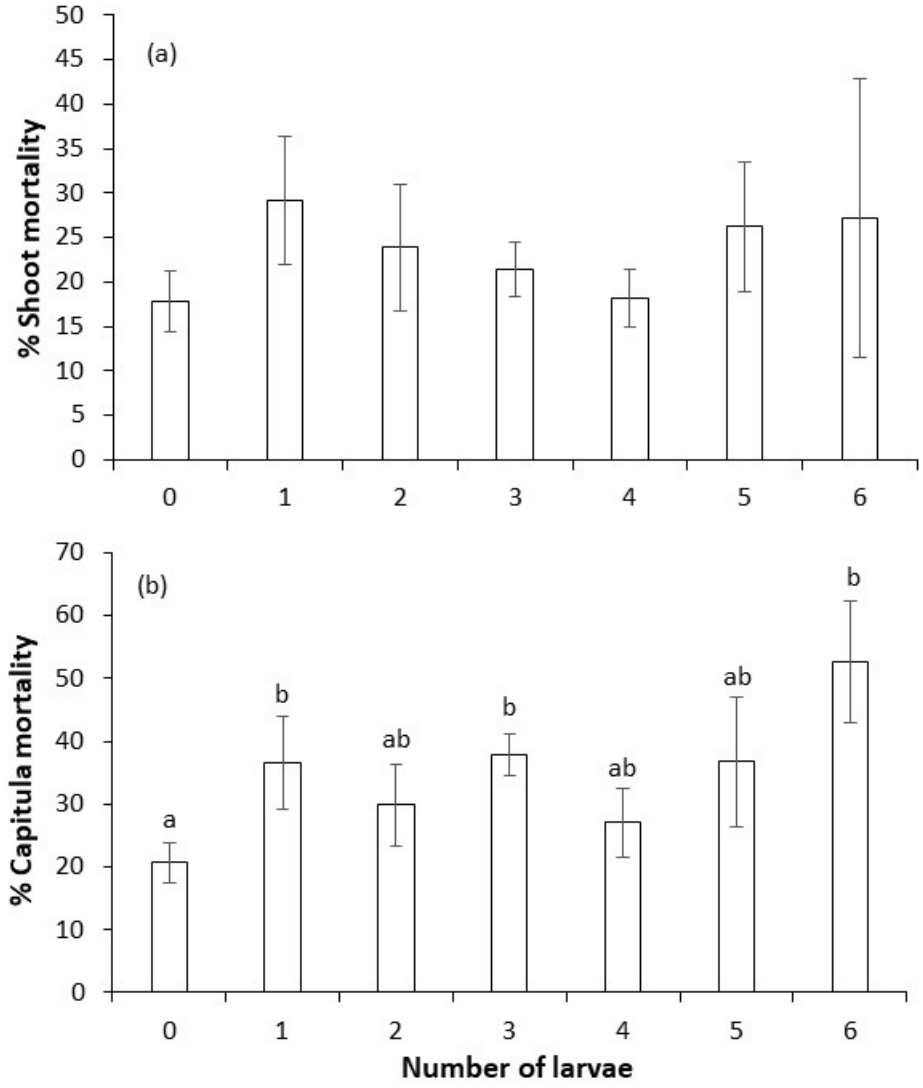

Figure 4. Percentage mortality suffered by the (a) shoots and (b) capitula of *Senecio madagascariensis* plants in relation to increasing numbers of *Gasteroclisus tricostalis* larvae. Different letters above the bars indicate significant differences ($P < 0.05$).

Discussion

Similar to other stem-boring weevils deployed against invasive Asteraceae (e.g. Briese, 1996; Dhileepan, 2003), herbivory by *G. tricostalis* on *S. madagascariensis* plants is not always obvious. Besides stem breakage observed in the field (Singh et al., 2022) and laboratory, infested plants often appear healthy despite high larval loads. Consequently, the weevil was initially deemed to have low biocontrol potential (Marohasy, 1991). In addition, adult feeding damage on the leaves is negligible, in contrast to the heavy damage inflicted by *Gasteroclisus rhomboidalis* (Boheman) on the leaves of cultivated *Amaranthus* species in Nigeria (Ekechukwu and Eluwa, 2008). We found that the impacts of larval stem boring are more

subtle, however, with significant negative effects on the shoot production and reproductive output of mature flowering plants. While the effects of larval damage on increased plant height and leaf production were not significant, there was no evidence of compensatory growth by *S. madagascariensis* plants to attack by larvae (see Gerber et al., 2008). Maternal effects, which included differential larval fitness due to the variable ages and vigour of the field-collected females, were likely a strong contributor to the high degree of variation observed in larval development and plant responses amongst the different density categories.

Although reductions in the production of new shoots and capitula were negligible at low larval densities, six or more larvae per mature plant exerted significant pressure on plant fitness. In particular, the production of new capitula was curtailed at a density of six larvae. *Senecio madagascariensis* invasion is driven by the production of copious wind-dispersed seeds (up to 30 000 per plant per year) and dense seed banks (Wijayabandara et al., 2022). As such, sustained reduction in seed set because of stem attack by *G. tricostalis* may result in gradual depletion of the seed bank and invasion potential at broad scales.

Impact studies involving stem-boring weevils (e.g. Briese, 1996; Dhileepan, 2003; Kluge and Zachariades, 2006; Gerber et al., 2008; Cuda et al., 2016) have typically involved the exposure of the same or varying numbers of adults to the experimental plants for fixed periods, followed by later comparisons of plant parameters recorded on the exposed versus control plants. Our study differed in that we intended to measure plant responses across varying numbers of larvae, the stage most damaging to plant growth. A challenge to this approach is achieving the desired numbers of larvae within a plant (which is only confirmed by dissection several weeks later), and thereby ensuring sufficient and comparable replications across larval densities. Given the high egg/larval mortality experienced, it was fortunate that our initial intention to use fixed numbers of eggs per plant (i.e., squashing excess eggs) was not adopted, since this may have resulted in very few or no larvae per plant and thereby prevented an accurate analysis of larval impacts.

The lower number of replications at the higher larval densities was indeed a constraint. In addition, the final larval counts did not account for any damage caused by larvae that died during the trials. Although we expected that plants with higher egg densities would suffer higher levels of larval damage, linear regressions using egg densities instead of larval densities displayed considerably weaker relationships with the response variables. Presumably, the uncertainty of larval survival, which decreased with increasing egg densities (see below),

confounded these relationships. Prior to this study, transfers of early-instar larvae into stems resulted in excessive mortality and proved ineffective as a means of ensuring known larval loads on the test plants. However, the technique was since refined in quarantine in Australia (P. Jones, CSIRO, pers. comm., 2023), ensuring that further testing involving larval transfers could be considered to produce more robust data. In particular, accurate data on initial larval loads will enable more effective monitoring of larval development/mortality in relation to plant performance. Since this study involved less than a single generation of *G. tricostalis*, additional testing involving multiple generations of the weevil can assess the ultimate impact of sustained damage on plants. Because *G. tricostalis* occurs throughout most of the year in the field in South Africa (Singh et al., 2022), *S. madagascariensis* plants are likely to suffer damage from multiple generations of the weevil. Multi-generational studies could also determine the fitness of later generations of the weevil that develop on previously damaged host plants.

The low percentage of eggs that produced late-instar larvae during these trials (around 35% across all females) and strong negative relationship between egg load and larval recovery per plant has implications for the culturing of *G. tricostalis* in the laboratory. Indeed, results from quarantine trials in Australia indicated that 34% of the original number of eggs laid produced either late-instar larvae, pupae or adults, thereby confirming our low oviposition success. Although larval competition for resources or differential egg viability due to the age of the females are likely to play a role, aggressive larval interactions may be responsible for mortality at higher larval densities. Indeed, endophagous larvae bearing mandibulate mouthparts often exhibit aggressive behaviour and inflict mortality on conspecific individuals (e.g., Klötzli et al., 2023). It thus seems prudent to reduce the number of weevils exposed to caged plants in quarantine, as well as their exposure times, to reduce egg densities on plants and hence increase larval survival. Short exposures (e.g., 3-5 days) of a single mating pair per plant can improve weevil numbers in cultures, as later experienced with the quarantine culture in Australia (P. Jones, CSIRO, pers. comm., 2023).

The low number of plants that hosted 5-6 mature larvae in this study may suggest that realization of the critical larval loads in the field (i.e., 6 and 12 to curtail flowering and shoot production, respectively) is unlikely. However, subsequent culturing of the weevil in quarantine in Australia has produced more larvae per plant than recorded in this study (P. Jones, CSIRO, pers. comm., 2023). This could be the result of higher quality of post-exposure plants maintained under optimal conditions in the quarantine laboratory *versus* those maintained under outdoor conditions in South Africa. In addition, Australian *S. madagascariensis* plants

are likely to display increased vigour due to their escape from specialist insect herbivores for more than a century (Harvey et al. 2015). Consequently, despite aggressive intraspecific interactions, larval loads in excess of six larvae (even up to 12) may be possible in the field in Australia, particularly in the absence of parasitism.

In conclusion, we have demonstrated moderate negative effects of increasing numbers of larvae on shoot development and reproductive outputs in mature *S. madagascariensis* plants. Given that high floral production is a significant driver of invasion by *S. madagascariensis* (McFadyen and Morin, 2012; Wijayabandara et al., 2022), any reduction in flowering could make a significant contribution to the management of fireweed populations. In addition, since we used mature flowering plants in these trials, the impact of *G. tricostalis* on younger pre-flowering plants could be substantially higher. For example, the stem-boring larvae of *Listronotus setosipennis* (Hustache) (Coleoptera: Curculionidae) were significantly more damaging to young pre-flowering plants versus mature plants of *Parthenium hysterophorus* L. (Asteraceae) (Dhileepan, 2003). In any event, the negative impact of *G. tricostalis* on the flowering of mature *S. madagascariensis* plants provides support for its consideration as a biocontrol agent.

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Chapter 5: Biology and host specificity of the stem-boring weevil *Gasteroclisus tricostalis*, a candidate agent for the biological control of fireweed (*Senecio madagascariensis*) in Australia

ABSTRACT

Senecio madagascariensis Poiret (Asteraceae) is a target for biological control in Australia, due to substantial impacts on agriculture and native environments. Studies in the plant's native range in South Africa prioritized the stem-boring weevil *Gasteroclisus tricostalis* (Thunberg) (Curculionidae) as a candidate agent. In this study, we assessed the biology and host specificity of *G. tricostalis* during laboratory trials conducted in both South Africa and Australia. No-choice trials in South Africa revealed that, despite oviposition on 15 non-target *Senecio* species, there was significantly lower oviposition relative to *S. madagascariensis*, with no survival to pupation or adulthood on any non-target species. In contrast, no-choice tests in quarantine in Australia, recorded oviposition on 12 non-target Australian native *Senecio* species, with oviposition on two species not significantly different to that on *S. madagascariensis*. Furthermore in Australia, larvae developed to adulthood on five non-target species, in numbers that were not significantly different to *S. madagascariensis.* These discrepancies can be explained by the lower phylogenetic distance from *S. madagascariensis* to the susceptible non-target species tested in Australia and, likely, optimal plant growth conditions in quarantine. Although no-choice tests are renowned for their conservative nature, preliminary paired-choice trials in both South Africa and Australia failed to demonstrate clear oviposition preferences for *S. madagascariensis*. Despite field surveys in South Africa reporting a highly restricted host range for *G. tricostalis*, the laboratory trials determined that, given the high diversity of the genus *Senecio* in Australia, the weevil is likely unsuitable for release and further trials were suspended.

KEYWORDS: Agent host range, no-choice testing, stem borers, weed biocontrol

Introduction

Senecio madagascariensis (Poiret) (Asteraceae) is an invasive herb from southern Africa and Madagascar that compromises pastoral agriculture, animal health and native flora in several invaded countries across the world (Wijayabandara et al., 2022). Australia and Hawaii are worst affected by the weed, which is colloquially known as fireweed or Madagascar ragwort. Biological control was first implemented in Hawaii, with the release of a defoliating moth in

2013, but with limited success (Ramadan et al., 2011; Krushelnycky et al., 2018). Although Australia has considered biocontrol since the 1980s (McFadyen and Morin, 2012; Sheppard et al., 2013), no agents have been released and the project has been through various stages of shelving and reinstatement (Olckers et al., 2021). The project recommenced in Australia in 2010, with recent studies focused in South Africa.

Field surveys in the KwaZulu-Natal Province of South Africa, the purported origin of the Australian *S. madagascariensis* populations (Scott et al., 1998; Le Roux et al., 2006; Sheppard et al, 2013), identified several herbivorous insect species that displayed potential as candidate agents for Australia (Egli and Olckers, 2015; 2020). However, because Australia supports a diverse *Senecio* flora (Thompson and Orchard, 2015), many were rejected prior to any testing because they displayed broad host ranges in the field in South Africa (see Egli et al., 2020; Zuma et al., 2021; Mkhize et al., 2023). In contrast, the stem-boring weevil *Gasteroclisus tricostalis* (Thunberg) (Coleoptera: Curculionidae) displayed consistent abundance across seasons (Singh et al., 2022; Chapter 2), host-range restriction in the field (Singh et al., 2023a; Chapter 3) and sufficient impact on the weed's reproductive output (Singh et al., 2023b; Chapter 4) to warrant importation into Australian quarantine facilities for host-specificity testing. The good track record of weevils in weed biocontrol (Herrick and Kok, 2010; Winston et al., 2014) also enhanced the status of *G. tricostalis* as the most promising candidate agent for *S. madagascariensis*.

Gasteroclisus tricostalis adults feed on the shoot tips of *S. madagascariensis* plants, but cause only slight foliar damage. Females have a pre-oviposition period of between eight days and three weeks and excavate holes in the stems, into which they deposit single eggs that are concealed by faeces (Matsunga et al. 2020; Singh et al., 2023b; Chapter 4). Eggs hatch after around eight days (Matsunga et al. 2020) and the larvae bore into the stems, where they feed on the pith, tunnel down towards the roots, and pupate in the stems. Adults can survive for several months in culture, while the time from oviposition to adult emergence is highly variable and, depending on laboratory conditions and host-plant quality, can take between 45 and 120 days (Matsunaga et al., 2020). Larval tunnelling weakens the stems of *S. madagascariensis* plants and, depending on larval loads in the stems, reduces the production of side branches (shoots) and floral components (capitula) (Singh et al., 2023b; Chapter 4).

Prior to this study, *G. tricostalis* had been imported into Hawaii in 2011/12 and cultured in quarantine from a single female and two males. Although initial host-specificity trials during

2012-2015 suggested a restricted host range (Matsunaga et al., 2020), testing was not completed and the project was shelved (M. Ramadan, Hawaii Department of Agriculture, pers. comm., 2023). Since Hawaii does not support any native species in the tribe Senecioneae (Ramadan et al., 2011), it was imperative that *G. tricostalis* be exposed to the closest related native Australian *Senecio* species to confirm its purported narrow host range and hence suitability for release. Consequently, laboratory host-specificity testing was initiated in South Africa in 2022, with field-collected weevils shipped to Australia during 2021-2023 to establish a quarantine culture for concurrent testing.

The aim of this study was to elucidate the laboratory host range of *G. tricostalis* by exposing it to a range of test-plant species that vary in phylogenetic distance from *S. madagascariensis*. Testing in South Africa focused on more distantly related native South African species in the tribe Senecioneae, but also some native Australian *Senecio* species. The Australian trials focused on native Australian *Senecio* species, particularly those most closely related to *S. madagascariensis*.

Materials and methods

Field collections and laboratory culture

During 2020-2023, 25 sites in the KwaZulu-Natal Midlands region of South Africa were surveyed for adults of *G. tricostalis* that were used to initiate and sustain laboratory cultures in South Africa and Australia. Around 190 adult weevils were collected, with most originating from four sites that were visited numerous times, namely Ashburton (29°41’29” S, 30° 29’44” E; 777 m a.s.l), Emanzini (29°27’50” S, 30° 22’49” E; 658 m a.s.l), Mpophomeni (29°32’49” S, 30° 10’56” E; 1066 m a.s.l) and Ukulinga (29°39’29” S, 30° 24’26” E; 757 m a.s.l).

Field-collected weevils were transferred to the University of KwaZulu-Natal (UKZN) insectary on the Pietermaritzburg campus and placed in large glass Petri dishes (15 cm diameter) for mating. Mating pairs were removed and confined to single potted *S. madagascariensis* plants (30-50 cm height, > 0.5 cm stem diameter), which were covered by ventilated cylindrical plastic sleeves (65 × 16 cm) that fitted into the 18 cm pots. The mating pairs were exposed to the plants for one week under LED plant-growth lights. Host plants were propagated in 18 cm plastic pots containing potting soil and were watered regularly. The insectary temperature was set at 24°C, with a 12:12 hr day: night lighting regime and ambient humidity. Oviposition scars on the plants were marked for observation.

After one week, the plants were removed from the insectary and placed outdoors in a shade house in the UKZN Botanical Garden, where they were watered using an automatic timer. Plants were kept in the shade house for seven weeks, then returned to the insectary, where they were kept in BugDorm-2® insect cages (60 × 60 × 60 cm; MegaView Science) for an additional four weeks under plant-growth lights. Plants were watered as required and inspected regularly for the emergence of F_1 adults, which were removed and kept individually in small plastic Petri dishes (7 cm diameter) where they were fed *S. madagascariensis* shoot tips. Once several F_1 weevils had emerged, they were placed in a large glass Petri dish for pairing. Weevils that did not pair up were transferred back to the smaller plastic Petri dishes. Reproductively-active adult pairs were used to sustain the laboratory culture and for later host-specificity testing.

After four weeks, the *S. madagascariensis* plants were dissected to detect any larvae, pupae, or adults trapped in the stems. Stems with living larvae or pupae were resealed with Parafilm™ and kept in a glass Petri dish to facilitate adult emergence. The duration of development from egg deposition to adulthood was determined from 130 larvae that developed to adulthood.

Quarantine culturing in Australia

During 2021-2023, six field-collected consignments comprising 120 adults were shipped to the CSIRO Black Mountain quarantine laboratory in Canberra, Australia. Due to travel restrictions as a result of the COVID-19 pandemic, most shipments failed to sustain a quarantine culture due to high in-transit mortality. The culture was finally sustained in 2023, following hand delivery of the last consignment. Upon receipt, the weevils were confined to cages (40 × 40 × 60 cm) containing healthy and reproductively mature *S. madagascariensis* plants and monitored for adult foliar feeding, male and female pairing, and oviposition. Adults that paired up were moved to separate cages containing fresh *S. madagascariensis* plants. Several oviposition holes were individually tagged and monitored over time to record the number of days to adult emergence. Twenty-five larvae were monitored at laboratory temperatures of 17°C (n = 7) and 21°C (n = 18) to determine the duration of development to adulthood. Thereafter, the culture was maintained at an average temperate of 23.8°C, relative humidity of 64.6%, and under LED lights set at 4,000 Kelvin (equivalent to natural sunlight) at a 14:10 hr day: night cycle.

Host-specificity testing

A list of test-plant species was compiled (Table 1), following a revision of the phylogenetic associations between *S. madagascariensis* and native Australian *Senecio* species (Schmidt-

Lebuhn et al., 2020; 2022). In particular, Australia harbours some 96 native and 22 introduced species of Senecioneae, with most belonging to the genus *Senecio* (Thompson and Orchard, 2015). Species were prioritised at different phylogenetic distance levels based on their biogeographical overlap and functional similarity with *S. madagascariensis* (e.g. growth form, reproductive life history, habitat preferences), with a focus on endemic native Australian species. Testing of Australian species was undertaken in both Australia and South Africa, following the shipment of test-plant seeds to South Africa. In addition, native South African species within the tribe Senecioneae, some more distantly related to *S. madagascariensis*, were included for testing in South Africa (Table 1).

Table 1. List of test-plant species in the tribe Senecioneae used in the host-specificity testing of *Gasteroclisus tricostalis* during trials in Australia (AU) and South Africa (SA).

Test-plant species	Rank[1]	Status	Trials
Senecio madagascariensis Poir.	0	Target weed	AU, SA
Senecio brigalowensis I. Thomps.	7	Native (AU)	AU
Senecio pinnatifolius A. Rich.	7	Native (AU)	AU
Senecio pinnatifolius var. *pinnatifolius* A. Rich.	7	Native (AU)	AU
Senecio spanomerus I.Thomps.	7	Native (AU)	AU
Senecio bathurstianus (DC.) Sch. Bip.[2]	13	Native (AU)	AU, SA
Senecio diaschides D.G. Drury	13	Native (AU)	AU, SA
Senecio hispidulus A. Rich.	13	Native (AU)	AU, SA
Senecio linearifolius var. *arachnoideus* I. Thomps.	13	Native (AU)	SA
Senecio linearifolius var. *latifolius* I. Thomps.	13	Native (AU)	AU, SA
Senecio macrocarpus F. Muell. ex Belcher	13	Native (AU)	AU
Senecio minimus Poir.	13	Native (AU)	SA
Senecio picridioides (Turcz.) M.E. Lawr.	13	Native (AU)	AU
Senecio gunnii (Hook.f.) Belcher	16	Native (AU)	SA
Senecio phelleus I. Thomps [2]	16	Native (AU)	AU
Senecio prenanthoides A. Rich.	16	Native (AU)	AU
Senecio quadridentatus Labill.	16	Native (AU)	AU, SA
Curio ficoides (L.) P.V. Heath	23	Native (SA)	SA
Curio talinoides (DC.) P.V. Heath	23	Native (SA)	SA
Kleinia fulgens Hook.f.	23	Native (SA)	SA
Kleinia galpinii A. Berger	23	Native (SA)	SA
Senecio tamoides DC.	23	Native (SA)	SA

Euryops chrysanthemoides (DC.) B. Nord.	24	Native (SA)	SA
Senecio bupleuroides DC.	Nd	Native (SA)	SA
Senecio polyanthemoides Sch. Bip.	Nd	Native (SA)	SA
Senecio retrorsus DC.	Nd	Native (SA)	SA

[1] Based on phylogenetic distance from *S. madagascariensis*, as determined by Schmidt-Lebuhn et al. (2020). Nd = not determined.

[2] Species identity uncertain.

South African trials

Host-specificity trials were conducted in the UKZN insectary, under the same conditions that the weevil cultures were maintained (see above). No-choice trials were conducted on 17 non-target species, whereby a single mating pair of *G. tricostalis* was exposed for four days to a single potted test plant, confined within a ventilated plastic sleeve. The reproductive status of each mating pair was confirmed by first confining them to a *S. madagascariensis* plant for four days, as a pre-trial control. All exposed plants were observed for oviposition scars, with each scar tagged to facilitate detection of the ensuing larval activity. After returning the weevils to the laboratory culture, the exposed plants were transferred to a shade house in the UKZN Botanical Garden.

After 66 days, the plants were returned to the laboratory and kept in the sleeves under plant-growth lights. Plants were checked daily for the emergence of adult weevils. After 80 days, the plants were dissected to assess larval feeding and record the presence of living weevil larvae and pupae, or parasitoids, within the stems. The number of adults and surviving immature stages, in relation to the number of oviposition scars, was recorded for each individual plant and the combined length of stem bored by the larvae was measured. Plants exhibiting signs of death or senescence were dissected earlier. With one exception, each non-target species was tested at least three times (Table 2).

Paired-choice trials were carried out on three non-target Australian *Senecio* species to assess the weevil's oviposition preferences. A single mating pair was confined with one plant each of *S. madagascariensis* and the test species in a BugDorm-2® cage (60 × 60 × 60 cm) for seven days. Oviposition scars were recorded as before. Two replicates of these trials were conducted for each test-plant pair.

Australian trials

Host-specificity trials were conducted in the quarantine laboratory of the CSIRO in Canberra, Australia. Preference was given to native Australian *Senecio* species within the group most closely related to *S. madagascariensis* (i.e., those in phylogenetic distance clade 7). No-choice trials were conducted on 13 non-target species, in which a single mating pair of *G. tricostalis*, drawn from the lab-reared culture, was exposed to a single potted non-target test plant, confined within a cage (40 × 40 × 60 cm). Prior to these trials, the reproductive viability of the weevils was confirmed on *S. madagascariensis* plants within the lab-reared culture. During their exposure to the test plant, adult feeding damage (i.e., percentage leaf surface area damaged) and the number of oviposition scars were recorded. Oviposition scars were tagged on the plants to enable the monitoring of subsequent larval feeding damage.

After four days, the non-target plants were removed from the cages and replaced with single *S. madagascariensis* plants for a further four days. After their exposure to the weevils, all non-target and control plants were monitored weekly for signs of larval damage. Trials with non-target species were only considered valid if the weevils oviposited on the control plants, followed by larval development over multiple instars. As in the South African trials, the plants were dissected after 80 days to assess larval feeding and the presence of living larvae and pupae in the stems. The number of adults and surviving immature stages, in relation to the number of oviposition scars, was recorded for each individual plant and the combined length of stem bored by the larvae was measured. Dying or senescing plants were dissected earlier. With some exceptions, each non-target species was tested at least three times (Table 3).

Paired-choice trials were carried out on four non-target Australian *Senecio* species that supported larval development to assess the weevil's oviposition preferences. Two mating pairs were confined with one plant each of *S. madagascariensis* and the test species in a cage (40 × 40 × 60 cm) for eight days. Oviposition scars were recorded as before. Given the decision to suspend further research on *G. tricostalis* (see below), only one replicate of these trials was carried out.

Statistical analyses

Non-parametric tests or generalized linear modelling were used to analyse the data derived from the no-choice tests, since these did not conform to the assumptions of normality or equality of variances. Data from the South African and Australian trials were analysed separately. Overall differences between the test-plant species in percentage leaf damage were

analysed using Kruskal-Wallis tests. Where overall differences were significant ($P < 0.05$), differences between each non-target species and *S. madagascariensis* were analysed with Mann-Whitney tests.

Differences in the counts of oviposition scars, surviving immature stages and adults reared were analysed using models incorporating a Poisson distribution and log link function. The proportion of eggs that survived to either late immature stages or adulthood were analysed using models incorporating a binomial distribution and logit link function. Differences in the extent of larval tunnelling were analysed using models incorporating a Tweedie distribution and log link function. All models were corrected for over-dispersion and used Wald chi-square statistics. Where overall differences were significant ($P < 0.05$), differences between each non-target species and *S. madagascariensis* were analysed with Least Significant Difference pairwise comparisons.

Spearman's rank-order correlation was used to determine the relationship between the mean oviposition levels and the phylogenetic distance of the test-plant species used in the no-choice tests. Oviposition data recorded during the paired-choice trials were not analysed statistically, due to insufficient replication arising from the project's early termination.

Results

Biology of G. tricostalis

The biology of the weevil is highly variable in terms of adult longevity, female fecundity and developmental duration, presumably because of host-plant quality as well as culturing conditions. Field-collected weevils are typically long-lived, with some individuals surviving for more than one year under laboratory conditions. While some mating pairs of weevils produced over 20 eggs per week on average, others produced fewer than five eggs per week. The survival and duration of development of the immature stages varied substantially between the two temperatures used initially to culture the weevil in quarantine in Australia. At 17°C, only 43% of larvae reached adulthood after a mean (± S.D.) of 110 (± 14) days, while at 21°C, 83% of larvae reached adulthood after a mean of 93 (± 12) days. Subsequent rearing at 23.8°C, 64.6% relative humidity and a 14:10 hr day: night cycle resulted in development to adulthood of around 76 days. Consequently, the host-specificity trials were terminated after 80 days (see above).

Development to adulthood was similarly variable during the trials in South Africa, where plants containing larvae were maintained in the temperature-controlled insectary (24°C) for five weeks versus seven weeks in the outdoor shade house. Developmental duration varied from 63 to 119 days with a mean (± S.D) of 90 (± 9) days and was presumably influenced by the variable outdoor rearing conditions. In addition, the *S. madagascariensis* plants were infested by other stem-boring taxa, notably dipteran and lepidopteran species that were previously recorded (see Singh et al., 2022; Chapter 2) and which may also have reduced the quality of the host plants.

Although larval stem boring typically results in the weakening of plants through tunnelling in the pith, in quarantine in Australia this often elicited the formation of gall-like structures on the stems. These comprised distinct swellings at the junctions of the main and lateral stems, beyond which the larvae did not tunnel further and often pupated in these structures. These structures were unique to Australian *S. madagascariensis* plants and were not recorded on South African conspecifics, whether in culture or in the field, possibly suggesting genotypic differences between Australian and South African *S. madagascariensis* populations.

Host specificity of G. tricostalis

No-choice tests

While adult feeding was not monitored during the South African trials, oviposition scars were recorded on all except two of the 17 non-target species, but with significantly higher levels of oviposition ($\chi^2 = 94.402$; df = 15; $P < 0.001$) on *S. madagascariensis* (Table 2). At the end of the trials, late-instar larvae, pupae or adults were recovered from *S. madagascariensis* only, with none of the non-target species supporting any larval development (Table 2). Parasitism of the immature stages of *G. tricostalis* was recorded on *S. madagascariensis* plants, causing around 10% mortality (Table 2). Although few adults were reared from the control plants, this was presumably also due to the termination of the trials before there was sufficient time to develop to adulthood (see above). The length of stem material bored by the larvae was significantly higher ($\chi^2 = 47.013$; df = 4; $P < 0.001$) on *S. madagascariensis* than on the four non-target species where some larval boring was recorded (Table 2).

Table 2. Response of *Gasteroclisus tricostalis* to non-target Australian and South African species of Senecioneae, relative to the target weed *Senecio madagascariensis*, during no-choice trials in South Africa. Values comprise means (± S.E.) and asterisks denote significant differences ($P < 0.05$) between each non-target species and the target weed. Significance was not determined where no immature stages or adults were recorded on any of the non-target species.

Test-plant species	Rank[1]	No. plants	Oviposition scars	Larvae/pupae recovered	Adults reared	% Survival[2]	Length bored (cm)[3]
S. madagascariensis	0	64	5.6 ± 0.3	2.2 ± 0.2[4]	0.2 ± 0.1	49.7 ± 4.0[4]	31.5 ± 3.0
S. bathurstianus?	13	3	1.3 ± 0.3*	0	0	0	0*
S. diaschides	13	9	1.1 ± 0.4*	0	0	0	0.6 ± 0.6*
S. hispidulus	13	5	1.4 ± 0.5*	0	0	0	0*
S. linearifolius var. *arachnoideus*	13	3	0*	0	0	0	0*
S. linearifolius var. *latifolius*	13	3	1.0 ± 0.6*	0	0	0	0*
S. minimus	13	6	1.3 ± 0.3*	0	0	0	2.3 ± 2.1*
S. gunnii	16	1	1.0*	0	0	0	0*
S. quadridentatus	16	5	2.4 ± 1.1*	0	0	0	9.8 ± 6.2*
C. ficoides	23	3	1.3 ± 0.7*	0	0	0	0*
C. talinoides	23	3	1.0 ± 0.6*	0	0	0	0*
K. fulgens	23	3	1.0 ± 1.0*	0	0	0	0*
K. galpinii	23	3	0*	0	0	0	0*
S. tamoides	23	3	0.3 ± 0.3*	0	0	0	0*

E. chrysanthemoides	24	3	1.0 ± 0.6*	0	0	0	0*
S. bupleuroides	Nd	3	1.3 ± 0.7*	0	0	0	0*
S. polyanthemoides	Nd	3	1.0 ± 0.6*	0	0	0	0.3 ± 0.3*
S. retrorsus	Nd	3	1.0 ± 0.6*	0	0	0	0*

[1] Based on phylogenetic distance from *S. madagascariensis*. Nd = not determined.

[2] Includes late immature and adult stages as a percentage of oviposition.

[3] Total length of stem bored by all larvae.

[4]Parasitized larvae recorded as 'alive' for the purpose of host suitability; when included as a mortality factor, these values decrease to 1.8 ± 0.2 for larvae/pupae recovered and 39.7 ± 3.7 for percentage survival.

During the quarantine trials in Australia, adults of *G. tricostalis* fed on the leaves of nine of the 13 non-target species. Although adult feeding was slight, there was significantly higher levels of feeding ($H = 40.954$; $df = 13$; $P < 0.001$) on *S. madagascariensis* than on five of the nine species (Table 3). Oviposition scars were recorded on all except one non-target species, with significantly higher levels of oviposition ($\chi^2 = 42.663$; $df = 12$; $P < 0.001$) on *S. madagascariensis* than on 10 species (Table 3). Oviposition on two non-target Australian species, *S. brigalowensis* and *S. phelleus*, was not significantly different to that on *S. madagascariensis*. At the end of the trials, late-instar larvae and pupae were recovered on seven non-target species, in numbers that were not significantly different ($\chi^2 = 2.230$; $df = 7$; $P = 0.946$) to *S. madagascariensis* (Table 3). Development to adulthood was recorded on five non-target species, also in numbers that were not significantly different ($\chi^2 = 3.267$; $df = 5$; $P = 0.659$) to *S. madagascariensis* (Table 3). Survival to late-instar larvae, pupae or adulthood (as a proportion of eggs deposited) was not significantly higher ($\chi^2 = 5.154$; $df = 6$; $P = 0.524$) on *S. madagascariensis* than on the non-target species and was even higher on some species (e.g. *S. brigalowensis*, *S. hispidulus*, *S. pinnatifolius* var. *pinnatifolius*) and *S. prenanthoides*, although this was presumably influenced by low replication in some species (Table 3). The length of stem material bored by the larvae was significantly higher ($\chi^2 = 24.735$; $df = 9$; $P = 0.003$) on *S. madagascariensis* than on seven of the 12 non-target species that supported oviposition (Table 3).

Table 3. Response of *Gasteroclisus tricostalis* to non-target Australian species of *Senecio*, relative to the target weed *Senecio madagascariensis*, during no-choice trials in Australia. Values comprise means (± S.E.) and asterisks denote significant differences ($P < 0.05$) between each non-target species and the target weed.

Test-plant species	Rank[1]	No. plants	% Leaf damage	Oviposition scars	Larvae/pupae recovered	Adults reared	% Survival[2]	Length bored (cm)[3]
S. madagascariensis	0	14	5.1 ± 0.4	8.2 ± 1.3	1.4 ± 0.6	0.9 ± 0.4	25.4 ± 5.8	30.9 ± 6.5
S. brigalowensis	7	5	1.6 ± 0.6*	8.0 ± 1.3	0.8 ± 0.4	1.0 ± 0.4	33.5 ± 28.9	33.2 ± 6.1
S. linearifolius var. *latifolius*	7	3	1.3 ± 0.9*	0.7 ± 0.7*	0	0	0	2.0*
S. pinnatifolius	7	2	3.0 ± 2.0	2.0 ± 2.0*	2.0	1.0	75.0	25.0
S. pinnatifolius var. *pinnatifolius*	7	3	1.3 ± 0.3	2.3 ± 1.2*	1.0 ± 1.0	0	33.3 ± 33.3	12.0 ± 11.0*
S. spanomerus	7	6	1.2 ± 0.5*	2.6 ± 1.1*	0.5 ± 0.2	0.4 ± 0.2	14.9 ± 9.8	3.5 ± 1.5*
S. bathurstianus?	13	1	0*	1.0*	1.0	0	100	9.0*
S. diaschides	13	6	0*	0.3 ± 0.2*	0	0	0	0*
S. hispidulus	13	7	0.7 ± 0.4*	2.1 ± 0.7*	1.2 ± 0.6	0.2 ± 0.2	41.7 ± 20.1	18.4 ± 3.2
S. macrocarpus	13	3	0.7 ± 0.7*	1.3 ± 0.7*	0	0	0	8.0 ± 8.0*
S. picridioides	13	1	0*	2.0*	0	0	0	0*
S. phelleus?	16	1	5.0	14.0	0	0	0	40.0
S. prenanthoides	16	3	2.7 ± 1.2	2.7 ± 1.5*	1.5 ± 0.5	1.0	63.3 ± 3.3	43.5 ± 5.5
S. quadridentatus	16	1	0*	0*	-	-	-	-

[1] Based on phylogenetic distance from *S. madagascariensis*.

[2] Includes late immature and adult stages as a percentage of oviposition.

[3] Total length of stem bored by all larvae.

There was a significant negative relationship (r_s = -0.433; n = 28; P = 0.021) between the mean numbers of eggs deposited per test-plant species and the phylogenetic distance of these species from *S. madagascariensis* (Figure 1). Plants ranked in the 7-13 distance range (Table 3) were largely more susceptible to oviposition by *G. tricostalis* than those that were more distantly ranked (Table 2).

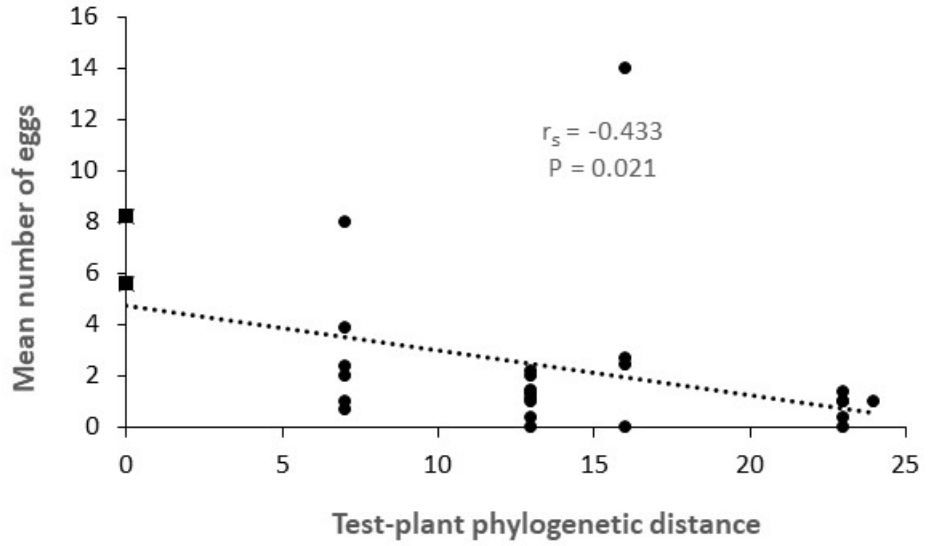

Figure. 1. Mean number of eggs deposited by *Gasteroclisus tricostalis* during no-choice tests conducted in South Africa and Australia that involved test-plant species (circles) at increasing phylogenetic distances from *Senecio madagascariensis* (squares).

Paired-choice tests

Female *G. tricostalis* did not display clear oviposition preferences for *S. madagascariensis* in the case of two of the three non-target species tested in South Africa (Table 4). *Senecio minimus* was the only species that did not support oviposition. The same trend was recorded in the single replicate of the Australian trials, where all four non-target species supported levels of oviposition that were similar to those on *S. madagascariensis* (Table 4).

Table 4. Numbers of eggs oviposited by *Gasteroclisus tricostalis* during adult paired-choice tests in South Africa and Australia involving *Senecio madagascariensis* and Australian non-target *Senecio* species.

Country	Test plant pair	Eggs
South Africa[1]	*S. madagascariensis*	3.5 ± 2.5
	S. linearifolius var. *arachnoideus*	3.0 ± 0.0

	S. madagascariensis	2.5 ± 2.5
	S. diaschides	1.0 ± 1.0
	S. madagascariensis	3.0 ± 2.0
	S. minimus	0.0 ± 0.0
Australia[2]	*S. madagascariensis*	14
	S. brigalowensis	8
	S. madagascariensis	10
	S. pinnatifolius	6
	S. madagascariensis	9
	S. phelleus	7
	S. madagascariensis	0
	S. spanomerus	1

[1] Means (± S.E.) since each test was replicated twice.

[2] Totals since each test was conducted once.

Discussion

During no-choice host-specificity tests in South Africa, none of the non-target test-plant species supported the development of *G. tricostalis* larvae, thereby supporting the results of the field host-range study (Singh et al., 2023a), which suggested that the weevil has a restricted host range. However, no-choice testing in Australia determined a broader host range in which five Australian *Senecio* species supported development to adulthood. Earlier testing in Hawaii (Matsunga et al., 2020), which included more distantly related test plants in the Asteraceae, reported survival to adulthood only on *Senecio vulgaris* L., an exotic weed native to Europe, North Africa and temperate Asia. Preliminary paired-choice trials in both South Africa and Australia also failed to demonstrate clear oviposition preferences, with females often ovipositing on non-target *Senecio* species. The indiscriminate oviposition behaviour of *G. tricostalis* females was also reported from no-choice trials in Hawaii, where eggs were recovered on several non-target species, including endemic species in the genus *Bidens* L. (Asteraceae), albeit with no larval survival (Matsunga et al., 2020). Further testing in Hawaii was suspended due to concerns that female probing and oviposition may inflict non-target damage due to secondary infection by plant pathogens.

The differences in the host-specificity results recorded during the South African and Australian trials could be explained by two considerations. Firstly, four of the six susceptible non-target plant species tested in Australia were phylogenetically closer related to *S. madagascariensis* than all those tested in South Africa. The trend of decreased oviposition with increasing phylogenetic distance from *S. madagascariensis* can explain why none of the non-target plants tested in South Africa were deemed to be susceptible. Secondly, the quarantine conditions under which the test plants were maintained post-exposure in Australia were likely more favourable than the largely outdoor conditions under which the plants were maintained in South Africa. Indeed, Ghebremariam et al. (2014) demonstrated that leaf-feeding by a chrysomelid beetle was significantly higher on non-host plants grown under controlled conditions (e.g., glasshouses) than on those grown in full sun. While these host-range expansions were attributed to morphological changes (i.e., reduced leaf trichome density), chemical changes mediated by improved plant growth conditions (Ghebremariam et al., 2014) could similarly cause changes in host acceptability.

The low numbers of adults reared from the *S. madagascariensis* controls during the trials in South Africa were presumably influenced by the outdoor conditions under which the plants were maintained for much of the weevils' life cycle. Indeed, the average duration of development from egg to adulthood was 90 days, some 10 days longer than the time at which the plants were dissected to record larval survival. Hence, survival was recorded as the number of all surviving life stages at the conclusion of the trials. However, despite potentially improved growth conditions in quarantine in Australia, the survival of *G. tricostalis* larvae on the *S. madagascariensis* controls (25.4%) was lower than that recorded in South Africa (39.7% including 10% mortality due to parasitism). This may well relate to differences in the chemical profile of Australian versus South African *S. madagascariensis* populations, where significantly higher alkaloid concentrations were detected in Australian populations (see Egli et al., 2022). In addition, the mechanisms underpinning gall formation in only Australian *S. madagascariensis* plants (e.g., possible plant defence responses) may further suggest intrinsic differences in how *G. tricostalis* interacts with plants from native versus introduced *S. madagascariensis* populations (see Wang et al., 2011). Furthermore, recent genetic analyses have revealed that Australian populations of *S. madagascariensis* may be more closely related to South Africa populations from the Eastern Cape, rather than KwaZulu-Natal, Province (A.N. Schmidt-Lebuhn, pers. comm., 2024).

The results of the Australian trials may have overestimated the actual host range of *G. tricostalis* (see Balciunas et al., 1996), particularly since the weevil was not recovered on species of *Senecio* in the field in South Africa that are in fact more closely related to *S. madagascariensis* than the seemingly susceptible Australian species (Singh et al., 2023a). However, considering the high diversity of the genus *Senecio* in Australia (Schmidt-Lebuhn et al., 2020; 2022), the rearing to adulthood of the weevil on five non-target species, and the native *S. brigalowensis* proving as suitable a host as *S. madagascariensis,* the decision was taken to suspend further studies on *G. tricostalis* and reject it as a candidate agent for Australia at this stage.

The ranking of the non-target test plants in relation to their phylogenetic distance from *S. madagascariensis* (Schmidt-Lebuhn et al., 2020; 2022) proved useful in test-plant selection and the overall interpretation of the host-specificity tests. Several countries that are invaded by *S. madagascariensis* vary in their diversity of native *Senecio* species and include none in the State of Hawaii (Ramadan et al., 2011), 19 in New Zealand (Sullivan et al., 2008), 67 in Brazil (Hind, 1999) and more than 230 in Argentina (Cabrera, 1966). Despite its rejection by Australia, countries with a low diversity of taxonomically distant *Senecio* species may thus be able to consider *G. tricostalis* as a candidate agent. The possibility that Australian *S. madagascariensis* may originate from the Eastern Cape Province of South Africa needs confirmation, so that any future surveys for biocontrol agents could be focused in this region. Similarly, genetic matching of *S. madagascariensis* populations in other invaded countries with those in South Africa should be a prerequisite to any future biocontrol efforts.

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Chapter 6: General Discussion and Conclusion

Introduction

Invasion by alien plants has become one of the leading drivers of changes in ecosystem and ecological functioning (Ehrenfeld, 2003; Brooks et al., 2004; Gaertner et al., 2009; Hulme et al., 2013). Alien plants have a range of negative impacts on their invaded areas, such as reducing species diversity (Hejda et al., 2009; Vilà et al., 2011), disrupting hydrological processes (Gorgens and Van Wilgen, 2004; Macdonald, 2004) and nutrient cycling (Allison and Vitousek, 2004; Liao et al., 2008) and changing fire regimes (Levine et al., 2003; Brooks et al., 2004). Due to these considerable negative impacts, the control of invasive alien plants is a high priority (Culliney, 2005). Physical and mechanical control, either in isolation or together with chemical control, are the most common methods used to manage invasive alien plants (Mack et al., 2000; Culliney, 2005). However, these are often ineffective and typically incur high costs, negatively affect the environment and require continuous follow-up procedures (Bellows and Fisher, 1999; Mack et al., 2000; Culliney, 2005). Consequently, biological control has been viewed as a more long-term, cost-effective and sustainable solution for alien plant invasions (Culliney, 2005; Sheppard et al., 2006; Clewley et al., 2012).

Senecio madagascariensis (fireweed), which is native to southern Africa, has become an extremely aggressive invader in many areas around the world and is especially problematic in the pastures of Australia (Sindel, 2009; Tsutsumi, 2011; Sheppard et al., 2013). In Australia and Hawaii, the plant causes a tremendous amount of damage as it poisons livestock and increases the cost of agricultural production due to unsustainable control options (Ramadan et al., 2011; Sheppard et al., 2013). Furthermore, current models predict that it could become a more serious threat and could likely spread to other regions in its current invaded range, or around the world, if not managed properly (Wijayabandara et al., 2022; Hooda and Chauhan, 2024). Thus, biological control of the weed has been viewed as a more permanent and sustainable control option (Ramadan et al., 2011; McFadyen and Morin, 2012; Sheppard et al., 2013).

Although Australia had initiated a biological control programme against fireweed in the early 1990s, no agent had proved suitable for release (McFadyen and Morin, 2012; Sheppard et al., 2013). Two insect species were imported into Australia and tested as candidate agents, but both were shown to have an unsuitably broad host range and were thus rejected (McFadyen and Morin, 2012; Sheppard et al., 2013). The primary reason for the difficulty in finding a

suitable control agent, is that there are some 96 native *Senecio* species in Australia, some of which are taxonomically closely related to fireweed (Sindel, 2009; McFadyen and Morin, 2012; Sheppard et al., 2013; Thompson and Orchard, 2015; Schmidt-Lebuhn et al., 2022). One such species is *Senecio pinnatifolius*, which can hybridize with fireweed (Prentis et al., 2007; Sindel, 2009). The vast amount of environmental, ecological and agricultural damage caused by the plant and the fact that fireweed was declared as a Weed of National Significance in Australia created a renewed interest in finding a suite of control agents for the plant (Sheppard et al., 2013). This led to the collaboration between the UKZN and CSIRO, which continued from 2010 to 2023. The work reported in the current study forms a major part of this collaboration.

Biological control research in South Africa

Surveys and agent prioritisation

Since the start of the collaboration between the CSIRO and UKZN in 2010, a significant amount of work has been conducted on fireweed in an attempt to find suitable biocontrol agents. During the initial field surveys which were conducted by Egli and Olckers (2015; 2017; 2020) in KwaZulu-Natal Province, the purported region of origin of the Australian populations, some 15 taxa were shortlisted as promising agents for biological control, with four main species prioritized as candidate agents. These primary agents were selected based on their abundance and the amount of damage inflicted on the plant's floral, stem and root tissues (Egli and Olckers, 2020). These included the flower-feeding moth *Homoeosoma stenotea* Hampson (Pyralidae), stem-boring weevil *Gasteroclisus tricostalis* (Thunberg) (Curculionidae), stem-boring moth *Metamesia elegans* (Walsingham) (Tortricidae) and root-feeding flea beetle *Longitarsus basutoensis* Bechyné (Chrysomelidae: Alticinae).

Conducting field host-range surveys in the native range of any potential control agent is a vital step in any biological control program (Schaffner, 2001; Van Driesche et al., 2008; Sutton et al., 2021). This allows researchers to reject unsuitable agents at an early stage and thus reduces the time and resources invested in them (Kuhlmann and Mason, 2003; Van Driesche et al., 2008; Sutton et al., 2021). Field host-range surveys that were conducted in South Africa involved native *Senecio* plants in conjunction with fireweed to determine the ecological (field) host range of the root-feeding flea beetle (Zuma et al., 2021), flower-feeding moth (Mkhize et al., 2023) and stem-boring weevil (Singh et al., 2023a; Chapter 3: Figure 1). DNA barcoding was then used to match adults to immature stages, but also determine the host range of these

candidate agents by comparing the sequences of immature and adult stages associated with fireweed plants to those associated with native *Senecio* species.

The flea beetle (*L. basutoensis*) was initially considered to be the most promising candidate agent, but was recovered on four native *Senecio* species during field host-range surveys (Zuma et al., 2021). Though two species (*Senecio oxyriifolius* and *Senecio. aff. serratuloides*) were thought to be occasional hosts, as only adults were recovered, the presence of both adults and larvae on *Senecio inornatus* and *Senecio polyanthemoides* suggested that the flea beetle had a broader host range than expected (Zuma et al., 2021). This was confirmed by laboratory multi-choice tests, during which late instar larvae were recovered on four non-target species (*Senecio polyanthemoides*, *Senecio pinnatifolius*, *Delairea odorata* and *Gynura procumbens*) (Zuma et al., 2021). Thus, due to the unacceptably broad host range of the flea beetle, it was rejected as a potential agent for Australia (Zuma et al., 2021).

A similar situation was experienced with the flower-feeding moth *H. stenotea*. During initial field surveys, *H. stenotea* larvae were recovered on six non-target *Senecio* species, namely S. *bupleuroides*, *S. coronatus*, *S. glaberrimus*, *S. inaequidens*, *S. oxyriifolius* and *S. polyanthemoides* (Mkhize et al., 2023). Although the moth was not tested in the laboratory, its broad field host range similarly disqualified it as a potential agent for Australia (Mkhize et al., 2023).

Stem-boring agents

Contrary to *L. basutoensis* and *H. stenotea*, the stem-boring weevil *G. tricostalis* showed a narrow field host range and was only found on two other *Senecio* species, *S. skirrhodon* and *S. inaequidens*, both of which are in the same species complex as fireweed (Singh et al., 2023a; Chapter 3: Figure 1). Due to its more restricted host range, the good track record of weevils in weed biological control programmes (Clewley et al., 2012) and its relatively high abundance in the field (Singh et al., 2022; Chapter 2: Figure 5), *G. tricostalis* was considered to be the most promising agent currently available for Australia.

During the course of the seasonal abundance study (Singh et al., 2022; Chapter 2) on stem-boring insects, larvae of *G. tricostalis* were recorded for nine months of the year and were only absent during mid to late winter (July to September) (Singh et al., 2022; Chapter 2: Figure 5), when insect abundance is typically low (Solbreck et al., 2022). The highest abundance of *G. tricostalis* larvae was during January (mid-summer) and May (late autumn) (Singh et al., 2022; Chapter 2: Figure 1 and 5) suggesting that season plays a significant role in determining the

abundance of the weevil. Of the four field sites that were sampled seasonally, the majority of *G. tricostalis* individuals originated from the Ashburton site (Singh et al., 2022; Chapter 2: Figure 1 and 5). This site was then prioritized for the collection of weevils during the remainder of the study, which focused specifically on *G. tricostalis*.

Molecular studies

DNA barcoding has become widely utilised in the determination of species identity, since it is both quick and efficient (Lopez-Vaamonde et al., 2021; Antil et al., 2023). Often it is impossible to identify insect specimens, especially immature stages, to species level, when using morphological features (Ball and Armstrong, 2006; Molina et al., 2017; Caterino and Recuero, 2024). Thus, DNA barcoding has become very useful in species identification, especially when species are cryptic, lack a range of morphological characteristics or include immature life stages that need to be matched with adults (Molina et al., 2017; Jin et al., 2022; Antil et al., 2023; Caterino and Recuero, 2024).

This was especially true during the course of determining suitable agents for the biological control of fireweed. All prioritised agents, including *G. tricostalis*, required the matching of endophagous immature stages with adults to enable the identification of species and their host-plant associations, which would have been practically impossible without DNA barcoding. During the field host-range study, 17 native *Senecio* species as well as additional samples (Egli et al., 2020) were used to determine the host-plant associations of the stem-boring weevils (Singh et al., 2023a; Chapter 3: Figure 1). The results from the analysis of the CO1 gene region revealed a total of 19 different weevil species associated with fireweed and native *Senecio* species (Singh et al., 2023a; Chapter 3: Figure 1). Of these *G. tricostalis* was shown to comprise two maternal lineages and was associated with only two other *Senecio* species, both in the *S. madagascariensis* species complex (Singh et al., 2022; 2023a; Chapter 3: Figure 1). This was viewed as sufficient to warrant further investigation into the impact of larval stem boring on fireweed plants (Singh et al., 2023b; Chapter 4) and a more intensive evaluation of the fundamental (i.e., laboratory) host range of *G. tricostalis* (Chapter 5).

Although *G. tricostalis* was originally revealed to comprise two lineages (Singh et al., 2022), subsequent analyses using additional sequences revealed a single lineage (Singh et al., 2023a). This precluded the need for additional studies with nuclear markers to determine the exact number of species or whether cryptic species were involved. Since individuals from these

two lineages were also revealed to be taxonomically identical and comprise a single species, the decision was taken to not pursue further genetic work.

Agent impact assessment

Pre-release impact trials are a vital part of any biological control program and allow researchers to quantify the effect that a candidate agent is likely to have on the target plant (McClay and Balciunas, 2005; Conrad and Dhileepan, 2007; Morin et al., 2009). This is a crucial step because it allows researchers to determine if the candidate agent has enough of an impact on the target plant to warrant further consideration and can prevent the release of ineffective agents (McClay and Balciunas, 2005; Conrad and Dhileepan, 2007; Morin et al., 2009). During the course of the impact trial, it was clear that *G. tricostalis* larvae have a subtle, but significant, impact on fireweed plants and in many cases, especially with late instar larvae, the plants exhibited signs of stem breakage and a reduction in health. In a few cases, the damage to the plant resulted in its death. The most significant impact of larval stem boring was a reduction in floral (and consequently seed) production and a reduction in side branches (Singh et al., 2023b; Chapter 4: Figure 2). Both of these factors are important in reducing the plant's ability to reproduce, which is a key factor in the invasive success of fireweed. This finding thus supported further studies on *G. tricostalis*, notably its importation into quarantine in Australia for host-specificity testing, as well as concurrent host-range testing in South Africa.

Laboratory host-range testing

Host-range testing is the most important pre-release study and is completed before a candidate agent can be considered for release (Schaffner, 2001; Van Driesche et al., 2008). These tests play a vital role in allowing researchers to determine the likelihood of any non-target effects, and to prevent the release of agents that have broader host ranges than anticipated (Sheppard et al., 2005; Van Driesche et al., 2008; Van Wilgen et al., 2013; Hinz et al., 2020). Although the ecological (field) host-range study revealed that *G. tricostalis* was restricted to the fireweed species complex (Singh et al., 2023a; Chapter 3: Figure 1), the fundamental (laboratory) host-range trials (no-choice trials), which were conducted concurrently in South Africa and Australia, revealed a different pattern. Late instar larvae and pupae of *G. tricostalis* were recovered on seven native Australian *Senecio* species, whilst adults were reared on four species that are more closely related to fireweed than the remaining test-plant species (Chapter 5: Table 3). It was clear that the greater the phylogenetic distance between the test-plant species and fireweed, the lower the likelihood of these supporting *G. tricostalis* development (Chapter 5:

Figure 1). Although the weevil developed on some closely related Australian *Senecio* species, it did not develop on any native South African species in the genus *Senecio* or tribe Senecioneae (Chapter 5: Table 2). Although no-choice tests are notoriously conservative, further testing (i.e. choice tests) was terminated prematurely because of the decision taken in Australia to reject *G. tricostalis* as a candidate agent.

Future of fireweed biological control

Since earlier genetic studies (Scott et al., 1998; Le Roux et al., 2006) showed that fireweed populations in Australia and Hawaii are closely related to populations in KwaZulu-Natal Province, all field surveys were conducted on fireweed populations in this province. However more recent, albeit preliminary, genetic work has suggested that fireweed populations in Australia may be more closely related to populations in the Eastern Cape Province of South Africa (A.N. Schmidt-Lebuhn, pers. comm., 2024). Consequently, if this is true, any future field surveys should be conducted in the Eastern Cape or other provinces in South Africa to determine the possibility of any new agents for Australia.

Future work should also look at determining any genetic differences between *S. madagascariensis* populations across South Africa, in order to match these with populations in Australia but also other invaded countries. This would aid in determining the most suitable areas to locate candidate agents for any countries considering biological control. It would also be useful to determine the phylogenetic relationships (i.e. distances) between South African native *Senecio* species and *S. madagascariensis*, as was done for the native Australian species (Schmidt-Lebuhn et al., 2022; 2024). This is because the laboratory host-range studies (Chapter 5: Figure 1) revealed that the species most closely related to fireweed were more susceptible to non-target attacks by *G. tricostalis*. Consequently, any future field host-range surveys could focus on native South African species that are most closely related to fireweed to determine the likelihood of non-target damage at an early stage.

Other research possibilities could include more studies on the two stem-boring moths, *Metamesia elegans* and *Platyptilia* sp. (Singh et al., 2022; Chapter 2: Figure 3; Singh et al., 2023a; Chapter 3: Figure 2), but also a group of root-feeding weevils that have not been studied as yet. Both stem-boring moths displayed a narrow field host range (Singh et al., 2023a; Chapter 3: Figure 2) that, like *G. tricostalis*, was restricted to species in the fireweed species complex. While *M. elegans* was found in higher abundance in the field than *Platyptilia* sp. (Singh et al., 2022; Chapter 2: Figure 3), it was also recovered on the closely related *Senecio*

harveianus and *Senecio inaequidens*, with *Platyptilia* sp. recovered on *Senecio harveianus* only (Singh et al., 2023a; Chapter 3: Figure 2). Although both moth species have potential as candidate agents, they are considerably more difficult to collect and culture compared to the weevil, which also proved challenging to culture given its extended life cycle. However, *Platyptilia* sp. is a better candidate since it can be collected more easily from floral material, being both a capitulum and stem borer (Egli & Olckers, 2020), and displayed a narrower field host range.

The root-feeding weevils are currently unexplored as possible agents, largely due to the earlier focus on the flea beetle (Zuma et al., 2021) and the promise initially shown by *G. tricostalis*. In the event of future funding, or interest in extending the project, the root-feeding weevils should be subjected to further investigation, which includes field surveys, molecular studies to determine their taxonomic status and host-plant relationships, and ultimately studies on their impact and host range.

Conclusion

Senecio madagascariensis has indeed become one of the most problematic invasive species in the rangelands of Australia (McFadyen and Morin, 2012; Sheppard et al., 2013), with current predictions expecting the plant to become invasive in areas where it was previously absent (Wijayabandara et al., 2022; Hooda and Chauhan, 2024). This can already be seen as the plant is now found in many other countries around the world (Chapter 1: Figure 1.2), most notably in Argentina (Lopez et al., 2008), Brazil (Panziera et al., 2018), New Zealand (Schmidt-Lebuhn et al., 2024), and Japan (Tsutsumi, 2011). Fireweed is a difficult target for biological control due to the vast number of native *Senecio* species found in many of these invaded areas (see below). Thus, potential biocontrol agents for these countries need to display an extremely narrow host range, which was not the case during either the current study or earlier studies arising from surveys in Madagascar (McFadyen and Morin, 2012). Thus, the prospects for biological control in countries such as Australia, Argentina and Brazil, which support some 96, 230 and 67 native *Senecio* species, respectively (Cabrera, 1966; Hind, 1999; Thompson and Orchard, 2015), are very limited.

The use of molecular tools has become a predominant force in species identification and phylogenetic studies. During the course of this study, DNA barcoding has played a major role in screening candidate agents, by facilitating comparisons of insect larvae between host plants and linking these with the adults. Insect species which displayed broad host ranges in the field

were thus excluded at an early stage (Egli et al., 2020; Zuma et al., 2021; Mkhize at al., 2023), thereby saving time and resources which would have been spent on their importation, culturing and laboratory testing.

Though many of the candidate biocontrol agents prioritised by research in South Africa were rejected by Australia due to unacceptably broad host ranges, countries with a less diverse *Senecio* flora could still consider biological control. In particular, the State of Hawaii has no native species in the tribe Senecioneae (Ramadan et al., 2011), while New Zealand hosts only 19 native *Senecio* species (Sullivan et al., 2008). A first step for these and other countries would be to determine the phylogenetic relationship between fireweed and their native plants, as phylogenetic distance was influential in explaining the non-target effects of *G. tricostalis* during laboratory host-range tests. Countries with few native *Senecio* species that are phylogenetically more distant to fireweed than observed in Australia, would be better placed to consider a biological control programme against *S. madagascariensis*.

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Appendix: Supplementary Table 1 for Chapter 3

Non-target *Senecio* species surveyed for stem-boring larvae at sites around KwaZulu-Natal, South Africa. Voucher specimens collected at each location are lodged in the John Bews Herbarium (NU) at the University of KwaZulu-Natal.

Species	Date	Site	Accession number	Coordinates (S, E)	Elevation (m)	Habitat
Senecio adnatus DC.	05/03/2018	Mount Gilboa	NU0092484	29°17'15" S 30°17'36" E	1751	Grassland
	04/04/2018	Sevontein Prison	NU0092469	29°45'47" S 30°03'01" E	1456	Dry savanna
Senecio affinis DC.	27/03/2018	Cumberland Nature Reserve	NU00924771	29°34'06" S 30°27'25" E	658	Grassland
Senecio bupleuroides DC.	19/03/2018	Hilton College	NU0092478	29°28'47" S 30°17'14" E	738	Savanna
Senecio coronatus (Thunb.) Harv.	13/12/2017	Ashburton Model Airplane Club	NU0092500	29°40'57" S 30°30'19" E	759	Dry savanna
	04/03/2018	Umgeni Valley	NU0092481	29°28'32" S 30°14'22" E	1012	Grassland
Senecio glaberrimus DC.	05/03/2018	Mount Gilboa	NU0092483	29°17'15" S 30°17'36" E	1751	Grassland
	19/03/2018	Hilton College	NU0092477	29°28'47" S 30°17'14" E	738	Savanna
Senecio inaequidens DC.	20/02/2018	Drakensberg 1	NU0092490	29°23'06" S, 29°54'08" E	1542	Roadside
	20/02/2018	Drakensberg 2	NU0092488	29°27'09" S, 29°45'55" E	1711	Roadside
	20/02/2018	Drakensberg 3	NU0092486	29°31'23" S, 29°38'46" E	1569	Roadside
Senecio inornatus DC.	20/02/2018	Drakensberg 4	NU0092489	29°24'11" S, 29°51'52" E	1510	Roadside

	20/02/2018	Drakensberg 5	NU0092485	29°38’47” S, 29°32’46” E	1472	Roadside
Senecio oxyriifolius DC.	05/03/2018	Mount Gilboa	NU0092499	29°17’15” S 30°17’36” E	1751	Grassland
Senecio panduriformis Hilliard.	04/04/2018	Sevontein Prison-	NU0092468	29°44’42” S 30°09’55” E	1481	Grassland
Senecio polyanthemoides Sch.Bip	16/12/2017	Ground Cover	NU0092498	29°23’20” S 30°10’31” E	1280	Grassland
	04/03/2018	Umgeni Valley	NU0092480	29°28’32” S 30°14’22” E	1012	Grassland
	19/03/2018	Hilton College	NU0092475	29°30’24” S 30°18’43” E	1108	Grassland
	19/04/2018	Sevontein Prison	NU0092467	29°46’09” S 30°08’52” E	1443	Grassland
Senecio retrorsus DC.	13/12/2017	Ashburton Model Airplane Club	NU0092499	29°40’57” S 30°30’19” E	759	Dry savanna
Senecio serratuloides DC.	19/03/2018	Hilton College	NU0092475	29°30’24” S 30°18’43” E	1108	Wetland
	04/04/2018	Sevontein Prison	NU0092466	29°45’23” S 30°09’21” E	1484	Wetland
Senecio skirrhodon DC.	06/02/2018	Mtwalume	NU0092495	30°29’29” S 30°37’60” E	9	Edge of sand dune
Senecio sp. nr *adnatus* DC.	16/12/2017	Ground Cover	NU0092497	29°23’38” S 30°10’46” E	1290	Dry grassland
	20/02/2018	Drakensberg 3	NU0092487	29°27’09” S 29°45’55” E	1569	Roadside
Senecio sp. nr *affinis* DC.	04/03/2018	Umgeni Valley	NU0092479	29°28’32” S 30°14’22” E	993	Grassland
Senecio sp. nr *conrathii* N.E.Br.	16/12/2017	Ground Cover	NU0092496	29°23’42” S 30°10’42” E	1237	Dry grassland
	19/03/2018	Hilton College	NU0092474	29°30’11” S 30°18’38” E	1141	Roadside/ grassland

Senecio sp. nr *serratuloides* DC.	19/03/2018	Hilton College	NU0092472	29°30’24” S 30°18’43” E	1108	Wetland
	04/04/2018	Sevontein Prison	NU0092465	29°45’05” S 30°09’32” E	1416	Edge of river/ grassland