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Genetic assessment and performance evaluation of *Lixus carinerostris* Boheman (Curculionidae): evaluating a potential biocontrol agent for invasive *Cryophytum crystallinum* L. (Aizoaceae)

By

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A thesis submitted in fulfilment of the requirements for the degree of Master of Science in Entomology

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January 2026

This thesis is dedicated to the future Dr Jackey Mukhawana, who is to be, because I am.

Acknowledgements

I would like to express my gratitude to the following people and organisations:

The National Research Foundation (NRF) for funding my MSc degree.

The United States Department of Agriculture (USDA) and the US Navy for the running costs of this research.

Rhodes University and the Centre for Biological Control (CBC) for granting me this opportunity to complete my MSc studies.

My Supervisor, Prof. Iain D Paterson, for his valuable feedback and wisdom. Iain's level of knowledge and passion for biocontrol is inspiring.

My co-supervisor, Ms Emma Sandenburgh, for all her assistance with my fieldwork throughout my MSc.

My co-supervisor, Dr Clarke van Steenderen, for his valuable assistance with my data analyses.

Prof. Shelley Edwards for her kindness, wisdom, and valuable advice and guidance in the Zoology and Entomology Molecular Laboratory (ZEML) at Rhodes University.

Prof. Martin Hill for all his support and inspiring words. Any postgraduate should get a chance to interact with this man.

The staff at the Centre for Biological Control (CBC) and the Department of Zoology and Entomology (DZE) at Rhodes University, with a special thanks to Mrs Jeanne van der Merwe and Mrs Ticia Swanepoel, for their assistance in organising payments and other administrative processes.

The Central Analytical Facilities (CAF) at Stellenbosch University for the ISSR fragment analysis.

My office mates, Soso and Deric, for all their jokes and encouragement throughout this journey.

My Honours year classmates, Riaan, Hlumelo, Karla, and Ntokozo. Going through this journey knowing I'm doing it with them has been extremely comforting

A special thanks to my friends, Dr Siya Mnciva, Nwabisa Magengelele, and Katlego Mogale. I honestly wouldn't have made it through this journey without their support.

Lastly, my parents, aunts, Johnson, Blessing, and John-jr Mukhawana. They are the reason I'm still grinding.

Abbreviations, Symbols, and Units

DNA – Deoxyribonucleic acid

mtDNA – Mitochondrial DNA

COI – Cytochrome oxidase subunit I

IAPS – Invasive alien plant species

IAS – Invasive alien species

ERH – Enemy Release Hypothesis

NUMTs – Nuclear Mitochondrial DNA Segments

ILS – Incomplete Lineage Sorting

Indels – Insertions and deletions

WHO – World Health Organisation

CBOL – Consortium for the Barcode of Life

USA – United States of America

PCR – Polymerase Chain Reaction

ISSR – Inter-Simple Sequence Repeats

SSR – Simple Sequence Repeats

AFLP – Amplified Fragment Length Polymorphism

RFLP – Restriction Fragment Length Polymorphism

nMDS – Non-metric multidimensional scaling

DAPC – Discriminant Analysis of Principal Components

PCA – Principal Component Analysis

GLM – General Linear Model

ZIP – Zero-inflated Poisson

ZINB -Zero-inflated Negative Binomial

MCMC – Markov Chain Monte Carlo

AIC – Akaike Information Criterion

ΔAIC – Delta AIC (difference in AIC values)

DF – Discriminant function

SE – Standard Error

CI – Confidence Interval

CSV – Comma-separated values

RFU – Relative Fluorescent Units

pp – Posterior Probability

β – Beta (regression coefficient, log-scale)

θ – Theta (dispersion parameter in negative binomial models)

α – Alpha (significance level)

p – p-value

n – Sample size

r – Correlation coefficient

exp (β) – Exponential of beta (multiplication effect)

R^2 – R-squared (coefficient of determination)

± – Plus or minus (For standard errors/confidence intervals)

% – Percent

∞ – Infinity

5’ – Five prime end (DNA/RNA)

3’ – Three prime end (DNA/RNA)

°C – Degrees Celsius

µl – Microliters

ml – Millilitres

bp – Base pairs

kb – kilobases

mm – Millimetres

cm – Centimetres

km – Kilometres

min – Minutes

h – Hours

s – Seconds

V/cm – Volts per centimetre

V – Volts

Abstract

Invasive alien plant species present a major threat to global biodiversity, ecosystem function, and economic stability. *Cryophytum crystallinum* L. (Aizoaceae) (Crystalline ice-plant), native to South Africa, has become invasive in California (U.S.A.), Mexico, Australia, and the Mediterranean, where it causes ecosystem degradation by increasing environmental salt concentration, displacing native species, and altering habitat structure. Classical biocontrol could offer a sustainable management strategy, but success depends on the proper characterisation of potential biocontrol agents. This thesis evaluated *Lixus carinerostris* Boheman (Coleoptera: Curculionidae), a stem mining weevil associated with *C. crystallinum* in its native range, as a potential biocontrol agent.

This thesis addressed three main objectives: 1. Determine whether *L. carinerostris* represents a single oligophagous species or a cryptic species complex across its four known *Cryophytum* host plant species (*C. crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi*); 2. Characterise the genetic structure of *L. carinerostris* populations; and 3. Assess weevil performance across all four host plant species.

Cytochrome oxidase subunit I analyses (69 specimens, 11 localities, 4 host plant species) revealed that *L. carinerostris* was a single panmictic species with low genetic diversity (0.77% nucleotide diversity) and no host plant species-specific or geographic structuring. Nuclear Inter-Simple Sequence Repeats analyses revealed that weevils associated with *C. pellitum* are genetically distinct from the other populations, while populations collected off other host plants showed significant genetic admixture. Phylogenetic analysis confirmed *L. carinerostris* to be a single species while also highlighting taxonomic inconsistencies within the broader *Lixus* genus. The absence of isolation by distance and high gene flow demonstrates strong dispersal capabilities across the three Cape provinces.

The field performance assessments revealed a mismatch between genetic homogeneity and developmental success across the four weevil/plant associations. Adult *L. carinerostris* demonstrated non-discriminatory feeding and oviposition behaviour. However, developmental outcomes varied considerably. *Cryophytum crystallinum* supported significantly higher pupal densities and adult emergence, *C. guerichianum* exhibited intermediate success, while *C. pellitum* and *C. barklyi* showed significantly reduced developmental success despite supporting larval establishment. This pattern exemplifies a partial evolutionary trap phenomenon, where oviposition efforts do not predict offspring performance.

The genetic differentiation observed in weevils associated with *C. pellitum* suggests that source populations for biocontrol should be collected preferentially from *C. crystallinum*, with *C. guerichianum*-associated populations serving as an alternative if needed, while *C. pellitum*-associated populations should be completely avoided.

The results suggest that *C. crystallinum* is *L. carinerostris*'s primary reproductive host plant species, supporting its potential as a biocontrol agent. The absence of other *Cryophytum* species in invaded regions minimises potential non-target risks to congeners. However, the resilience on a single host plant species in invaded regions necessitates careful consideration of plant phenology for successful weevil establishment. This research provides important baseline data for evidence-based biocontrol decision-making and emphasises the importance of integrating population genetics with ecological performance assessments in agent evaluation programs.

Table of Contents

List of Figures

51.28% of the between-group variance, followed by DF2, which captures 25.45% of the variance.

Figure 5. Bayesian clustering results from STRUCTURE analysis (MCMC = 50,000, burn-in = 100,000) of *Lixus carinerostris* ISSR data, showing assignment probabilities for K=2, K=3, and K=4. Each vertical bar indicates the individual's estimated membership coefficient (Q) in each of the inferred clusters. Samples are grouped by their host plant species: *Cryophytum crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi*. Replicate runs consistently support K=4 as the most likely number of genetic clusters.

Figure 6. Scatter plot showing the correlation between genetic distance (Jaccard index) and pairwise geographic distance (km) among *Lixus carinerostris* samples, utilising ISSR data. Each data point corresponds to a unique pairwise comparison. The dashed line represents the linear regression fit (Mantel r = 0.008, P = 0.435).

Figure 7. Map of performance data collection sites by host plant species.

Figure 8. *Cryophytum crystallinum* stem exhibiting severe chlorosis due to *Lixus carinerostris* developing inside.

Figure 9. Internal stem damage caused by developing *Lixus carinerostris.*

Figure 10. Descriptive summary plots of *Lixus carinerostris* performance between *Cryophytum barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C, pellitum* (yellow). Bar plots represent the mean counts from each *Cryophytum* host plant species across all measured performance parameters.

Figure 11. Comparative negative binomial GLM plot of the number of feeding holes created by *L. carinerostris* on the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant

observations with fitted regression lines from the negative binomial GLM. No significant host plant species effect was found ($p = 0.464$).

Figure 12. Comparative negative binomial GLM plot of the number of oviposition holes produced by *Lixus carinerostris* on the stems of *Cryophytum barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. No significant host plant species effect was found ($p = 0.297$).

Figure 13. Comparative negative binomial GLM plot of the number of *Lixus carinerostris* larvae inside the stems of *Cryophytum barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected ($p = 0.00144$).

Figure 14. Comparative negative binomial GLM plot of the number of *Lixus carinerostris* pupae inside the stems of *Cryophytum barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected ($p = 1$).

Figure 15. Comparative negative binomial GLM plot of the number of *Lixus carinerostris* pupae inside the stems of *Cryophytum barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations. Regression lines from the negative binomial GLM were not fitted due to model prediction failure. Statistically significant host plant species effects were detected ($p = 1$).

Figure 16. Comparative negative binomial GLM plot of the number exit/emergence holes created by *Lixus carinerostris* on the stems of *Cryophytum barklyi* (purple), *C. crystallinum*

(black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected ($p = 0.0228$)

List of Tables

Chapter 1 - General introduction

1.1 Invasive alien species

Biological invasions have become a major global concern, costing the world over $95.3 billion in management and $1130.6 billion in damages since 1960 (Seebens et al. 2018, Cuthbert et al. 2021). The IPBES Thematic assessment on invasive alien species (IAS) and their control has further reported that IAS have caused global economic losses of approximately $423 billion in 2019 alone, and the number of alien species are expected to increase by at least 36% by 2050, with economic costs also expected to continue rising (Thomas 2023). By definition, invasive alien species are those that establish, spread, and persist in new geographic areas outside their native distribution (Thomas 2023). Several plant species have been intentionally and unintentionally spread out of their native ranges into new regions, where some become invasive and cause major impacts on biodiversity, ecosystem services, and environmental quality (Richardson and Rejmánek 2011). More than 37000 alien species have been recorded globally, of which over 3500 are recognised as invasive, affecting terrestrial, freshwater, and marine systems, with impacts reported from biodiversity hotspots to landscapes that are heavily modified, and from low to high-income countries (Thomas 2023).

Over the past few decades, the world has experienced an alarming surge in the number of invasive alien plant species (IAPS) (Seebens et al. 2018, Rai and Singh 2020). This surge results from the expansion of anthropogenic movement, global trade, and the destruction of natural habitats (Vilà and Ibáñez 2011). Many plant species are introduced into novel regions every year; however, not all become invasive. Whether a plant becomes invasive depends on several factors, such as the biology of the plant, the abiotic characteristics of the exotic landscape, and the biotic interactions that occur with pre-existing organisms in novel regions (Mitchell et al. 2006).

1.2 Success of invasive alien species

Understanding the mechanisms behind the successful invasion of IAPS is a crucial component in attempting to control them and minimise their impacts. Most biologists concerned with IAPS adopt the framework set out by Richardson et al. (2000), which describes biological invasion as a series of barriers that a species negotiates and overcomes to become either naturalised or invasive in a novel environment (Richardson et al. 2000). The Richardson framework is individual-based and focuses on barriers that would prevent an individual from arriving at a novel location, its growth, and reproduction. This framework considers these three factors as hindrances to plant naturalisation and evaluates whether individuals can survive, set viable seeds, or spread and persist through vegetative means.

Although this is a useful framework, it does not consider propagule pressure as a significant factor in the success of IAPS. As a result, a unified framework that merges multiple approaches, including those that consider propagule pressure as a major influence on IAPS success was proposed (Blackburn et al. 2011). The Blackburn et al. (2011) unified framework recognises that the biological invasion process can be divided into a series of stages and that in each stage, there are barriers that a species or population needs to overcome to enable it to progress. Although this was apparent in other frameworks, the unified framework makes it explicit that these biological invasion process stages are separated by barriers that must be overcome for a species or population to pass on to the next stage, and how IAPS overcome these barriers. This unified framework brings about a more comprehensive understanding of how species with certain advantageous characteristics can successfully navigate through invasion stages. Blackburn et al's (2011) framework also shows the best course of action at each stage of the invasion process. It also highlights the point where neither eradication nor containment is possible.

Successful IAPS are characterised by high net primary production, phenotypic plasticity, rapid growth, high fecundity, long-range seed dispersal, and resistance to pests and pathogens that already exist in the invaded areas (Davidson et al. 2011, Jelbert et al. 2015, Zhang et al. 2023). These characteristics enable IAPS to exploit available resources and establish themselves more effectively in new environments than native species do. This is further perpetuated by the absence of their coevolved natural enemies that keep them in check in their native distribution, as outlined in the Enemy Release Hypothesis (ERH) (Keane and Crawley 2002). This success occurs despite the genetic paradox associated with biological invasions, which posits that invasive populations should suffer from experiencing a reduction in genetic diversity due to founder effects and genetic bottlenecks during the introduction process (Dlugosch and Parker 2008a, Estoup et al. 2016). However, many IAPS maintain enough genetic variation or compensate using alternative mechanisms like phenotypic plasticity and rapid evolutionary changes, enabling them to thrive even with potentially limited genetic diversity (Dlugosch and Parker 2008a, Davidson et al. 2011).

The ERH posits that IAPS should experience relatively decreased control by herbivores and other natural enemies in exotic/introduced regions, compared to their native range (Heger and Jeschke 2014). This hypothesis explains the success of plant invasion on the basis that natural enemies are important for regulating plant populations, they have a higher impact on native than on exotic plant species, and that plants are capable of capitalising on a reduction in enemy regulation, leading to increased population growth (Heger and Jeschke 2018). Although several factors contribute to the successful invasion of a plant in a new environment, the consensus findings in the literature show that the concept of enemy release plays a major role in most successful IAPS (Theoharides and Dukes 2007).

1.3 Drivers of invasion

Understanding the drivers of biological invasion is crucial in developing effective control and prevention strategies. Drivers of biological invasion are multifaceted and involve direct and indirect factors that operate at various scales. Climate similarity between the exotic and native ranges of a species' distribution is considered an essential requirement for successful invasions (Gallardo et al. 2015). Other abiotic factors can also play important roles in determining invasion success. Geomorphology, climate, and soil characteristics play key roles in determining the nature and intensity of land use and the influence that land use has on biological invasions (Bellard et al. 2016). Global climate change is one phenomenon that aids biological invasion and has led to drastic shifts and changes in climatic patterns and conditions, rendering environments unsuitable for the survival of native plant species, thus opening niches for potential biological invaders (Pauchard et al. 2016, Hulme 2017).

The presence and abundance of IAPS is influenced by socioeconomic activities (Cuthbert et al. 2021) such as transport and tourism, which are strongly associated with the pathways of introduction (Hulme 2009) or the intensity of environmental disturbances caused by anthropogenic activities (Bellard et al. 2016). Anthropogenic activities such as land clearing, urbanisation, agriculture, and landscaping can cause disturbances that favour the establishment of IAPS (Potgieter et al. 2017, 2018).

The diversity invasibility hypothesis was first proposed by Charles Elton over six decades ago, stating that species-rich communities are less susceptible or more resistant to biological invasion because greater species richness means fewer available niches for newcomers to occupy (Feng et al. 2019). Darwin posited that alien species are more likely to successfully invade native communities if they are phylogenetically distantly related to them (Darwin's naturalisation hypothesis), because the distance in phylogeny may indicate differences in

niches, which may favour coexistence (Darwin and Peckham 2006). Both hypotheses have been confirmed in grassland communities (Feng et al. 2019). In a study by Feng et al. (2019), both Elton's diversity invasibility hypothesis and Darwin's naturalisation hypothesis were clearly shown to be interlinked in that it is highly likely that even though there is high species diversity in the native region if the alien species is phylogenetically distant, it stands a much better chance of occupying new niches or invading already occupied niches by outcompeting the species that already occupy those niches.

1.4 Impacts of Invasive Alien Plant Species

Although some IAPS can be beneficial to communities in the invaded areas (Hayes and Holzmueller 2012), research has shown that the negative impacts on local biodiversity and ecosystem processes outweigh the societal benefits in most cases (Carboni et al. 2021, Sobuj and Byun 2023). Invasive alien plant species cause negative impacts across multiple dimensions: ecological impacts via competition and habitat modification, economic impacts via agricultural and management costs, and social impacts via effects on human communities and cultural practices (Van-Wilgen and De-Lange 2011, Vilà et al. 2011). It has been suggested that in some systems, invasive species create long-lasting changes in soil chemistry, alter fire regimes, or establish dense monocultures that prevent the reestablishment of native species even after the removal of the invasive species (Gibbons et al. 2017).

1.4.1 Ecological Impacts of Invasive Alien Plant Species

Biological invasions are one of the main drivers of biodiversity loss in many parts of the world (Hester and Harrison 2007). In some cases, the introduction of non-native plant species to new regions endangers or even leads to the extinction of indigenous organisms (Lowe et al. 2000). Several experimental studies have demonstrated how IAPS succeed in depleting native biodiversity by competition or hybridisation with native species and disturbing ecological

processes in natural habitats by altering nutrient cycles, hydrology, and fire regimes (Vilà et al. 2011).

The magnitude of this problem is particularly concerning, given the alarming rate at which the world is losing its biodiversity (Briggs 2016). One of the most serious ecological impacts of IAPS is their contribution to the ongoing global biodiversity crisis, with a major concern being that IAPS threaten species already on the verge of extinction (Pimentel et al. 2005). This impact is quantified by the fact that invasive alien species are the primary factor in listing approximately 42% of the United States' species as threatened or endangered (Pimentel et al. 2005, Mikeladze 2015). Globally, invasive alien species impact approximately 25% of all threatened species (Smith, 2020).

The case of European purple loosestrife (*Lythrum salicaria* L. (Lythraceae)), introduced in the United States in the 19th century as an ornamental plant (Malecki et al. 1993), exemplifies how the ecological impacts of a single IAPS can cascade through an entire ecosystem. The species has reduced the biomass of 44 native plants and endangered wildlife, including the bog turtle and several duck species that depend on these native plants for food (Pimentel et al. 2005). The ripple effect of such displacement signifies how IAPs can fundamentally alter food webs and ecological relationships that have coevolved over millennia. Hawaii has experienced similar effects from invasive alien species, with over 800 native species endangered and over 200 already extinct (Pimentel et al. 2005). These examples represent just a fraction of the many IAPs that have catastrophic global impacts on ecosystems and communities (for more examples, see (Vilà et al. 2011).

Beyond direct species-to-species competition, IAPS alter ecosystem structure and function through habitat modification. Alien trees and shrubs displace native vegetation over large areas, resulting in native species diversity loss, soil property alterations, and changes in ecosystem

dynamics (Borgmann and Rodewald 2005, Pimentel et al. 2005, Mikeladze 2015). Such changes usually bring about positive feedback loops that favour further invasion while making it increasingly difficult for native species to recover.

Many IAPS form dense monocultures, which are a particularly problematic form of habitat modification. *Chromolaena odorata* (L.) R.M. King and H. Rob. (Asteraceae) exemplifies such patterns. This species is of concern in agricultural lands and commercial tree plantations, as it forms dense stands that prevent the establishment of other plant species (Andreu and Vilà 2010). Other IAPS with effects comparable to *C. odorata* include *Phragmites australis* (Cav.) Trin. Ex Steud (Common reed), *Hakea drupacea* (C.F. Gaertn.) Roem. & Schult., and *Ailanthus altissima* (Miller) Swingle. (tree of heaven) (Lazzaro et al. 2020). This monopolisation of space and resources fundamentally alters the structure and diversity of plant and microbial communities, with cascading effects throughout the ecosystem (Andreu and Vilà 2010, Lazzaro et al. 2020).

1.4.2 Economic Impacts of Invasive Alien Plant Species

Invasive alien plant species cause significant economic losses in the agriculture, pastures, and forestry sectors (Hanley and Roberts 2019, Sobuj and Byun 2023). *Lythrum salicaria*, for example, now occurs in over 48 states in the U.S and costs over $45 million per annum in control measures and forage losses (Pimentel 2009). Additionally, occurrences of IAPS in grazing lands pose profound economic challenges for livestock-dependent communities. For example, the South American Mesquite *(Prosopis juliflora* (Sw.) DC. (Fabaceae)), has invaded pastures in Asia, Australia, and Africa, competing with native vegetation and significantly reducing livestock feed (William and Jafri, 2015). Such widespread degradation of pastures not only affects immediate livestock production but also undermines the long-term

sustainability of pastoral systems that support many community livelihoods across the globe (William and Jafri 2015).

1.4.3 Social Impacts of Invasive Alien Plant Species

Invasive alien species can indirectly affect human well-being by disrupting natural environments and the provision of ecosystem services (Jones 2017). The United Nations Environment Program (2016) has identified environmental shocks resulting from invasive species as a significant threat to human well-being. Furthermore, the World Health Organisation (WHO) (2015) has indicated that the ecological impacts of invasive species pose an ongoing threat to human lifestyles and community interactions. These impacts are evident in instances of extensive water invasions by IAPs, such as water hyacinth (*Eichhornia crassipes* (Mart.) Solms (Pontederiaceae)), which significantly reduce or even prevent recreational activities like boating, fishing, and various water sports (Coetzee et al. 2021). The invasion of water hyacinth in Hartbeespoort Dam serves as a pertinent example, where the plant has formed dense mats over the water surface, trapping boats and obstructing access to the dam (Moffat et al. 2024). It is clear that effective, sustainable, and cost-efficient control methods for IAPS are necessary from ecological, economic, and social perspectives.

1.5 Control of Invasive Alien Plant Species

“Prevention is better than cure”, and so the primary goal of invasive species management should be to prevent the introduction of IAPS into novel environments (Sobuj and Byun 2023). Prevention strategies focus on robust biosecurity frameworks, including border control, prohibited species lists, and quarantine systems, that have proven to be effective for preventing invasions at the earliest stages (Reaser et al. 2020). However, when prevention measures fail, and IAPS have successfully invaded a new environment, eradication is considered the best course of action (Culliney 2005).

A set of conditions must be met for the successful eradication of any population (Bomford & O'Brien, 1995). These include proper planning, a commitment to complete the eradication process, ensuring the entire population of the target species is removed, and preventing re-invasion. Additionally, it is important to obtain support from local community members and to have the ability to show how eradicating invasive species will be beneficial (Mack and Lonsdale 2002).

Plants are difficult to eradicate, even in small populations, because they have dormant life stages, such as soil seed banks and high intrinsic rates to increase even in isolated populations (Mack and Lonsdale 2002). The eradication of plant populations requires a long campaign that involves the sustained removal of individuals before they set their seeds, but there are no cases in the literature where this was successfully performed, except on islands (Hulme 2020). This could be because it is difficult to detect IAPS in their early stages, and usually, by the time they are noticed to be problematic, they have already dispersed their seeds (Mack and Lonsdale 2002). Furthermore, the rate at which most invasive species establish greatly outstrips the available resources for their eradication (Green and Grosholz 2021).

If a species has already extensively invaded an environment, eradication is often not economically feasible owing to limited resources for control. Therefore, different control measures/methods for IAPS can be employed, namely, mechanical (includes both physical and cultural methods (assisted revegetation, burning, grazing, clipping, mowing, hand pulling, etc.)), chemical (herbicides) and biological control (pathogens, parasites, phytophagous insects, etc.) (Van-Wilgen and De-Lange 2011, Sobuj and Byun 2023). These control methods aim to reduce IAPS populations to levels where their negative impacts are mitigated to acceptable thresholds, rather than achieve complete eradication.

Selecting an appropriate and effective control method is not an easy process because different IAPS display different ecological and physical processes. Furthermore, different components of the control strategies must be considered and optimised before selecting the most effective control measure or strategy. These include the frequency of control treatment application, the combination of different control measures, and the duration of the monitoring period following treatment application (Sobuj and Byun 2023).

Chemical and mechanical control measures are usually not sustainable in the long term, as follow-up applications remain necessary over the extended periods of time (Li et al. 2020). Although the effects of chemical control on native plant species may be temporary, so are its effects on the IAPS, as reinvasion has been seen to occur after the "successful" removal of the IAPS through chemical control (Kaiser-Bunbury et al. 2015). Given the limitations of both mechanical and chemical control, specifically regarding their short-term effects and need for repeated applications, biological control often emerges as a more sustainable alternative (Abbas et al. 2018).

1.6 Biological control

Biological control (biocontrol) is a method that uses living organisms, typically natural enemies of the target IAPS sourced from its native range, to suppress IAPS populations in areas where they have become problematic. The practice encompasses multiple approaches for managing and controlling invasive species using their natural enemies. Classical biocontrol can be defined as the study and use of natural enemies of an invasive species from one geographic region to another to permanently suppress populations of invasive species, and is the most widely used method to control IAPS (Olckers et al. 2021, Lesieur et al. 2023). Other forms of biocontrol include conservation biocontrol (enhancing existing natural enemy populations through habitat management), augmentative biocontrol (mass-rearing and periodic release of

natural enemies), and inundative biocontrol (repeated releases of large numbers of biocontrol agents for immediate suppression) (Eilenberg et al. 2001, Van Driesche and Hoddle 2009). Biological control offers the potential for long-term self-sustainability and effective management, requiring minimal to no maintenance intervention, particularly in sensitive environments like protected areas or riparian habitats (Ballal 2022). Biocontrol is both cost-effective and economically sustainable (Van-Wilgen and De-Lange 2011, Ballal 2022).

It has been over 200 years since the release of the first insect biocontrol agent against prickly pear (*Opunctia ficus-indica* (L.) Mill (Cactaceae)) in India (Harris 1991). Since then, biocontrol of invasive alien plants as a scientific practice has grown and developed exponentially, with considerable success (Hokkanen and Sailer 1985). By 2012, 468 species of biocontrol agents had been released against 175 IAPS globally, with 65.7% of target IAPS experiencing some level of control and over 50% of established agents causing substantial damage to target IAPS (Schwarzländer et al. 2018).

Like any other control method, classical biocontrol has its shortcomings. The unpredictable nature of ecological interactions means that biocontrol outcomes can differ significantly across different environments and time scales, rendering it difficult to guarantee consistent results. Climate change and habitat modification can further alter the effectiveness of biocontrol programs by affecting the physiology and distribution of both the target species and the biocontrol agents, as well as how they interact with one another (Thomas et al. 2001, Hellmann et al. 2008).

Other limitations include direct and indirect non-target impacts, host shifts by biocontrol agents, dispersion to unwanted areas, and disagreements about its success in the field (Walsh et al. 2023). Direct non-target impacts occur when biocontrol agents attack and damage closely related native species. For example, *Rhinocyllus conicus* Frolich (Coleoptera: Curculionidae),

an insect that was introduced into North America to control exotic thistles, subsequently has attacked several native thistle species, reducing seed production. This type of impact can be avoided through the use of host-specificity testing before releasing the biocontrol agent. On the other hand, indirect non-target impacts involve the disruption of ecological interactions and relationships without direct physical damage to the non-target species. These may include the disruption of pollinator networks, food web dynamics, or competitive relationships and are more difficult to predict (Suckling and Sforza 2014).

For an organism to be considered a potential biological control agent, it must be host-specific (i.e. have a limited host range) to minimise the risk of non-target effects (Briese 2000b). If a biocontrol agent is host-specific, its safety will not be compromised by the presence of alternative hosts (Stiling and Cornelissen 2005). An ideal biological control agent primarily attacks and damages the target weed while causing minimal to no impact on native and/or economically important plants (Briese 2000b, Müller-Schärer and Schaffner 2008).

1.7 Host specificity in biological control

The success of any given biocontrol program can be attributed to several factors, including the characteristics of the IAPS, the biocontrol agent, the target ecosystem, and the methods of selecting, propagating, and introducing the biocontrol agent (Hokkanen and Sailer 1985). Given that biocontrol involves the deliberate introduction of non-native species to combat IAPS while conserving native biodiversity, conservationists are interested in biocontrol as a tool for conservation biology; however, they are concerned about the possible risks of non-target effects of biocontrol agents (Gurr et al. 2002). This is where the concept of host specificity becomes a crucial component of biocontrol programmes.

Generalist phytophagous insects are usually not considered for the biocontrol of weeds as they are more likely to pose risks to non-target plant species (Driesche and Hoddle 2017, Loomans

2021). Phytophagous insects used for biological control must be restricted in their host range to feeding on the target weed (i.e. be host-specific) or a small group of closely related plant species that are absent or not important in the region where the agent is intended to be released (Hinz et al. 2014, Nawaz et al. 2023). The host specificity of a biocontrol agent is a very important component of weed biocontrol (Marohasy et al. 1992, Pearson and Callaway 2003, 2005) and can be defined as a measure of how selective/specific biocontrol agents are to the target weed (Van Klinken and Edwards 2002).

Biological control agents are meticulously selected and rigorously tested to evaluate and ensure their degree of host specificity before they are released in the field (Sheppard et al. 2005). This is done by bringing potential biocontrol agents from their indigenous distribution to quarantine facilities where their behaviour and host affinity/specificity are evaluated and monitored before they can be approved for field release (Buckingham 1992). This is done by exposing the potential biocontrol agent to the target plant species and other non-target species (usually close relatives of the target species) to evaluate the agent's host preference and affinity (Briese and Walker 2002, Heard 2002). Host specificity testing involves an organised series of laboratory and/or field studies where the potential biocontrol agent is presented with a range of plant species in controlled conditions to assess feeding behaviour, reproductive success, and survival rates (Van Klinken and Edwards 2002).

The host specificity protocols include no-choice tests (whereby the insects are given no choice but to feed on one plant species or die, with the target IAPS included as a control), choice tests (whereby the insects are exposed to multiple plant species simultaneously), and open field tests in the insect's indigenous range to evaluate the potential biocontrol agent's host range (Briese and Walker 2002, Heard 2002, Paynter et al. 2015). These tests are conducted on a carefully

selected range of host plant species that represent potential non-target plants at risk (Nechols et al. 1992, Heard 2002, Driesche and Hoddle 2017).

Host specificity testing provides relevant and valuable information that is used to assess the safety of potential biological control agents before their release (Marohasy 1998). Although laboratory host-specificity testing does not replicate or realistically simulate field conditions, it can inform risk analysis by showing which native plants or valued exotic plants are at risk or within the natural fundamental host range of the biocontrol agent. Selecting relevant and feasible non-target species for host-specificity testing can be very challenging, especially for a weed with many closely related species. It is impractical to implement host-specificity testing on all close relatives of the target weed; therefore, test plant species must be prioritised based on criteria such as biogeographic overlap, phylogenetic affinity, and ecological similarity (Briese and Walker 2002). A biocontrol agent's host affinity and efficacy can be easily influenced by its adaptation to host plant species, geographic climate conditions, or both, a concept known as local adaptation (Fowler et al. 2012, Müller-Schärer et al. 2020, Hinz et al. 2024). While host specificity testing provides valuable safety information, it is crucial to recognise that the host range and preferences of potential biocontrol agents are not static; they can be influenced by evolutionary processes that happen over time (Hanks and Denno 1994, Mopper and Strauss 2013). This has significant implications for biocontrol success and safety.

1.8 Plant-insect coevolution: Local adaptation and host specialisation

Local adaptation is driven by natural selection, whereby insects with traits that increase their fitness are more likely to survive and reproduce, leading to the overall accumulation of those advantageous traits in the population over time in a particular geographic region and/or host plant species (Dudaniec et al. 2018, Wadgymar et al. 2022). This evolutionary process has been observed in many insect groups. For instance, *Drosophila suzukii* Matsumura (Diptera:

Drosophilidae), an invasive fruit fly, exhibits rapid local adaptation to seasonal host fruits. Populations demonstrate a heightened preference for oviposition and improved offspring performance on the fruit species from which they originated, indicating strong genetic adaptation to their local hosts (Olazcuaga et al. 2022). Beyond genetic changes, the gut microbiota of *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae), the Fall webworm moth, facilitates adaptation to novel host plants, enhancing larval survival and compatibility with previously unsuitable plants (Zhang et al. 2024a). Furthermore, *Henosepilachina niponica* Lewis (Coleoptera: Coccinellidae), the ladybird beetle, exhibits asymmetric local adaptation to different thistle species, with populations performing optimally on their native host plants, suggesting early-stage population divergence driven by host specialisation (Nakasone et al. 2024). These examples highlight the capacity of insects to swiftly adapt to local conditions and specific hosts, typically through physiological, genetic, and microbial mechanisms, which is a critical factor in their success as biocontrol agents or invasive species.

1.8.1 The concept of coevolution and reciprocal adaptation

The term “coevolution” was only coined in the mid-1960s in attempts to explain how insects adapt to their host plants (Ehrlich and Raven 1964). This concept was groundbreaking in that it recognised that plants and their associated herbivores do not evolve in isolation but rather shape each other’s evolutionary trajectories through reciprocal selection pressures (Futuyma 1986). A prevailing theory suggests that locally occurring herbivores (including phytophagous insects) with shorter generation times relative to their host plants have the opportunity to adapt more rapidly to their local hosts than insects with longer generation times or those without regular exposure (Kniskern and Rausher 2001).

Several hypotheses have emerged attempting to explain coevolutionary mechanisms. One proposed hypothesis states that a plant lineage that evolves a novel defence trait against the

herbivores feeding on it may escape into an enemy-free “adaptive zone”, leading to diversification. Additionally, the associated herbivores were hypothesised to then evolve counter-defence characteristics and diversify along with the diversifying plant lineages, therefore, resulting in bursts of speciation in both the plants and their associated phytophagous insects (Ehrlich and Raven 1964). This hypothesis was very challenging to test, especially since Ehrlich and Raven provided no mechanisms for how to test it (Agrawal and Zhang 2021).

Ehrlich and Raven’s hypothesis was not tested until 19 years later in a study that evaluated the relationship between plants in the parsley family (Apiaceae) and swallowtail butterflies (Papilionidae) (Berenbaum, 1983). In this study, Berenbaum (1983) analysed the stepwise process laid out by Ehrlich and Raven (1964), evaluated the evidence for each, and found that the biosynthesis and counter-adaptive physiological traits between the different members of the parsley family and their associated phytophagous insects were evolutionarily complementary. Historically, the plants synthesised linear furanocoumarins, but the insects were able to feed on them, resulting in them evolving toxic angular furanocoumarins. Furanocoumarins are phototoxic chemical compounds that get incorporated into the DNA of insects or pathogens and inhibit DNA replication, leading to the insect/pathogen’s demise (Berenbaum, 1978).

Further studies found that cytochrome P450 monooxygenases were key to understanding how these insects detoxify themselves (Berenbaum et al. 1996, Calla et al. 2020). This research demonstrated that coevolution is not simply about congruent and parallel phylogenies of plants and their associated herbivores but rather involves the convergence of defence-offence interaction evolution. If an insect coevolves with a plant species with a certain set of defence mechanisms, they can potentially use other distantly related plants with similar defences as hosts. But as closely related species are more likely to have similar defence mechanisms, it is

closely related plant species that usually make up the host range of specialist herbivorous insects.

Bernays and Chapman (2007) demonstrated how important it is to understand why and how insects select their hosts. The authors demonstrated how the morphological characteristics of plants may influence their acceptability as hosts for insects by presenting visual appeal and/or providing a suitable substrate for insect locomotion and palatable plant tissues. Furthermore, phytophagous insects use volatile organic compounds and other chemicals as cues to locate and evaluate potential host plants (Poldy 2020, Binyameen et al. 2021). These chemicals can serve as repellents or attractants that allow insects to differentiate unsuitable host plants from suitable ones (Bernays and Chapman 2007, Poldy 2020, Binyameen et al. 2021).

Plants evolve particular physical and chemical traits in attempts to defend themselves against their associated herbivores. In turn, the insects develop adaptive traits that allow them to tolerate and even become resistant to these defensive traits (e.g. toxins), a phenomenon called reciprocal adaptation (a mechanism of how coevolution occurs) (Futuyma 1986). For example, *Heliconius* Kluk (Lepidoptera: Nymphalidae) larvae develop and feed on *Passiflora* L. (Passifloraceae). *Passiflora* evolved toxic physical and chemical defence traits, such as the production of cyanogenic glycosides, to defend against *Heliconius* herbivory (Agrawal and Kotanen 2003). In turn, some species of *Heliconius* evolved the capacity to tolerate or sequester high amounts of these potent compounds (Cavin and Bradley 1988, Agrawal and Zhang 2021). Therefore, in cases where an insect species has multiple host species with different defence mechanisms, different populations found on different host plant species may develop tolerance/resistance to the defences of their respective host plant species, provided the host plant species occur in allopatry. If the host plant species exist in sympatry, insects may be

exposed to multiple host plant species and their defence compounds, potentially allowing them to evolve tolerance to the defences of all of their host plant species.

1.8.2 Host range classification in phytophagous insects

There is a spectrum between insects that feed only on one plant species and those that feed on a wide range of plant species (Bernays and Chapman 2007). Many authors group insects into categories based on their host-plant ranges. Commonly recognised categories are: monophagous, oligophagous, and polyphagous (Björkman and Larsson 1991, Thompson 1998, Ge et al. 2025).

1.8.2.1 Monophagous insects

Monophagous insects are those that feed on one species or a group of species within a single genus (Cates 1981, Ge et al. 2025). These are insects that likely use similarities in the traits of these plant species to select and determine the acceptability of the plants (Bernays and Chapman 2007). Examples of monophagous insects include *Agrilus planipennis* Fairmaire. (emerald ash borer) which exclusively feeds on *Fraxinus* L. (Oleaceae) spp. (ash trees) and has become a serious invasive species in eastern North America (Gandhi and Herms 2010), and *G. calmariensis*, a biocontrol agent for *L. salicaria* (Loosestrife) (Blossey et al. 1994).

1.8.2.2 Oligophagous insects

Oligophagous insects are those that feed on a group of plants in different genera, but the same family (Ge et al. 2025). Similar to monophagous insects, this group may also use similarities between the different plants within the same family to determine their acceptability (Bernays and Chapman 2007). For example, the thistle biocontrol agent, *Cassida rubiginosa* Muller (Coleoptera: Chrysomelidae), feeds on several thistle and knapweed species of the *Cardueae* tribe (Hettiarachchi et al. 2023).

1.8.2.3 Polyphagous insects

Polyphagous insects feed on plants from a wide range of families. These insects probably utilise several cues to select and determine the acceptability of plants (Bernays and Chapman 2007). For example, *Spodoptera frugiperda* J.E. Smith (fall armyworm) feeds on over 350 plant species across multiple families and has become invasive in many parts of the world (Togola et al. 2025). Other terms used to categorise insects based on their feeding include stenophagous for insects with restricted host ranges, and euryphagous to group insects with a wide host range (Bernays and Chapman 2007). These terms will not be used in this study; instead, monophagous, oligophagous, and polyphagous will be used.

1.8.3 Cryptic species and Host Ranges

Allozyme studies of the host-specific *Apiomorpha* Rubsaamen (Hemiptera: Eriococcidae) cryptic species complex demonstrated that speciation of phytophagous insects parallel to host specificity on genotypes of the same plants is not always accompanied by morphological changes, resulting in the emergence of cryptic species (Cook and Rowell 2007). Cryptic species are species that are morphologically indistinguishable but genetically distinct (Beheregaray and Caccone 2007, Bickford et al. 2007, Rouxel et al. 2013, Marchán et al. 2018). In fact, the actual number of species on earth is most likely far greater than the current tally of nominal species, most of which are classified using the typological/morphological species concept.

Over the past decade, studies began to address the misclassification of certain species, facilitated by the advent of increasingly accessible, cost-effective, and rapid DNA sequencing technologies available to biologists (Bickford et al. 2007, Smith et al. 2018). One of the most notable applications of molecular techniques came from the analysis of the neotropical skipper butterflies *Astraptes fulgerator* Welch (Lepidoptera: Hesperiidae), which showed that the presumed single species actually represented at least ten genetically and somewhat ecologically

distinct cryptic species (Hebert et al. 2004). These new molecular tools are also adding to the discovery of new species that are prospective biological control agents by revealing monophagous populations within species that were previously believed to be polyphagous (Smith et al. 2018).

The abundance of cryptic species may have a major positive impact on the improvement of finding safe and effective biocontrol agents, as seemingly polyphagous insect species may in fact comprise of several monophagous insects, each of which may be suitably host-specific to be used as an agent against a particular host plant (Smith et al. 2018). The presence of cryptic species may also have some negative implications for biocontrol. Two or more allopatric populations may hide differences that are not morphologically visible; therefore, it is very important to incorporate genetic screening when identifying potential biocontrol agents, as at times subtle morphological differences (if any at all) can be easily overlooked as intraspecific variation (Paterson et al. 2016). This is very important because within these allopatric populations, there may be groups that are more host-specific than others, and populations that may be more effective at controlling an IAPS than others due to local adaptation to specific plant genotypes (Smith et al. 2018), making them more suitable for biological control.

1.8.4 Local adaptations in weed biocontrol

Potential biocontrol agents are adapted to the climatic conditions of their native range, which may enhance their establishment and effectiveness when introduced into similar environmental conditions (Stevens and Rizzo 2008, Harms et al. 2021, Gibson et al. 2024). Therefore, it is sensible to export them from regions with similar climates to the region they are to be released to increase the probability of successful establishment and spread. Furthermore, empirical research shows that parasites (pathogens, parasitoids, and phytophagous insects) are often adapted to the local populations of their hosts, to the extent that they perform better on

genotypes from their local populations than others (Lively et al. 2004, Greischar and Koskella 2007). This, however, is not always the case, as local adaptation can fluctuate through time and space (Lajeunesse and Forbes 2002). Therefore, it may be questionable to assume that potential biocontrol agents will be adapted and specific to certain populations of the target weed in the native range, as environmental conditions – including host plant availability, climate, and ecological interactions - can significantly vary across different locations and time periods (Stevens and Rizzo 2008, Hinz et al. 2024). This spatial and temporal variation in adaptation means that biocontrol agents collected from one area of the native range may not perform equally well against target IAPS from other areas, even within the same native geographic region (Sutton et al. 2021, Hinz et al. 2024, Istas and Szűcs 2025). Regarding phytophagous insects and parasitoids, the insect develops on a single host per generation. In such a case, the selection pressures exerted on one generation to perform well on a host genotype may differ from those of the generations before. This can lead to negative frequency-dependent selection and dynamically changing patterns of local adaptation and maladaptation of the phytophagous insect or parasitoid (Hufbauer and Roderick 2005).

These evolutionary dynamics present significant challenges for biocontrol practitioners in selecting potential biocontrol agents. They must decide where they should search for potential biocontrol agents. One option is to prioritise searching for potential biocontrol agents in regions with a similar climate to the introduction area, which might maximise establishment success but risk introducing agents poorly matched to the local host plant genotype (Goolsby et al. 2006). Alternatively, biocontrol practitioners could focus on the geographical origin of the target IAPS to find insects that are adapted to the genotype. A third option involves searching away from the area of origin to find new associations that might offer different advantages (Goolsby et al. 2006). Finally, they could consider searching for biocontrol agents on distantly

related plant species with similar chemical profiles as the target IAPS, which might reveal novel agent-plant associations.

Studying population structures of insects (specifically dispersion and breeding behaviour) through molecular techniques can assist in detecting phenomena such as local adaptations of insects to their hosts and even differentiation at a local scale. These genetic approaches can uncover cryptic species complexes, identify locally adapted populations, and assist biocontrol practitioners in selecting the most appropriate populations for biocontrol (Goolsby et al. 2006, Cook and Rowell 2007, Paterson et al. 2016). Understanding the genetic structure and ecological behaviours of potential biocontrol agents is important for both maximising efficacy through selection of appropriately adapted populations and ensuring safety through identification of host-specific lineages.

1.9 Study System

Cryophytum crystallinum (L.) N.E.Br. (Aizoaceae) (formerly called *Mesembryanthemum crystallinum* L.), commonly known as the Crystalline ice plant, is a prostrate succulent annual plant that is indigenous to Southern Africa (Gerbaulet 2017). *Cryophytum crystallinum* has invaded several parts of the world: Australia, Mexico, Chile, the Caribbean, the Mediterranean, and the Western United States of America (Figure 1) (Adams et al. 1998, Olckers et al. 2021). This plant has epidermal bladder cells (modified trichomes) in which it accumulates extensive amounts of salt, giving it an ice-like appearance (Olckers et al. 2021). In addition to being a halophyte, *C. crystallinum* can survive and thrive under adverse environmental conditions that have shown to be lethal to most plants (Kloot 1983, Adams et al. 1998). Furthermore, *C. crystallinum* is cold and drought-tolerant, making it a successful invader of arid environments across the globe (Vivrette and Muller 1977, Kloot 1983, Olckers et al. 2021). Additionally,

Olckers et al. (2021) reported that *C. crystallinum* responds rapidly to rain and has a swift growth rate that accelerates under stressful conditions.

Outside its native range, *C. crystallinum* is considered a major threat to indigenous ecosystems and biodiversity (Moran 1950, Adams et al. 1998). After establishing itself in a novel environment, *C. crystallinum* accumulates large quantities of salts in its bladder cells. When it reaches a state of senescence, all the salt leeches out of the dying plant and into the surrounding environment, increasing the topsoil salinity and rendering the environment unsuitable for other plants to grow (Kloot 1983, Olckers et al. 2021). This plant has significant adverse effects on the environments it invades. It has led to the degradation of habitats in California (Vivrette and Muller 1977), deterioration of pastures in Australia (Kloot 1983), and has led to the reduction of the coastal cover of Egypt's indigenous annual plants by approximately 68%, and total plant cover by approximately 48% (El-Ğhareeb 1991). Olckers et al. (2021) reported that surveys for potential biological control agents for *C. crystallinum* commenced in 2019, with at least three promising candidates (*Lixus carinerostris* Boheman (Coleoptera, Curculionidae), *Calodemas prolixus* Faust (Coleoptera, Curculionidae) and one Urodontidae species).

According to the enemy release hypothesis (ERH), *C. crystallinum* has a competitive advantage over native plant species of the exotic geographic locations it invades because of the absence of its natural enemies that control its spread and growth rate in its native distribution (Keane and Crawley 2002). In South Africa, *C. crystallinum* is naturally controlled by at least two weevil species, *L. carinerostris* and *C. prolixus,* which are the plant's most abundant natural enemies (Louw 1993). Adult *Lixus carinerostris* feed on the surfaces of mature stems and leaves, and create pin-sized holes in the epidermal tissues of the plant internodes where they oviposit, and subsequently cover the eggs with faecal matter (Louw 1993). The eggs hatch into larvae, which feed and develop in the stems of *C. crystallinum* (destroying the vascular tissues

of the plant, rendering it unable to transport water and nutrients from the roots to the rest of the plant, leading to loss of turgor, permanent wilting point, and death) until they reach adulthood and create exit holes using their mandibles (Louw 1993). *Calodemas prolixus* larvae feed on the roots, girdling the taproot and consuming smaller roots before creating pupation chambers (Louw 1993). Louw (1993) also reported that both weevils can be found on an individual plant and appear to cause sufficient damage. This thesis specifically focuses on the stem-mining weevil, *L. carinerostris*.

Lixus carinerostris was found feeding on close relatives of *C. crystallinum*, namely, *Cryophytum guerichianum* (Pax) Schwantes, *Cryophytum pellitum* (Friedrich) Gerbaulet (synonymous with *Mesembryanthemum pellitum)*, and *Cryophytum barklyi* N.E.Br (synonymous with *Mesembryanthemum barklyi* Hook.f.). Field observations have shown that *C. guerichianum* is the most widely distributed species of the four, growing across all three South African provinces. *C. barklyi* and *C. pellitum* mainly grow in the Karroo. *C. crystallinum* typically grows towards the South of the Western Cape, with some populations observed further north in the Northern Cape. *Lixus carinerostris*' usage of multiple hosts raised the potential existence of populations that are locally adapted to different host plants and/or geographic locations. These weevils were first morphologically identified as *L. carinerostris* in the early 1990s (Louw 1993). Morphological identification for insects with a wide host range cannot be completely reliable, because some studies have genetically detected cryptic species in insects with multiple hosts, and if *L. carinerostris* is to be considered as a biocontrol agent for *C. crystallinum*, there needs to be certainty that it is a single species. In the case that *L. carinerostris* is a cryptic species complex, the correct species needs to be carefully selected and reared for further host specificity testing and impact assessments on the target IAPS. This selection is important because different cryptic species or populations may demonstrate different levels of host specificity, with some potentially being safer for release due to narrower

host ranges. Furthermore, some populations may be more effective as a result of local adaptation to the invasive genotype of *C. crystallinum*, potentially leading to better establishment, higher damage rates, and greater long-term control (Müller-Schärer et al. 2020).

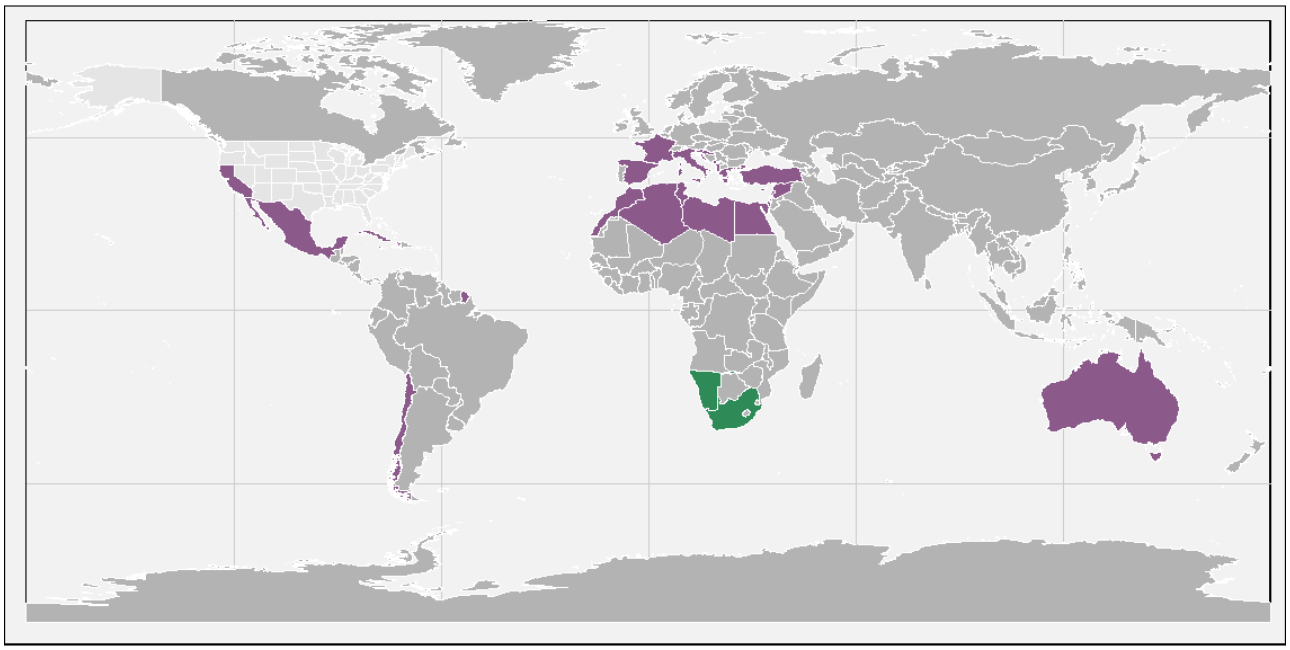

Figure 1. Global distribution of *C. crystallinum*. Areas in green are where the plant is native, and areas in purple are introduced areas as stated by Moran, 1950; Adams et al. 1998; Olckers et al. 2021; and Unpublished genetic analyses by the Centre for Biological Control

Aims

The aims of this Master's thesis were to:

1. Use molecular markers to determine whether *L. carinerostris* populations across different host plant species represent a single oligophagous species or a cryptic species complex.

2. Evaluate the population genetic structure of *L. carinerostris* populations to identify patterns of local adaptation and population differentiation that may influence both host specificity and efficacy.

3. Evaluate the impact of *L. carinerostris* on the various plants it utilises as hosts.

To date, there are no studies that genetically identify *L. carinerostris* or assess its genetic structure across the four host plants within its native range. Addressing this knowledge gap is crucial for the global biological control of the invasive *C. crystallinum*, as it enables the selection of populations that are both highly host specific and well adapted to damage the target plant species.

Chapter 2 – Genetic assessment of *Lixus carinerostris* across multiple host plant species in South Africa

2.1 Introduction

The *Lixus* Fabricius genus belongs to the Lixini Schoenherr tribe, in the Lixinae Schoenherr subfamily (Curculionidae Latreille), with over 500 species described globally (Trnka et al. 2016). *Lixus* species feed and develop inside the stems or roots of herbaceous plants, but occasionally develop in the seed capsules or petioles (Gültekin 2007, Trnka et al. 2016). Although some *Lixus* species, like *Lixus incanescens* Boheman, are considered agricultural pests, others have been used for biocontrol, such as *Lixus cardui* Olivier (Coleoptera: Curculionidae), for musk thistle in Australia (Nikulina and Gültekin, 2011; Arbabtafti et al. 2012). While many studies have morphologically described the different species of *Lixus* (Zotov 2009, Nikulina and Gültekin 2011, Trnka et al. 2016), not as many studies have sought to genetically study these weevils, whether to genetically barcode them or understand their population dynamics. This has left a gap in knowledge concerning the population genetics of the *Lixus* genus, leaving the possibility of cryptic species in the group.

Lixus carinerostris is one species that has been morphologically described with no complementary genetic studies in the literature and no genetic sequences on any of the online databases (GenBnk and BOLD). This weevil has been observed to be the most abundant natural enemy of *C. crystallinum* and is being considered for biocontrol in areas where the plant has invaded (Olckers et al. 2021).

The possible existence of unknown cryptic species complexes and locally adapted populations within potential biocontrol agent populations underscores the critical importance of conducting population genetic studies prior to host specificity testing. While cryptic species (morphologically indistinguishable, but genetically distinct taxa) offer some opportunities

(Smith et al. 2018), they also pose a significant challenge in biocontrol programs globally (Bickford et al. 2007, Paterson et al. 2016, Canavan et al. 2020, Ricciardi et al. 2021). Cryptic species or genetically distinct populations may exhibit different host preferences, physiological tolerances, and ecological adaptations (Roderick and Navajas 2003, Paterson et al. 2019). Conducting biocontrol programmes while unaware of the presence of cryptic species could result in the release of ineffective and potentially unsafe agents (Paterson et al. 2019).

The discovery of cryptic species within the stem-mining weevil *Mecinus janthinus* Germar (Coleoptera, Curculionidae), fundamentally changed the understanding of the toadflax biocontrol program in North America and demonstrated how molecular tools can explain variable field outcomes (Toševski et al. 2011). Toševski et al (2011) used an integrated taxonomic approach, combining mtDNA sequencing, morphological analyses of subtle differences in rostrum shape and male genitalia, and ecological observations of host plant associations across native European and introduced North American ranges. This study revealed that the presumed single species, *M. janthinus,* comprised two genetically distinct cryptic species: *M. janthinus* sensu stricto, which specialised on the yellow toadflax (*Linaria vulgaris* (L.) Mill. (Plantaginaceae)), and the newly described *M. janthiniformis* Toševski & Caldara (Coleoptera: Curculionidae), which specialised on Dalmatian toadflax (*L. dalmatica* (Plantaginaceae)). The genetic analysis revealed p-distances ranging from 1.3% to 2.4% with 20 distinct haplotypes identified from *M. janthinus* and 29 from *M. janthiniformis* (Toševski et al. 2011). Toševski et al's (2011) discovery explained the long-term variable establishment success: releases where the right species was matched with its preferred host plant species achieved better outcomes.

Similarly, a study that investigated *Eccritotarsus* (Hemiptera, Miridae) populations used for the biocontrol of water hyacinth (*Pontederia crassipes* (Pontederiaceae)) revealed two

reproductively isolated and genetically distinct cryptic species with different thermal physiologies and biogeographic adaptations (namely, i.e. Brazilian *E. catarinensis* Carvalho (Hemiptera: Miridae) and Peruvian *E. eichhorniae* Henry & Sosa (Hemiptera: Miridae) (Paterson et al. 2016, 2019)). Paterson et al (2016) analysed the COI barcoding gene region of the mtDNA, which revealed 5.2% sequence divergence, and nuclear ISSR markers, which showed two distinct groups within the presumed single species. The study also conducted cross-breeding experiments that demonstrated near-complete reproductive isolation. When individuals from the two different groups were forced to reproduce, it resulted in significantly lower offspring than when they bred with members of their own population (Paterson et al. 2016). Thermal studies showed that though the two cryptic species' fitness traits are at an optimum at 20-25 °C, the Peruvian *E. eichhorniae* appeared to perform significantly better at 30 °C (Ismail and Brooks 2016). These findings were further corroborated by Paterson et al (2019), which revealed that although the host specificity of the two cryptic species is similar, *E. eichhoniae* performed better at higher temperatures in terms of feeding damage compared to *E. catarinensis,* which performed better at lower temperatures. These studies had important implications for how the water hyacinth biocontrol program is strategised, by highlighting that climate matching based on species-specific thermal physiology is critical for the establishment success of the biocontrol agent.

Perhaps most notable is the Saltcedar (*Tamarix* (Tamaricaceae)) biocontrol program, which revealed that *Diorhabda* Weise (Coleoptera, Chrysomelidae) beetles were composed of multiple cryptic species with distinct genitalia, thermal physiologies and diapause requirements, directly affecting their establishment success and geographic distribution (Bean et al. 2014a). Through genetic and genital morphology analyses, the presumed single species, *D. elongata,* was revised and reclassified into five distinct cryptic species. These were the Asian desert *D. carinulata*, the Mediterranean *D. elongata*, the grasslands/desert *D. carinata,*

the subtropical *D. sublineata*, and the maritime subtropical *D. meridoinalis* (Tracy and Robbins 2009, Bean et al. 2013). Bean et al (2013) highlighted that interspecific breeding was almost impossible, resulting in non-viable eggs, and, at times, high female mortality due to incompatible genitalia between species. A series of diapause requirements and thermal physiology demonstrated how the different cryptic species have different thermal physiologies and have different diapause requirements (Tracy and Robbins 2009, Dalin et al. 2010, Bean et al. 2012, 2014a, 2014b). These studies demonstrated how pre-release species-specific characterisation of photoperiodic and thermal physiology are essential for predicting where biocontrol agents will successfully establish, that single-source introductions may inherently result in limited geographic ranges, thus requiring multiple ecotype or cryptic species releases matched to local climate zones (Bean et al. 2012).

There are currently no genetic studies of any sort on *L. carinerostris* in the literature. To avoid the challenges presented by the presence of cryptic species, it is essential to incorporate genetic analyses of *L. carinerostris* into the ice-plant biocontrol program. Therefore, to address this knowledge gap, *L. carinerostris*'s genetic variability and population genetic structure can be determined using molecular markers from both nuclear and mitochondrial genomes. Two complementary molecular techniques are particularly well suited for this purpose: DNA barcoding and Inter-simple sequence repeats (ISSR) analysis.

2.1.1 DNA barcoding

The emergence of molecular species identification can be traced back to the early 1980s, when scientists started exploring genetic approaches to differentiating microorganisms and other taxa (McAndrew and Majumdar 1983). The term 'DNA barcode' was formally introduced in 1993 during research on *Plasmodium falciparum* Welch protozoan isolates (Arnot et al. 1993). This method gained popularity in 2003 and has since evolved, especially after the establishment of

the Consortium for the Barcode of Life (CBOL) in 2004. The CBOL is an organisation that focuses on creating standard protocols for DNA barcoding applications across diverse taxa (Hebert et al. 2003a, Antil et al. 2023).

DNA barcoding represents a molecular method for identifying and discovering species by employing standardised short DNA sequences called barcodes (Antil et al. 2023). The technique has continued to advance significantly from conventional Sanger sequencing to powerful, high-throughput methods like next-generation sequencing (NGS), which has enhanced accuracy and cost efficiency (Jinbo et al. 2011).

2.1.1.1 Molecular Marker Selection

Mitochondrial loci are preferred targets for DNA barcoding due to several genomic characteristics, including higher mutation rates and more variation compared to nuclear genes, relatively few recombination events, and high abundance within cells (Ballard and Whitlock 2004, Hill 2016). The Cytochrome oxidase subunit I (COI) gene is the most commonly used barcode region for identifying animal taxa (Hebert et al. 2003b). The gene's utility stems from the fact that it shows few sequence insertions and deletions (indels) and has enough variation for distinguishing species, especially among invertebrates (Blaxter 2004, Andújar et al. 2018).

2.1.1.2 The Barcoding Gap and Taxonomic Thresholds

DNA barcoding operates on the idea that genetic variation between species is greater than variation within species, leading to what is called the 'barcoding gap' (Phillips et al. 2019). Recent large-scale studies have shown that genetic divergence thresholds for species delimitation are not universal; instead, they differ between taxonomic groups (Zhang & Bu, 2022). While early studies suggested genetic divergence thresholds of 3% for invertebrates and 2% for vertebrates, more recent analyses show that optimal genetic divergence thresholds can range between 1% and 4% depending on the taxonomic group under study (Kartavtsev 2011,

Chappell et al. 2012, Zhang and Bu 2022). This variation reflects differences in evolutionary rates and genetic diversity across taxa, highlighting the need for taxon-specific approaches rather than fixed thresholds in DNA barcoding and species delimitation studies (Chappell et al. 2012, Zhang and Bu 2022).

DNA barcoding is a crucial tool for taxonomists and is used in many areas of biological science. Recent advancements have increased its usefulness in assessing biodiversity and conservation efforts (Grant et al. 2021, Antil et al. 2023). Recent advancements include the combination of high-resolution melting analysis (HRM) with barcoding (Bar-HRM), DNA mini-barcoding for degraded samples, and DNA metabarcoding for environmental samples (eDNA) (Nazar et al. 2025). A major advantage of this technique is that it applies to all life stages of an organism, as the barcode sequence remains consistent regardless of age (Grant et al. 2021).

2.1.1.3 Limitations of DNA barcoding

DNA barcoding faces several limitations that can negatively affect its reliability as a sole taxonomic tool (Rubinoff et al. 2006). Heteroplasmy (multiple mtDNA variants within individuals) and the increasingly reported biparental mtDNA inheritance challenge conventional assumptions about maternal-only inheritance and can result in inconsistent genetic comparisons (Kmiec et al. 2006, Kaczmarczyk-Ziemba et al. 2025). The evolutionary rate of mitochondrial genes differs significantly across taxa, at times resulting in negative barcoding gaps where intraspecific variation is higher than interspecific variation (Rubinoff et al. 2006). Introgression through hybridisation and incomplete lineage sorting causes discordance between gene trees and species trees, thus making taxonomic boundaries unclear and leading to potential misidentifications (Funk and Omland 2003, Maddison and Knowles 2006). Nuclear mitochondrial DNA segments (NUMTs), mitochondrial DNA fragments incorporated into the nuclear genome, can co-amplify during PCR and create false impressions

of cryptic species or biparental mitochondrial DNA inheritance if not properly detected (Colombo et al. 2024).

2.1.1.4 Success despite limitations

Despite its limitations, DNA barcoding has achieved significant success across many insect orders, including Lepidoptera, Coleoptera, Orthoptera, Heteroptera, Plecoptera, Trichoptera, Ephemeroptera, and Diptera (Hausmann et al. 2013, Hendrich et al. 2015, Schmidt et al. 2017, Ćukušić et al. 2017). Large-scale initiatives such as the International Barcode of Life project have successfully barcoded millions of specimens, demonstrating DNA barcoding's practical utility in biodiversity assessment and species discovery, with advancements in sequencing technologies further improving reliability (deWaard et al. 2019, Ratnasingham et al. 2024). Therefore, DNA barcoding is a valuable tool when used for the right purpose and interpreted with a complete understanding of its limitations.

2.1.2 Inter-Simple Sequence Repeats (ISSRs)

Inter-Simple Sequence Repeats (ISSRs) are segments of DNA located between microsatellite sequences, also known as Simple Sequence Repeats (SSRs) (Wolfe 2005, Gaskin et al. 2011, Ng and Tan 2015, Lingaiah et al. 2024). These microsatellite sequences consist of repeated DNA motifs, ranging from one to six nucleotides, that appear randomly at various locations in the genome and show high mutation rates (Gaskin et al. 2011, Lingaiah et al. 2024, Arslan et al. 2025). Because they are polymorphic, ISSRs are useful for detecting genetic variation among species and offer a cost-effective solution with high levels of polymorphism compared to other molecular methods (Abbot 2001, Ng and Tan 2015, Gemmill and Grierson 2021). Each organism exhibits unique ISSR fragment patterns across its genome, which can be identified using techniques like agarose gel electrophoresis and capillary electrophoresis (Gemmill and

Grierson 2021). Generally, ISSR primers consist of dinucleotide or trinucleotide repeat sequences (Blair et al. 1999, Wolfe 2005).

The ISSR analysis technique was first established in the early 1990s for the identification of plant cultivars (Blair et al. 1999, Wolfe 2005, Ng and Tan 2015). Since its inception, the method has been applied across diverse taxonomic groups, including plants, vertebrates, invertebrates, and fungi, for assessing genetic diversity (Abbot 2001, Lingaiah et al. 2024). In biocontrol, the ISSR technique has been used to effectively match invasive species with their native populations and identify cryptic species (along with other genetic techniques) among biocontrol agents, like *Eccritotarsus* species, used for controlling water hyacinth (Taylor et al. 2011, Paterson et al. 2016). Inter-Simple Sequence Repeats have also been successfully applied to cochineal scale insects (*Dactylopius* spp.) used to control invasive cacti, where the technique contributed to effectively distinguishing cryptic species and intraspecific lineages, which is essential for appropriate biocontrol agent selection (van Steenderen et al. 2021).

Advantages of using the ISSR technique include the use of universal primers that do not require prior genomic sequence information of the subject taxon, widespread occurrence of ISSRs across diverse taxonomic groups providing high-resolution data, time and cost efficiency relative to alternative methods such as Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP) (Bornet and Branchard 2001, Bornet et al. 2004). Furthermore, the stability of inherited microsatellite alleles across generations makes them suitable for both individual-level and population-level analyses (Ng and Tan 2015, Gemmill and Grierson 2021).

2.1.2.1 Limitations of Inter-Simple Sequence Repeats

The AFLP technique typically demonstrates greater performance compared to ISSRs and RAPDs by exhibiting relatively lower genotyping errors, enhanced robustness, and superior

informativeness, as supported by multiple comparative studies on agricultural cultivars and plant species (Meudt and Clarke 2007, Costa et al. 2016). The primary sources of errors in band-based methodologies arise from allele homoplasy (non-homologous fragments migrate to identical positions) and scoring inaccuracies, with homoplasmy becoming more common in smaller fragments, complex profiles, and at higher taxonomic distances (Bonin et al. 2004, Holland et al. 2008, Whitlock et al. 2008). Multiple comparative studies on agricultural cultivars and plant species support these findings (Jeung et al. 2005, Kafkas et al. 2006, Tiwari et al. 2013, Costa et al. 2016, Vasconcelos et al. 2016, Medraoui et al. 2024). Genotypic errors are the inconsistencies in genetic data from identical samples, typically arising from subjective data scoring, but can be minimised through PCR replication (Bonin et al. 2004, Whitlock et al. 2008). These errors are calculated as Average Euclidean error rates (mismatches/total band instances) and complementary Average Jaccard error rates that exclude shared band absences, with AFLP Euclidean error rates typically ranging between 2-5% though ideally shouldn't exceed 1%, though interstudy comparisons remain difficult because of methodological differences (Bonin et al. 2004, Pompanon et al. 2005, Holland et al. 2008).

2.1.2.2 Inter-Simple Sequence Repeats as High-Resolution Molecular Identification Tools

Simple Sequence Repeats have a relatively higher mutation rate compared to many other DNA regions (Zietkiewicz et al. 1994). In contrast, ISSRs are relatively more variable; as such, they can provide relatively more enhanced resolution that reveals more distinct differences between groups compared to conventional microsatellites (Zietkiewicz et al. 1994, Pradeep Reddy et al. 2002). This enhanced resolution is particularly valuable when standard DNA barcoding regions cannot distinguish between closely related taxa, including intraspecific lineages and distinct population groups. Therefore, fragment analysis techniques like ISSRs can serve as molecular barcodes by generating unique fingerprint profiles, with recent applications demonstrating their effectiveness in genetic diversity studies of various species (Jeung et al. 2005, Kafkas et al.

2006, Tiwari et al. 2013, Costa et al. 2016, Vasconcelos et al. 2016, Zhao et al. 2020, Medraoui et al. 2024).

Inter-simple sequence repeat analysis has been successfully used in genetic variability and population structure studies across different insect orders, with continued applications in contemporary molecular studies (Taylor et al. 2011, Barbosa et al. 2014, Wang et al. 2016). Furthermore, the COI gene in mtDNA has been utilised in numerous population studies examining genetic variability, population genetic structure, phylogeny, phylogeography, and DNA barcoding across diverse insect taxa (Xu et al. 2019, Fernández et al. 2022). However, neither DNA barcoding not ISSR analysis had been previously applied to *L. carinerostris*.

This study aimed to determine whether *L. carinerostris* constitutes a cryptic species complex with multiple species that are differentiated by host plant species or geographic location, or if *L. carinerostris* represents a single species with multiple host associations within the *Cryophytum* genus. The findings of this study will inform *C. crystallinum* biocontrol as to which *L. carinerostris* populations to target for the source population or if any of the populations can be used for the biocontrol program.

2.2 Methods

2.2.1 Survey across *Cryophytum* Species

Between 2022 and 2024, a field survey was carried out using the distribution of *Cryophytum* species (Gerbaulet 2017) and existing citizen science data from iNaturalist (https://www.inaturalist.org/) to locate sites with various *Cryophytum* species in South Africa. Data from iNaturalist was verified by examining the images provided in each sighting to confirm plant identification. Upon arrival at each site, plant species identifications were verified using the Gerbaulet (2017) illustrated handbook for succulent plants, which includes a key to the genus. The stems, leaves, and ground beneath the plants were visually inspected for

Lixus weevils. Stems were checked for signs of chlorosis, weevil feeding holes, oviposition holes, and exit holes.

2.2.2 Sample collection

Between 2022 and 2025, weevil specimens were collected from *C. crystallinum*, *C. guerichianum*, *C. pellitum* and *C. barklyi* (from several different plant individuals per sampling site) across *C. crystallinum*'s native distribution (Steinkopf, Hondeklip Bay, Dwarskesbos, Port Nolloth, McDougalls Bay, N7, Baviaanskloof, Elands Bay, Robertson, Leipoldtville, Oudtshoorn, Vanrhynsdorp, Yzerfontein) (Figure 2). At each site, as many weevils as possible were collected with sample sizes ranging from 1 to 9 individuals per site, depending on weevil availability. Sampling sites spanned the Eastern, Western, and Northern Cape provinces of South Africa (Figure 2). Specimens were delivered to the laboratory in 70% and 99% ethanol for possible future morphological analyses and molecular analyses (current study), respectively. Each tube comprised either one adult or one larva. They were stored at -20 °C until needed for DNA extraction at Rhodes University, Department of Zoology and Entomology. Weevil specimens were assigned coded labels that indicated their collection site, host plant species, and specimen number using various naming conventions such as site-plant-number combinations (e.g., EC1) or numbered systems with additional identifiers (e.g., 831, 810i).

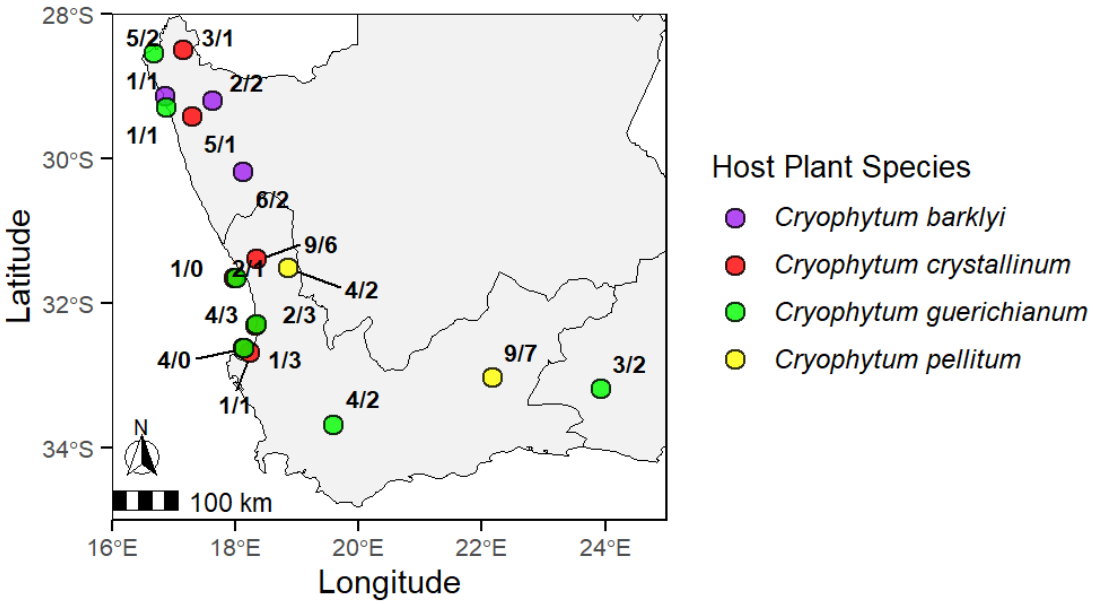

Figure 2. Map of the Northern, Western, and Eastern Cape provinces of South Africa, showing the different *Cryophytum* species sites depicted by different coloured points (see legend). The number next to each point indicates the number of *L. carinerostris* samples successfully genetically analysed from that site. The numbers are shown as "COI/ISSR", i.e., the first number is for samples successfully used for COI analysis, and the second number is for samples successfully used for ISSR analysis

Three legs from each weevil were used for DNA extraction from adults. The remaining parts of the specimens were stored at – 20 °C. Genomic DNA was extracted from each individual using a PureLink™ Genomic DNA Mini Kit, following the manufacturer's protocol.

2.2.4 Cytochrome c oxidase subunit I PCR and sequencing

2.2.4.1 PCR protocol, data capturing and data processing

Mitochondrial Cytochrome oxidase subunit I (COI) was amplified using Polymerase Chain Reaction (PCR) by using primers "LCO1490JJ" (5'-CHACWAAYCATAAAGATATYGG-3') and "HCO2198JJ" (5'-AWACTTCVGGRTGVCCAAARAATCA-3') (Astrin and Stüben 2008). This primer pair has been used to successfully amplify weevil DNA, and was used in the discovery of a new weevil species (Stüben et al. 2021). Each PCR reaction contained: 12.5 µl DNA polymerase (iTaq Universal SYBR® Green Supermix), 6.5 µl PCR-grade water, 2.0

µl forward primer (LCO1490JJ), 2.0 µl reverse primer (HCO2198JJ), and 2.0 µl DNA extract. A negative control with all the above constituents except DNA extract (replaced with PCR-grade water) was also included. Polymerase Chain Reaction conditions are outlined in Table 1.

Table 3. Polymerase Chain Reaction cycling conditions for amplification of *L. carinerostris* COI barcode gene region

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	94°C	5 minutes	1
Denaturation	94°C	45 seconds	38
Annealing	50°C	35 seconds	
Extension	72°C	45 seconds	
Final elongation	72°C	10 minutes	1
Hold	4°C	∞	1

DNA amplicons were visualised through agarose gel electrophoresis. A volume of 5.0 µl PCR product from each individual was homogenised with 1 µl of 6x DNA loading dye. The PCR product-loading dye mixture was loaded into an ethidium bromide-stained 1% agarose gel and electrophoresed at 90 volts for 40 min. The gel was then visualised under a UV light source and photographed. A GeneRuler 100-kb DNA ladder was used to estimate the relative size of amplified DNA. Positive agarose gel electrophoresis PCR products were outsourced to Macrogen-Europe (https://order.macrogen-europe.com/quotation/retrieveCesQuotation.do?menuCd=QUO100) in the Netherlands for forward direction Sanger sequencing.

2.2.5 Cytochrome c oxidase subunit I data processing and analysis

2.2.5.1 Sequence library preparation and sequence alignment

Chromatograms were visualised in MEGA version 11 (Tamura et al. 2021), and the beginning and ends of sequences were trimmed accordingly (±50–70 bp). Individual DNA sequences were used in BLAST searches on GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome#) to uncover closely related sequences in the database. Sequences displaying an identity percentage of greater than 85% were added to the sequence library. OneZoom Tree of Life Explorer (https://www.onezoom.org/) and BLAST results were used to select appropriate outgroups for the phylogenetic analysis. Outgroups were selected based on their phylogenetic proximity to the *Lixus* genus within the Lixinae subfamily (Table S1). These included representatives from the genera *Larinus*, *Pachycerus*, and *Cyphocleonus*. Sequence alignment was performed online using MAFFT v7, using default settings (Katoh et al. 2019).

2.2.5.2 Pairwise distance analysis

Sequences of the same species across all genera were grouped in MEGA version 11 (Tamura et al. 2021). Pairwise distance analyses were conducted within groups and between groups using the proportion of differences index/Hamming distance (number of differences divided by total number of sites) with pairwise deletion of gaps. Standard errors were estimated using 1000 bootstrap replicates.

2.2.5.3 Bayesian and Maximum Likelihood Inference

jModelTest v2.1.10 (Darriba et al. 2012) was used to select the appropriate evolutionary model for the COI gene region based on the Akaike Information Criterion (AIC), followed by the use of Bayesian inference and Maximum Likelihood methods to construct phylogenies. The Bayesian phylogeny was created using MrBayes v3.2.6 (Ronquist et al. 2012), and the

Maximum Likelihood phylogeny was created using the IQ-TREE web server (Trifinopoulos et al. 2016). MrBayes was run using random starting trees, four chains (three hot and one cold) and set to 1 million generations. Trees were sampled every 1000 generations with a burn-in of 1500. MCMC convergence was checked using Tracer v1.7 (Rambaut et al. 2018). Maximum likelihood analysis was run at 1000 bootstrap repeats with default settings.

Both Maximum Likelihood and Bayesian output trees were visualised on Figtree v1.4.4 (Rambaut 2018), and the appearance was modified for better presentation. The Bayesian phylogenetic tree served as the primary tree, with bootstrap values from the Maximum Likelihood tree superimposed on it in Microsoft PowerPoint. Species labels were subsequently incorporated into the tree via MS PowerPoint.

All DNA sequence details used in the genetic analyses are listed in the supplementary Tables S1 & S2, including sample identifiers, collection localities, host plant species, and GenBank accession numbers for sequences obtained from the database.

2.2.6 Inter-Simple Sequence Repeats PCR and sequencing

2.2.6.1 PCR protocol, data capturing and data processing

Three ISSR primers – HB13 (5'-GAG AGA GAG AGA GAG C-3') (Tabikha and Adss 2021), Universal primer 826 (5'-ACA CAC ACA CAC ACA CC-3') and 809 (5'-AGA GAG AGA GAG AGA GC - 3') from Primer set # 9 of the University of British Columbia Nucleic Acid Protein Service Unit were tested (Abbot 2001). The universal primer 809 was selected as it produced clear, consistent, and polymorphic bands when visualised using gel electrophoresis. This primer was labelled with 5'6-FAM^{TM} fluorescent dye. PCRs were run in 20 µL reactions, which consisted of 10 µL iTaq^{TM}, 1.5 µL primer, 3µL DNA template, and the rest of the volume was PCR-grade water. The PCR protocol is displayed in Table 2. Each sample was replicated (same DNA extract) in a different PCR machine (both of which were the Applied Biosystems

Veriti Model 384 Well Thermal Cycler Lab) to validate the occurrence of bands/peaks (Sutton et al. 2017; Taylor et al. 2011). As a preliminary validation stage, 4.5 µL of each PCR product was run on a 1% agarose gel at 6 V/cm for 3 hours. PCR products were subsequently sent to the Central Analytical Facilities (CAF) division in Stellenbosch, South Africa, where DNA fragment analysis was performed using capillary electrophoresis (Applied Biosystems Inc., 3130 genetic analyser, GS1200LIZ size standard).

Table 4. Polymerase Chain Reaction cycling conditions for amplification of *Lixus carinerostris* ISSR809

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95°C	7 minutes	1
Denaturation	95°C	45 seconds	38
Annealing	52°C	35 seconds	
Extension	72°C	45 seconds	
Final elongation	72°C	7 minutes	1
Hold	4°C	∞	1

2.2.7 ISSR data processing and analysis

2.2.7.1 Electropherograms to binary data

Electropherograms were analysed in Genemarker® v2.7.4 (SoftGenetics®). Binary data from GeneMarker were opened in RawGeno v2.0 (Arrigo et al. 2012). The analysis parameters were set at a maximum and minimum bin width of 1 and 0.5 base pairs (bp), respectively, scoring from 100 to 500 bp, and a low relative fluorescent units (RFU) of 100, as recommended by Applied Biosystems (2014) for data produced by the 3130 Series instrument. The resulting binary matrix was organised in Microsoft Excel.

2.2.7.2 Binary data processing

A GUI application called BINMAT (https: //clarkevansteenderen.shinyapps.io/BINMAT/) (van Steenderen, 2022) was used to consolidate the replicate pairs in the binary matrix. The method combined replicated samples in accordance with a predetermined rule: if a band appeared once in a set of duplicates (represented by "0" and "1"), it was defined as uncertain and marked by a "?". However, if two replicates showed the presence of a band or both revealed its absence, those were kept as "1" or "0" accordingly. Additionally, the program calculated mean error rates through Euclidean as well as Jaccard distance measures, including their standard deviation and produced output that revealed the mean and minimum to maximum peak count along with their standard deviation. Samples with fewer than 20 peaks were excluded from subsequent analysis to ensure data quality. The consolidated binary matrix was used to group sample pairs based on the host plant species they were collected from (*C. crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi*).

2.2.7.3 Genetic distance vs geographic distance

To evaluate the relationship between geographic and genetic distances among *L. carinerostris* samples, a Mantel test was conducted using the consolidated data matrix. Pairwise genetic distances were calculated using Binary ISSR profiles based on the Jaccard similarity index, while geographic distances (in kilometres) were determined as great circle distances using the Haversine formula, which relied on longitude and latitude coordinates recorded at each sampling location. The analysis was executed in Rstudio v2025.05.1, employing the vegan (Oksanen et al. 2007), geosphere (Hijmans 2019), and tidyverse packages (Wickham et al. 2017). A Mantel test with 999 permutations and Pearson's product-moment correlation was applied to assess the correlation between the geographic and genetic distance matrices.

2.2.7.4 Discriminant Analysis of Principal Components (DAPC)

To further investigate the genetic structure among the sampled *L. carinerostris* individuals based on the ISSR marker data, a Discriminant Analysis of Principal Components (DAPC) was performed using the R package adegenet (Jombart 2008, Jombart and Ahmed 2011) in R v2025.05.1. DAPC analysis is different from conventional Principal Component Analysis (PCA) in that it includes a priori grouping information, which maximises between-group variance and minimises within-group variance, rendering it especially suitable for identifying genetic structure when group assignments are known (Jombart et al. 2010). The ISSR dataset was imported to R as a binary presence-absence matrix, where rows represented individual *L. carinerostris* samples and columns represented scored loci.

The ISSR marker data were converted into a genind object using the df2genind() function of the adegenet package (Jombart 2008), with ploidy set to 1 and data type specified as "PA" (presence/absence). The grouping information was extracted from the "Host plant species" column of the dataset and used to define populations.

A principal component analysis (PCA) was performed without priori information on the dataset using the dudi.pca() function from the ade4 package (Dray and Dufour 2007) before the discriminant analysis. This was done to reduce dimensionality and avoid overfitting when the number of loci exceeded the number of individuals. The number of principal components (PCs) retained was selected according to a general rule of thumb (approximately one-third of the number of individuals), balancing power and overfitting risk. The number of discriminant functions (DAs) retained was set to the number of groups minus one. DAPC was then performed using the dapc() function of the adegenet package (Jombart 2008).

The resultant discriminant functions were visualised using scatterplots and density plots of individual coordinates along DF1 and DF2, implemented with both base R graphics and

ggplot2 (Wickham 2016). Group separation and individual clustering were examined, and variable contributions to discriminant functions were explored using loadingplot() of the adegenet package (Jombart 2008). Marginal density plots along DF1 and DF2 were produced using the ggExtra package (Attali and Baker 2019) to assess the distribution of individuals per group. The R code, along with the input file used for this analysis is included as supplementary material at https://github.com/mukhawanajackeyjackson-dot/MSc-supplementary.

2.2.7.5 STRUCTURE

Lixus carinerostris genetic clustering was further analysed using STRUCTURE v2.3.4 (Pritchard et al. 2000, Evanno et al. 2005, Falush et al. 2007). STRUCTURE uses Bayesian analysis and the Markov Chain Monte Carl (MCMC) estimation method to assign individuals to genotypic cluster groups (K) (Pritchard et al. 2000). The STRUCTURE analysis was run with 50 thousand burn-in iterations followed by 500 thousand MCMC iterations. Eight replicate runs were conducted for each K value, K=1 to K=8, using the admixture model with correlated allele frequencies. The analysis was run twice: once supervised with grouping information provided as priors ('PopData') and once unsupervised with no grouping information. The STRUCTURE results were uploaded to STRUCTURESELECTOR (Li & Liu, 2018), a program that finds the best estimate of the number of genetic clusters (K-value) present. There are four statistical tests used to estimate the optimal K-value for a dataset (MedMedK, MedMeaK, MaxMedK, MaxMeaK). In this analysis, the threshold was set to range between 0.5 and 0.8, as recommended (Puechmaille 2016). The K-estimation according to the conventional Evanno method was reported (Evanno et al. 2005). The results were visualised using the CLUMPAK software (Kopelman et al. 2015).

2.3 Results

A total of 69 sequences representing 19 sites spread across 11 localities (Steinkopf, Hondeklipbay, Holgat, Port Nolloth, Baviaanskloof, Elands Bay, Robertson, Leipoldtville, Oudtshoorn, Vanrhynsdorp, Yzerfontein) and four plant host species (*Cryophytum crystallinum, C. guerichienum,* and *C. barklyi*) were used for this genetic analysis. The COI barcoding gene region size for all the samples analysed was 523 base pairs long following sequence cleaning and trimming. Sixteen variable sites were identified from the aligned COI sequences of *L. carinerostris*. Nucleotide diversity across all the *L. carinerostris* samples was 0.00768 (0.77%), which indicates low genetic variation in the COI barcoding region of the mtDNA.

2.3.1 Phylogenetic analysis

The Bayesian analysis revealed that all *L. carinerostris* specimens formed a single well-supported clade (posterior probability/bootstrap support (pp) = 1/100) (Figure 3). *Lixus carinerostris* formed a sister clade to other *Lixus* species (*L. brevirostris* Capiomont (Coleoptera: Curculionidae), *L. purpurariensis* Petri (Coleoptera: Curculionidae), and *L. kraatzi* Cpiomont (Coleoptera: Curculionidae)). However, the analysis also uncovered unexpected relationships that challenge the current taxonomic classification of the *Lixus* genus. Some of the species (*L. cinerascens* Schonherr (Coleoptera: Curculionidae), *L. angustatus* Fabricius (Coleoptera: Curculionidae), and *L. punctiventris* Boheman (Coleoptera: Curculionidae)) previously assigned to the *Lixus* genus form well-supported clades (pp = 1/100) separate from the other members of the genus. *Lixus cinerascens* formed a clade next to *Larinus* individuals, while *L. punctiventris* formed a sister clade to members of the *Pachycerus* genus. *Lixus angustatus* forms a well-supported clade separate from both of these tribes. The phylogenetic tree also revealed two polytomies and a relatively low support value

near the base of the tree (pp = 0.55/49), further supporting the unresolved nature within this group.

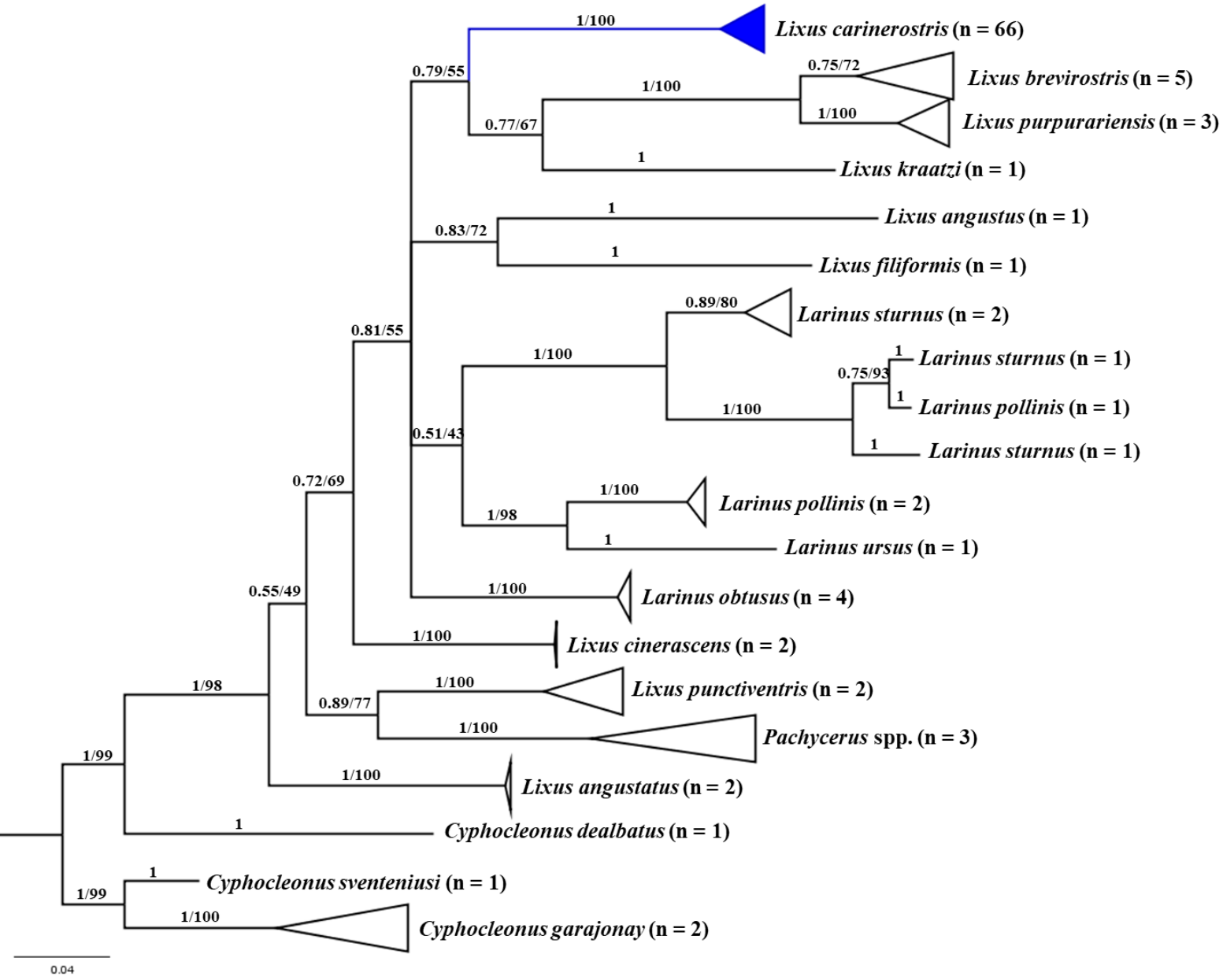

Figure 3. Bayesian phylogenetic tree showing *Lixus carinerostris* (in blue) forming a sister clade to other *Lixus* species. The values appearing first on each branch are Bayesian posterior probability support values, and the values appearing second (after "/") are maximum likelihood bootstrap support values. Collapsed clades are labelled by the species name followed by the number of sequences in brackets. Uncollapsed clades only had one sequence each and are labelled using the species name and the number of sequences in brackets. The scale bar represents the number of nucleotide substitutions per site. *Cyphocleonus* spp. were used as an outgroup to root the phylogenetic tree.

2.3.2 Pairwise sequence comparison (p-distance values)

The intraspecific COI p-distances of *L. carinerostris* and the other *Lixus*, *Larinus*, *Pachycerus*, and *Cyphocleonus* are outlined in Table 3. An intraspecific p-distance of 0.007 ± 0.0014 was observed among 69 *L. carinerostris* individuals collected from the four *Cryophytum* host plant species across 11 geographic locations in South Africa.

Table 3. Intraspecific pairwise distances of *Lixus* and closely related species. The d-values represent uncorrected p-distance values, and S.E. are the standard error values. Entries marked "n/c" indicate values that could not be computed due to only one representative sequence being available for the species.

Species name	d	S.E
Lixus carinerostris	0,007	0,001
Lixus angustatus	0,002	0,002
Lixus angustus	n/c	n/c
Lixus brevirostris	0,041	0,006
Lixus cinerascence	0	0
Lixus filiformis	n/c	n/c
Lixus kraatzi	n/c	n/c
Lixus punctiventris	0,044	0,009
Lixus purpurariensis	0,023	0,005
Larinus sturnus	0,084	0,011
Larinus pollinis	0,105	0,011
Larinus obtusus	0,006	0,002
Larinus ursus	n/c	n/c
Pachycerus segnis	0,078	0,011
Pachycerus madidus	n/c	n/c
Cyphocleonus garajonay	0,002	0,002
Cyphocleonus sventeniusi	n/c	n/c
Cyphocleonus dealbatus	n/c	n/c

The interspecific p-distance values between *L. carinerostris* and other *Lixus*, *Larinus*, *Pachycerus*, and *Cyphocleonus* species ranged from 0.15 to 0.20 (Table 4). These findings suggest a significant COI divergence of *L. carinerostris* from other members of the Lixini tribe.

Table 4. Interspecific pairwise distances between *L. carinerostris* and other *Lixus, Larinus, Pachycerus*, and *Cyphocleonus* species. Values on the bottom left half represent uncorrected p-distance values, and values on the top right half of the table represent the standard error values

	L._carinerostris	*L._angustatus*	*L._angustus*	*L._brevirostris*	*L._cinerascence*	*L._filiformis*	*L._kraatzi*	*L._punctiventris*	*L._purpurariensis*	*L._sturnus*	*L. pollinis*	*L._obtusus*	*L._ursus*	*P._segnis*	*P._madidus*	*C._garajonay*	*C._sventeniusi*	*C._dealbatus*
L._carinerostris		0,02	0,02	0,02	0,02	0,02	0,02	0,01	0,02	0,01	0,01	0,02	0,02	0,02	0,02	0,02	0,02	0,02
._angustatus	0,15		0,02	0,01	0,01	0,02	0,02	0,01	0,02	0,01	0,01	0,01	0,02	0,01	0,02	0,02	0,01	0,02
L._angustus	0,17	0,16		0,02	0,02	0,02	0,02	0,02	0,02	0,02	0,01	0,02	0,02	0,02	0,02	0,02	0,02	0,02
L._brevirostris	0,15	0,16	0,19		0,02	0,02	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,02	0,02	0,02	0,02
L._cinerascence	0,15	0,14	0,16	0,17		0,02	0,02	0,01	0,02	0,01	0,01	0,01	0,02	0,01	0,02	0,02	0,02	0,02
L._filiformis	0,17	0,16	0,17	0,17	0,17		0,02	0,02	0,02	0,02	0,01	0,02	0,02	0,02	0,02	0,02	0,02	0,02
L._kraatzi	0,16	0,17	0,20	0,16	0,16	0,15		0,02	0,01	0,01	0,01	0,01	0,02	0,01	0,01	0,02	0,02	0,02
L._punctiventris	0,15	0,14	0,16	0,15	0,12	0,16	0,17		0,01	0,01	0,01	0,01	0,02	0,01	0,02	0,02	0,01	0,02
L._purpurariensis	0,16	0,15	0,18	0,08	0,16	0,17	0,16	0,14		0,01	0,01	0,02	0,02	0,02	0,02	0,02	0,02	0,02
L._sturnus	0,16	0,16	0,18	0,18	0,15	0,17	0,16	0,16	0,18		0,01	0,01	0,01	0,01	0,01	0,02	0,01	0,01
L._pollinis	0,14	0,16	0,17	0,18	0,15	0,16	0,15	0,15	0,17	0,12		0,01	0,01	0,01	0,02	0,02	0,01	0,01
L._obtusus	0,15	0,13	0,18	0,17	0,12	0,15	0,15	0,14	0,17	0,14	0,14		0,02	0,01	0,02	0,02	0,02	0,02
L._ursus	0,15	0,15	0,20	0,16	0,15	0,18	0,17	0,16	0,17	0,17	0,13	0,14		0,02	0,02	0,02	0,02	0,02
P._segnis	0,17	0,15	0,20	0,18	0,14	0,17	0,16	0,14	0,17	0,18	0,17	0,16	0,18		0,01	0,01	0,01	0,02
P._madidus	0,18	0,16	0,21	0,16	0,15	0,17	0,15	0,16	0,16	0,18	0,18	0,16	0,19	0,10		0,02	0,02	0,02
C._garajonay	0,20	0,15	0,19	0,18	0,15	0,19	0,19	0,17	0,19	0,18	0,19	0,16	0,19	0,17	0,19		0,01	0,02
C._sventeniusi	0,17	0,13	0,18	0,16	0,14	0,18	0,17	0,14	0,18	0,16	0,17	0,15	0,16	0,17	0,18	0,08		0,02
C._dealbatus	0,18	0,18	0,19	0,20	0,15	0,20	0,19	0,16	0,20	0,17	0,17	0,15	0,16	0,17	0,18	0,15	0,15	

2.3.3 Inter-Simple Sequence Repeats Analysis

2.3.3.1 Discriminant Analysis of Principal Components

The DAPC analysis showed patterns of genetic differentiation among *L. carinerostris* populations associated with the different *Cryophytum* host plant species (Figure 4). DF1 explains 51.28% of variance, and DF2 explains 25.45% of variance. Weevil DNA samples collected from *C. pellitum* showed distinct separation from the other samples associated with the other three host plants, with minimal overlap with samples from *C. barklyi* along both discriminant functions. Weevil DNA samples collected from *C. guerichianum* exhibited extensive overlap with both *C. crystallinum* and *C. barklyi*. However, *C. crystallinum* and *C. barklyi* samples demonstrated some level of separation from each other in the ordination space.

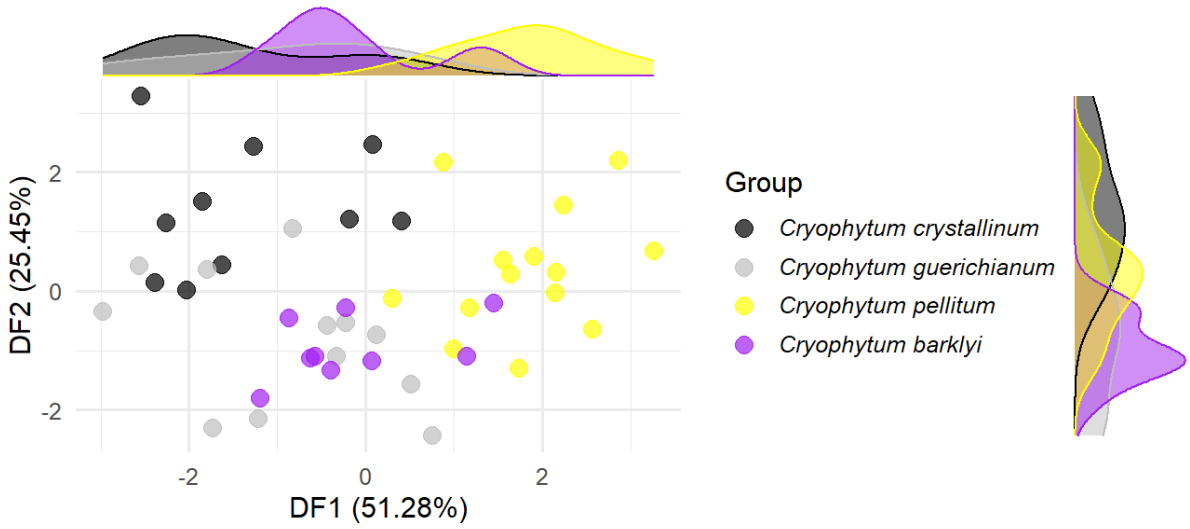

Figure 4. Discriminant Analysis of Principal Components (DAPC) scatter plot of ISSR809 marker data for *L. carinerostris* across four host plant species. The scatter plot shows individual samples plotted along the first two discriminant functions (DF1 and DF2), with colours representing the groupings based on host plant species (black: *C. crystallinum*, grey: *C. guerichianum*, yellow: *C. pellitum*, purple: *C. barklyi*). Density plots along the top and right margins show the distribution of individuals along DF1 and DF2, respectively. DF1 captures 51.28% of the between-group variance, followed by DF2, which captures 25.45% of the variance.

2.3.3.2 STRUCTURE Analysis

With population data not set as priors, the Puechmaille (2016) method revealed an optimal K-value of 1 (0.6 threshold) for all four tests (MedMedK, MedMeaK, MaxMedK, and MaxMeaK), which suggests that the most parsimonious interpretation of the ISSR data is a single panmictic (uniform random mating between groups) population. However, the Evanno et al. (2005) method revealed an optimal K-value of 4 according to both the ΔK and mean LnP(K) methods. When population data were set as priors, the same results were obtained.

The STRUCTURE plot findings are consistent with the findings of the DAPC analysis (Figure 5). At K=3 and K=4, *C. pellitum*-associated weevils show a distinct genetic cluster composition (indicated by a single dominant colour in the STRUCTURE plot). This observed *C. pellitum*-associated clustering pattern supports the genetic differentiation of this group observed in the DAPC analysis. On the other hand, weevils associated with *C. crystallinum*, *C. guerichianum*, and *C. barklyi* exhibited extensive genetic admixture across multiple clusters, with most individuals showing mixed ancestry. Although *C. guerichianum*-associated weevil DNA samples show considerable overlap with both weevil DNA samples from *C. crystallinum* and *C. barklyi*, there is evidence of subtle genetic differentiation between *C. crystallinum* and *C. barklyi*-associated weevil populations, consistent with the genetic differentiation revealed in the DAPC ordination space. While *C. pellitum*-associated weevils represent a genetically differentiated population within *L. carinerostris*, the other three host-associated populations show limited genetic structuring.

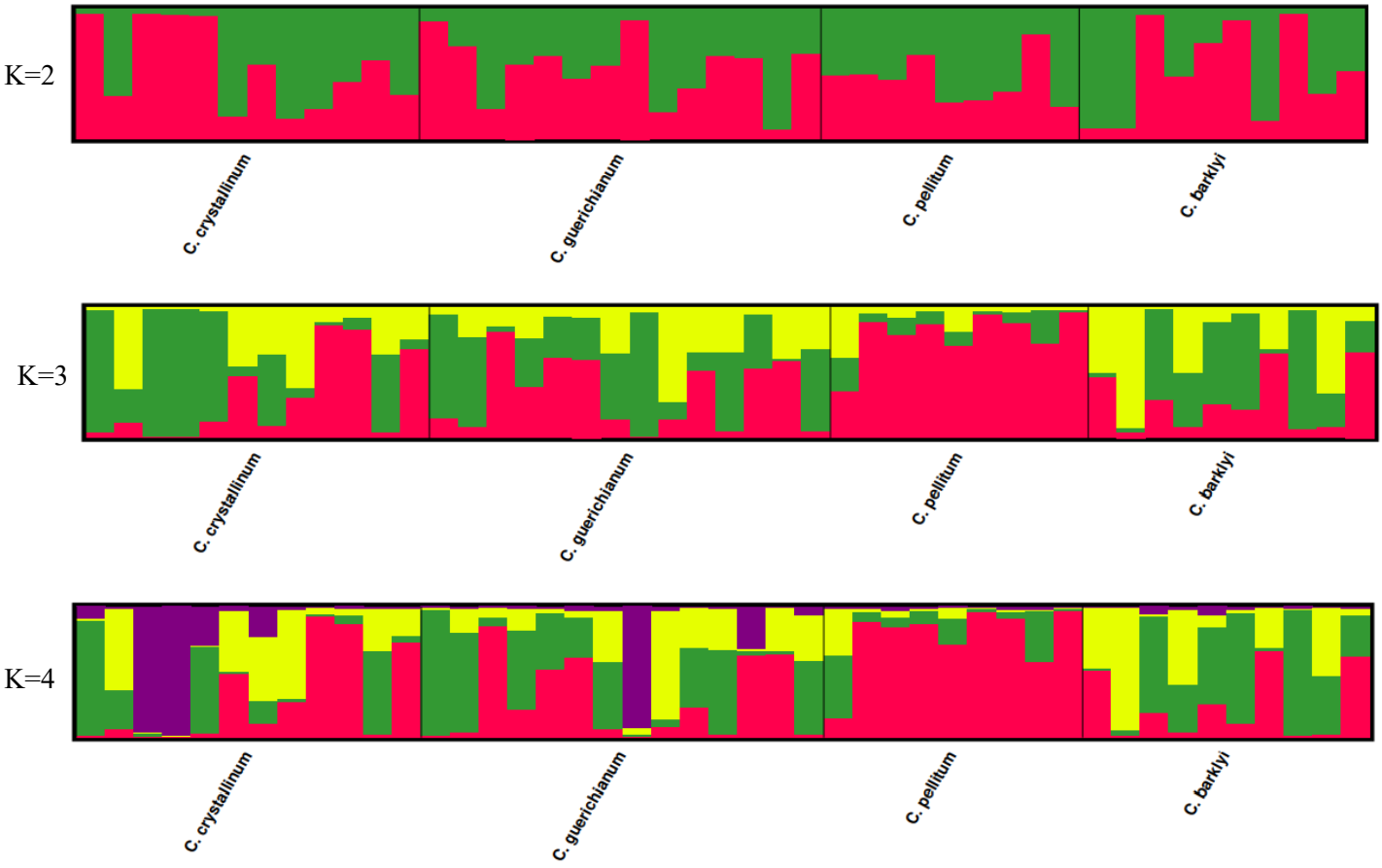

Figure 5. Bayesian clustering results from STRUCTURE analyses (MCMC = 50,000, burn-in = 100,000) of *L. carinerostris* ISSR data, showing assignment probabilities for K=2, K=3, and K=4. Each vertical bar indicates the individual's estimated membership coefficient (Q) in each of the inferred clusters. Samples are grouped by their host plant species: *C. crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi*. Replicate runs consistently support K=4 as the most likely number of genetic clusters.

2.3.3.3 Isolation by distance

The Mantel test revealed a broad range of genetic distances, with most values exceeding 0.7, across all geographic distances, indicating no clear pattern of genetic dissimilarity increasing or decreasing with geographic distance. The Mantel correlation coefficient (r=0.008) is nearly zero, and the P-value (0.445) indicates that this relationship is not statistically significant (Figure 6).

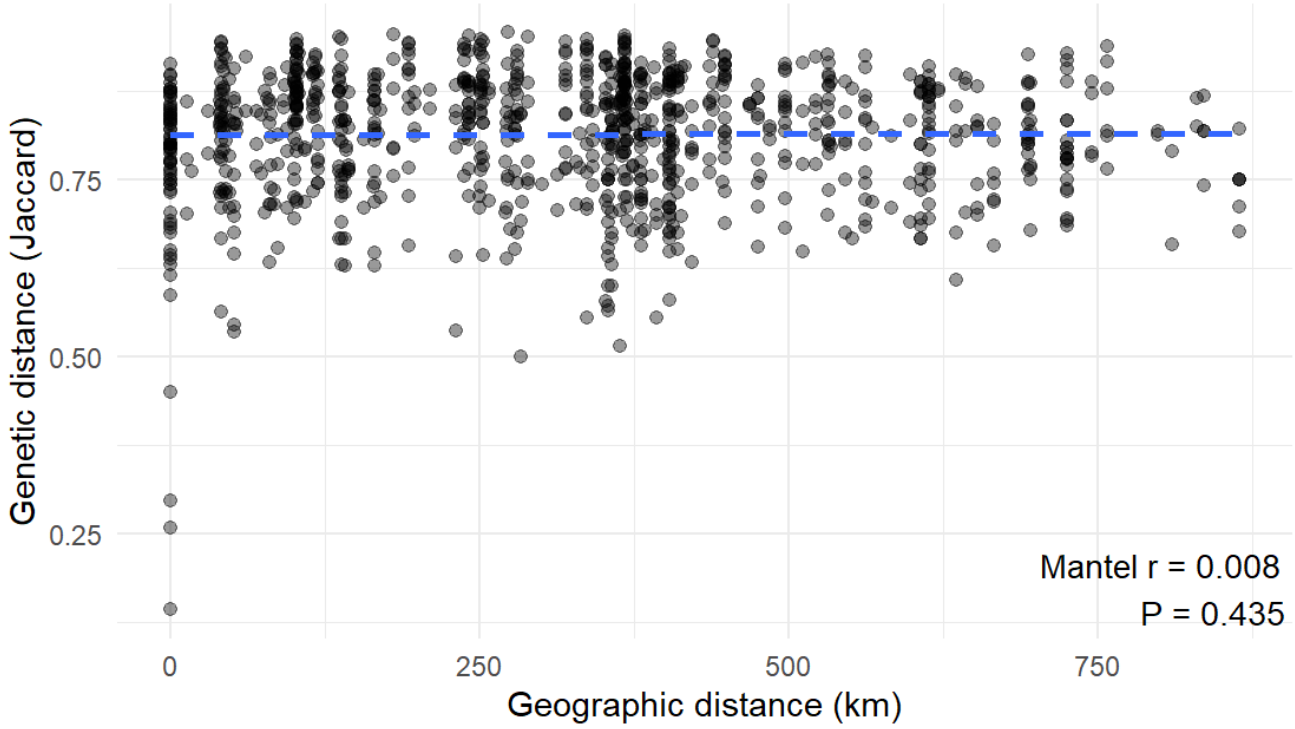

Figure 6. Scatter plot showing the correlation between genetic distance (Jaccard index) and pairwise geographic distance (km) among *L. carinerostris* samples, utilising ISSR data. Each data point corresponds to a unique pairwise comparison. The dashed line represents the linear regression fit (Mantel r = 0.008, P = 0.435).

2.4 Discussion

Identifying cryptic species complexes and local adaptation within potential biocontrol agents through population genetic studies is very important for biocontrol safety. Several studies have demonstrated the significance of cryptic species in biocontrol programs. The findings of this study contribute to addressing the knowledge gap regarding the genetic characterisation of *L. carinerostris*, a potential biocontrol agent for *C. crystallinum*. Contrary to the discovery of cryptic species complexes in other biocontrol programs, such as on the *Linaria*, *P. crassipes*, and *Tamarix* (L.) (Tamaricaceae) biocontrol programs (Toševski et al. 2011, Bean et al. 2014a, Paterson et al. 2016), the COI data in the present study indicate a panmictic population with significant gene flow across the three Cape provinces and host plant species, as evidenced by the low intraspecific p-distance and the lack of geographic or host-specific clustering. The low

intraspecific p-distance is consistent with established findings for DNA barcoding in Coleoptera, where intraspecific p-distances are typically less than 3.07% in Curculionidae (Ma et al. 2022). This low p-distance is comparable with other biocontrol program studies that discovered cryptic species complexes in their biocontrol agents, particularly the *Eccritotarsus* cryptic species complex, which revealed a p-distance of 5.2% (Paterson et al. 2016). Furthermore, this pattern aligns with a high dispersal throughout the Cape provinces, similar to patterns observed in other phytophagous insects (Roderick 1996, Peterson and Denno 1998, Sun et al. 2015).

The observed variable sites in the COI barcoding gene region did not reveal any grouping patterns associated with either host plant species or the geographic location of specimen collection. Such grouping patterns would be considered a sign of possible local adaptation to the geographic location or the host plant species. Despite the presence of variable sites in the sequenced COI mitochondrial gene region, the overall nucleotide diversity was low (0.77%), indicative of limited genetic divergence among the *L. carinerostris* individuals. Such a low level of intraspecific diversity is consistent with ongoing gene flow across the sampled geographic areas and host plant species, a pattern also observed in other population genetics assessment studies that used COI barcoding (Hupało et al. 2023, Khatun et al. 2024).

It is also important to consider the limitations of mitochondrial markers in this context. While the COI region is excellent for species identification and phylogeography (Pentinsaari et al. 2016), it is a protein-coding gene that is subjected to purifying selection at its first and second codon positions, which may limit its ability to detect adaptive divergence (Galtier et al. 2009). While the third codon position is more variable, it primarily reflects demographic history and neutral evolutionary processes rather than adaptive processes (Machado et al. 2020). The COI's selectively neutral nature means it mainly reflects population demographic history, but not

recent adaptive differentiation to different host plant species or different environmental conditions (Lunt et al. 1996, Funk and Omland 2003).

In contrast to the mitochondrial genome patterns, the nuclear ISSR data present a more complex scenario, as fragment analysis methods can offer greater sequence variation compared to mtDNA markers (Marwal et al. 2014). While the COI evolves at a relatively faster rate than nuclear genetic markers, it typically reflects only maternal lineage inheritance. On the contrary, ISSR markers assess multiple nuclear loci at once, offering patterns of biparental inheritance (Vijayan 2005). Although COI is a useful tool for identifying species, it often fails to reveal genetic structure within populations at finer geographical scales effectively (Abouseada et al. 2023). ISSR analysis, on the other hand, produces multilocus nuclear genome data that more effectively clarifies population structure and detects recent evolutionary changes because of its sensitivity to recombination and biparental inheritance (Bornet and Branchard 2001, Ng and Tan 2015).

The DAPC analysis revealed distinctive genetic differentiation patterns among *L. carinerostris* populations associated with the four *Cryophytum* host plant species. Most notably, *C. pellitum*-associated weevils exhibited clear genetic clustering, separated from populations associated with the other three *Cryophytum* hosts along both discriminant functions, with minimal overlap observed only with *C. barklyi*-associated weevils. This clustering pattern suggests that weevils associated with *C. pellitum* may represent a genetically differentiated lineage within *L. carinerostris*, potentially reflecting early stages of ecological specialisation or recent divergence associated with *C. pellitum*.

The DAPC findings revealed that *C. guerichianum*-associated weevil demonstrated extensive overlap with *C. crystallinum* and *C. barklyi*-associated weevils in the ordination space, which suggests that *C. guerichianum* may serve as a genetic bridge (interbreeds with both populations,

thus facilitating some genetic flow) between these two host associations. Despite both overlapping with *C. guerichianum*, weevil populations associated with *C. crystallinum* and *C. barklyi* exhibited distinct genetic differentiation. This pattern suggests that while there is gene flow among the three host plant associations, it may be primarily facilitated through *C. guerichianum*-associated populations, which appear to have an intermediate genetic position. This may be because *C. guerichianum* is the most widely distributed species of the four. Furthermore, the *C. barklyi* association may serve as a genetic bridge between the *C. pellitum* and the other *Cryophytum* host plant species.

The STRUCTURE analysis corroborated the patterns of genetic differentiation observed in the DAPC analysis. At K=3 and K=4, the STRUCTURE plot revealed that *C. pellitum*-associated *L. carinerostris* weevils demonstrated a distinct genetic cluster composition, which was absent in the other host associations. This distinct genetic signature supports the idea that weevils associated with *C. pellitum* represent a population that is genetically distinct within *L. carinerostris*. Additionally, this pattern may suggest that there is limited gene flow between weevils associated with *C. pellitum* and weevils associated with the other three *Cryophytum* species, or a more recent genetic divergence.

Contrary to the distinct genetic clustering observed in *C. pellitum*-associated weevils, the STRUCTURE plot revealed that weevils collected from *C. crystallinum*, *C. guerichianum*, and *C. barklyi* demonstrated extensive genetic admixture, with most *L. carinerostris* individuals showing mixed genetic composition. This genetic admixture indicates substantial historical and ongoing gene flow among these three associations. While these findings suggest that *C. pellitum*-associated weevils represent a clearly differentiated population, the other three *Cryophytum* host plant species associations form a genetically interconnected network with limited but detectable structure.

The genetic differentiation observed in the nuclear ISSR markers that was not apparent in the COI barcoding gene region of the mtDNA is not uncommon in insect population genetics. Similar patterns have been observed in other phytophagous insects, where mtDNA markers demonstrated panmixia, while nuclear markers revealed population structure (Malausa et al. 2007, Chu et al. 2011). These varying patterns in the two markers can arise from several factors, which include sex-biased dispersal where one sex disperses more than the other, or simply the different mutation rates and inheritance patterns of mtDNA relative to non-coding nuclear DNA. These findings highlight the importance of using multiple types of genetic markers with different inheritance patterns when assessing genetic structure in potential biocontrol agents.

Both the mitochondrial and nuclear genetic markers indicate that *L. carinerostris* is a *Cryophytum* oligophagous species rather than a species complex (Jaenike 1990, Forister et al. 2015). From an ecological perspective, the overall homogeneity across host plant species indicates that *L. carinerostris* possesses broad physiological tolerance rather than specialised adaptations to individual host plant species (Jaenike 1990). However, the genetic differentiation in *C. pellitum*-associated weevils, along with the subtle structure among weevils associated with *C. crystallinum*, *C. guerichianum*, and *C. barklyi,* suggests that the evolutionary trajectory toward host speciation may be in the early stages. This may be because *C. pellitum* typically occurs in isolation from the other host plants, thus potentially isolating the weevils associated with it to some extent. The absence of genetic isolation by distance patterns, coupled with genetic homogeneity across a broad geographic range, suggests effective and significant long-distance dispersal capabilities, possibly facilitated by active flight (Slatkin 1993, Roderick 1996, Hutchison and Templeton 1999).

2.4.2 Phylogenetic Analysis and Taxonomic Implications

Moving from population-level dynamics to broader taxonomic patterns, the phylogenetic findings have significant implications for the taxonomy of *L. carinerostris* and the *Lixus* genus

within the Lixinae subfamily. The Bayesian analysis showed that all *L. carinerostris* specimens clustered together in one well-supported clade, confirming that all *L. carinerostris* samples across all four *Cryophytum* host plant species and 11 geographic localities represent a single species.

The analysis also uncovered some notable taxonomic issues that challenge the current classification of the *Lixus* genus, revealing its polyphyletic nature. The phylogenetic analysis also revealed two polytomies and relatively low Bayesian and ML support values near the base of the tree, further supporting the unresolved nature within this group. Three species, namely, *L. cinerascens*, *L. angustatus*, and *L. punctiventris* (identified through GenBank submissions as *Lixus* species), clustered outside the main *Lixus* clade.

The proximity of *L. cinerascens* to *Larinus* species (Lixini tribe), and *L. punctiventris* to *Pachycerus* species (Cleonini tribe) suggests that these species may require taxonomic reassignment. The association of *L. punctiventris* with *Pachycerus* (Cleonini tribe) may also suggest a potential tribal reassignment. The *Pachycerus* genus is a group of weevils within the Cleonini tribe, typically associated with Asteraceae (Alonso-Zarazaga 1999). The polyphyletic nature of the *Lixus* genus is consistent with recent phylogenetic discoveries of the Lixinae subfamily, which have shown that none of the three recognised tribes within the subfamily are monophyletic (Volovnik et al. 2021). This suggests that the *Lixus* genus may require significant revision, provided further genetic and morphological assessments are conducted.

2.4.3 Implications for Biocontrol of *Cryophytum crystallinum*

These ecological and genetic findings also have practical implications for biocontrol efforts. The genetic homogeneity observed in the COI marker across *C. crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi* suggests that *L. carinerostris* is a *Cryophytum* specialist, rather than being specifically adapted to *C. crystallinum* alone. While the broad *Cryophytum* host range of

L. carinerostris may raise important concerns for risk assessment, *C. crystallinum* typically occurs as the only *Cryophytum* species in invaded regions, particularly in California and Mexico (Vivrette and Muller 1977, D'Antonio and Mahall 1991); therefore, *L. carinerostris* non-target risks are minimised in these regions. *Cryophytum* is endemic to southern Africa, so if *L. carinerostris* is a *Cryophytum* specialist, then it could be released anywhere in the invaded distribution as a biocontrol agent. However, the genetic differentiation observed in weevils associated with *C. pellitum* raises important concerns for source population selection in terms of agent efficacy. If this differentiation is a reflection of host plant species-specific adaptation or preferences, weevils associated with *C. pellitum* may perform differently on *C. crystallinum* compared to the others, possibly being less effective on the target weed.

When it comes to selecting appropriate populations for biocontrol, the COI analysis indicates that all sampled populations are genetically similar, suggesting that the geographic origin within South Africa may be less significant than previously assumed. However, the ISSR findings revealed host-associated genetic structure, particularly in weevils associated with *C. pellitum*, suggesting that source population selection should prioritise weevils already associated with *C. crystallinum* to maximise chances of successful establishment and efficacy on the invasive *C. crystallinum*. Weevils associated with *C. guerichianum,* which genetically overlap with those associated with *C. crystallinum* in the ISSR analyses, may represent a viable alternative source if *C. crystallinum*-associated populations are unavailable or insufficient.

In terms of dispersal ability, the mitochondrial genetic homogeneity across large geographic distances indicates a strong dispersal capability, which could facilitate rapid colonisation and spread following release as a biocontrol agent. The observed gene flow patterns suggest that introduced populations would likely maintain diversity through immigration from nearby established populations, reducing the risk of local extinction.

2.5 Conclusion

This study provides the first genetic characterisation of *L. carinerostris* and provides valuable insights for biocontrol decision-making. Furthermore, this study reveals that *L. carinerostris* is a genetically cohesive species with high dispersal capability across all four *Cryophytum* host plant species throughout all three Cape provinces of South Africa. The COI barcoding gene region of the mtDNA consistently showed genetic homogeneity with no evidence of cryptic species complexes or host plant species-specific lineages. The observed low divergence values, combined with the absence of genetic isolation by geographic distance, indicate significant ongoing gene flow across all *L. carinerostris* populations associated with the different host plant species. However, the ISSR data from the nuclear DNA revealed some host plant species-specific genetic differentiation, showing that gene flow may be mediated through specific host plant species associations.

From a biocontrol standpoint, *L. carinerostris* presents both opportunities and challenges. Its genetic uniformity at the mitochondrial level and high dispersal capability suggests good establishment potential. If *L. carinerostris* is found to pose no non-target risks outside the *Cryophytum* genus during host specificity testing, it would be safe to be released in areas with no other *Cryophytum* species, like California and Mexico (Vivrette and Muller 1977). Ultimately, these findings contribute to the already broad understanding of insect-plant coevolution and provide a crucial genetic baseline for evidence-based biocontrol decision-making.

Chapter 3 – Comparative feeding and reproductive performance assessment of *Lixus carinerostris* across multiple host plant species in South Africa

3.1 Introduction

The previous chapter found that *L. carinerostris* was a single panmictic species (based on the COI barcoding gene) across all four *Cryophytum* host plant species in South Africa. However, this does not automatically rule out the possibility of differential host plant species preference and performance, especially with the evidence of genetic differentiation observed in the nuclear ISSR marker data. Phytophagous insects typically demonstrate considerable differences in their feeding and reproductive performances across different host plant species, even among congeners (Surles 1972, Maddox and Sobhian 1987, Blossey et al. 1994, Mangan et al. 2024). These performance variations are displayed through varying levels of feeding damage, oviposition rates, larval survival, and successful complete development, ultimately creating hierarchies of host plant species suitability (Briese 1996, Kluge and Zachariades 2006). Such performance differences have been reported across several oligophagous insects, where genetic analysis revealed panmixia, but performance assessment exhibits clear host plant species preference and differential developmental success rates (Gripenberg et al. 2010, Thompson 2019). For instance, *Rhynocyllus conicus* Froehling (Coleoptera: Curculionidae), shows varying performances across multiple *Carduus* species, with higher survival rates on *Carduus nutans* (L.) (Compositae) versus *Carduus acanthoides* (L.) (Compositae), despite these plants being congeners (Surles 1972). Similarly, *Bangasternus orientis* (Coleoptera: Curculionidae) demonstrates differing developmental success among different *Centaurea* species, with higher performance on *Centaurea solstitialis* L. compared to *Centaurea diffusa* Lam. (Asteraceae) and *Centaurea calcitrapa* (L.) (Asteraceae) (Maddox and Sobhian 1987).

Most *Lixus* species display oligophagous feeding behaviour, often specialising on a few phylogenetically related plant species (Skuhrovec and Volovnik 2015, Trnka et al. 2016,

Mangan et al. 2024). The *Lixus* weevils' host specificity has important implications for weevil impact and performance assessment studies, as the level of specialisation can affect weevil performance and the potential for biocontrol applications. Comparative performance evaluations across all host plant species of a potential biocontrol agent provide valuable information for identifying preferred versus marginal hosts and identifying the most damaging weevil populations for biocontrol, which is crucial for understanding the population dynamics of a potential biocontrol agent and predicting efficacy (Mangan et al. 2024).

Oviposition behaviour and offspring performance can be decoupled in oligophagous phytophagous insects, representing an evolutionary trap phenomenon (Renwick 1989, Menacer et al. 2021). This means that female insects may oviposit on plant species that do not necessarily maximise offspring fitness or survival, contrary to the predictions of the preference-performance hypothesis (Menacer et al. 2021). This can be as a result of misleading environmental cues, plant chemistry, or even ecological constraints, and has been reported in several studies, showcasing that oviposition choices do not always align with optimal offspring success (Renwick 1989, Gripenberg et al. 2010, Menacer et al. 2021). *Pieris napi oleracea* Harris (Lepidoptera, Pieridae) butterflies exemplify insects that readily oviposit on the alien *Alliaria petiolata* (M.Bieb.) Cavara & Grande (Brassicaceae), yet larval survival is significantly varied across different populations, creating a demographic sink (Huang et al. 1994, Haribal and Renwick 2001). Similarly, *Euphydryas editha* Boisduval (Lepidoptera: Nymphalidae) shows oviposition preferences that do not correlate with larval performance across its different host plant species, resulting in suboptimal fitness outcomes (Rausher 1982). Therefore, it is possible that although *L. carinerostris* feeds and oviposits on all four *Cryophytum* host plant species, some of these species may not provide optimal conditions for the weevil's successful completion of development.

Extensive impact assessment methodologies have been developed through biocontrol programs, providing robust frameworks for evaluating host plant-insect interactions and comparing performance across multiple host plant species. Studies on weevils such as *Lixus aemulus* Petri (Coleoptera: Curculionidae) have demonstrated that stem-mining weevils can exhibit varying performance across multiple host plant species, with documented effects including stem mortality, reduction in dry mass of infested stems, and reduction in fruit production, which vary considerably among host plant species (Kluge and Zachariades 2006). Similarly, *L. cardui* has demonstrated that feeding damage can result in both biomass reduction and decreased seed production in *Onopordum* (Asteracea) host plant species, but with different degrees of impact depending on the host plant species characteristics (Briese et al. 2004). These varying performance patterns often manifest at different developmental stages, with some species showing successful early establishment but failure in later developmental stages, as exemplified by *Galerucella pusilla* (L.) (Coleoptera: Chrysomelidae) on different *Lythrum* species (Blossey et al. 1994). These studies provide valuable criteria for designing a comparative performance assessment for *L. carinerostris* across all its known *Cryophytum* host plant species.

The assessment of weevil performance on different host plants usually involves measuring how well the weevils survive, develop, and reproduce on the various host plants, as well as their impact on the plants (Briese 1996). Several mechanisms may be responsible for varying host plant suitability, including differences in nutrient quality, variations in chemical defences, and physical habitat structural differences (Scriber 1984). For example, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae) performance differs across cotton species due to variations in gossypol content and fibre characteristics (Scriber 1984). Some performance studies combine field and laboratory experiments, focusing on metrics such as feeding damage, weevil survival, reproductive success, and host plant resistance (Briese 1996, Gültekin 2006). Therefore,

evaluating external feeding and oviposition damage, as well as developmental success on and inside stems, could be valuable for assessing *L. carinerostris*' performance across different host plant species. The number of larvae and pupae inside stems can indicate reproductive investment. Pre-emergent adults and exit holes serve as measurable outcomes of successful development. These external performance indicators, along with internal developmental stage assessments, enable a comprehensive and comparative evaluation of *L. carinerostris*' feeding and reproductive performance across host plant species.

The oligophagous nature typical of the *Lixus* genus suggests that *L. carinerostris* likely has specialised adaptations to *C. crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi*, potentially resulting in host plant species-specific damage patterns. Such patterns of specialisation have significant ecological and evolutionary implications, as varying performance can create sink habitats and influence population dynamics. The asymmetric coevolutionary pressures resulting from variations in performance may lead to varying degrees of physiological compatibility between herbivores and their host plants (Thompson 2019).

Therefore, this study aimed to assess whether there is any variation in weevil feeding, reproductive, and developmental performance of *L. carinerostris* across all its known host plant species in the native range of South Africa. The results of this study have significant implications for the biocontrol program of *C. crystallinum*. Population of the weevils for biocontrol should be reared on the plant species that produces the highest number of healthy offspring, and could also help determine if the target weed is the primary host plant.

3.2 Methods

3.2.1 Sampling Site Selection and Data Collection

Sampling sites across the Western and Northern Cape provinces of South Africa for each plant species —*C. crystallinum*, *C. guerichianum*, *C. pellitum*, and *C. barklyi*—were selected based

on the Gerbaulet (2017) distribution guide and existing citizen science data from iNaturalist (Figure 7). To ensure comparability across host plant species, sampling was conducted at sites where plants were at similar phenological stages, specifically targeting plants with mature stems and producing fruit. Sampling efforts differed among host plant species due to variations in their distribution and abundance across study sites, resulting in 16, 9, 12, and 9 individual plants of *C. crystallinuum*, *C. barklyi*, *C. guerichianum*, and *C. pellitum,* respectively. Although *C. pellitum* and *C. barklyi* have relatively smaller sample sizes, all four *Cryophytum* host plant species were included in the subsequent analysis to provide a comprehensive assessment of *L. carinerostris*' performance across all its host plant species in South Africa. The sites did not cover a defined area because the density of plants at each site was different, and since some of the species (*C. crystallinum* and *C. pellitum*) are annual plants, they tend to grow in slightly different locations every year.

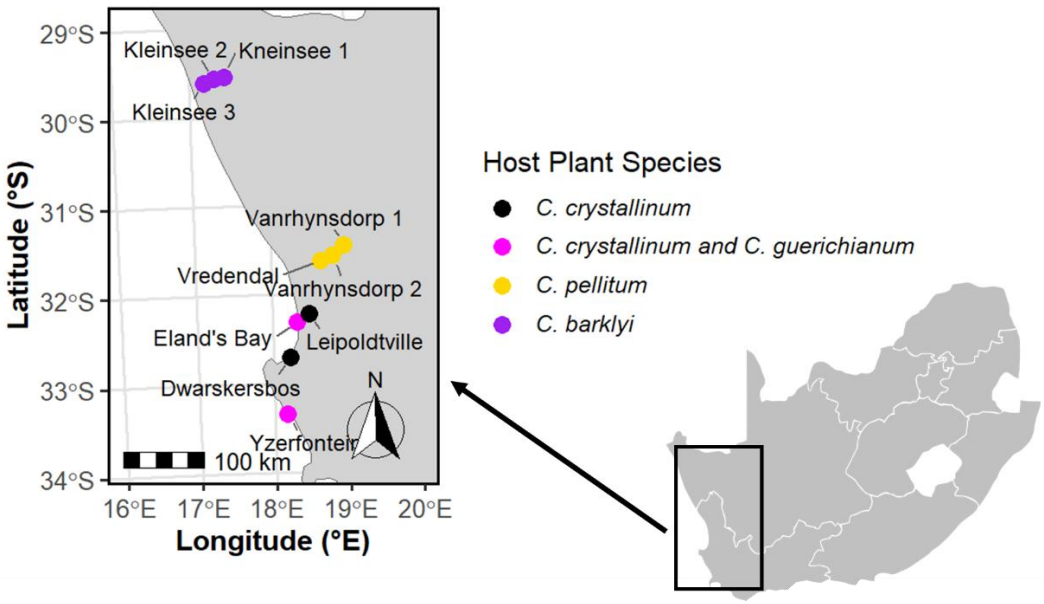

Figure 7. Map of performance data collection sites in South Africa, coloured by host plant species.

At each site, three adult plants of approximately the same age (determined by the presence of mature stems and fruits) were selected, and the three longest stems were measured and documented as indicators of the overall plant size. Each plant was thoroughly examined for

signs of stem chlorosis (Figure 8), indicative of weevil burrowing. Feeding holes (approximately 0.3 cm in diameter), oviposition holes (approximately 0.1 cm in diameter and covered by faecal matter), and exit holes (approximately 0.5 cm in diameter) were counted and recorded for each plant. Secateurs were used to dissect each stem carefully. *Lixus carinerostris* larvae, pupae, and pre-emergent adults (Figure 9) were identified using the Meregalli (2014) *Lixinae* morphology and systematics guide, counted, and recorded. A subset of samples was used in the COI barcoding analysis to confirm species identity.

Figure 8. *Cryophytum crystallinum* stem exhibiting severe chlorosis due to *L. carinerostris* developing inside.

Figure 9. Internal stem damage caused by developing *L. carinerostris.*

3.2.2 Statistical analysis

Statistical analyses were performed in R version 4.4.2 (R Core Team, 2024). All data were cleaned using the dplyr package (Wickham 2015). Plant size for each plant was calculated as the mean of the three longest measured stems. Host plant species were used as categorical

predictors with four levels i.e. *C. crystallinum*, *C. guerichianum*, *C. barklyi*, and *C. pellitum*, with *C. crystallinum* set as the reference category for model comparisons. Setting *C. crystallinum* as a reference category facilitated interpretation of performance differences relative to the target IAPS for biocontrol. The mean plant size was included as a continuous covariate to account for host plant species size differences. For each of the plants, there were six response variables recorded: (1) number of feeding holes, (2) number of oviposition holes, (3) number of exit holes, (4) number of larvae, (5) number of pupae, and (6) number of adults.

3.2.2.1 Zero-inflation evaluation

Before model selection was conducted, each of the response variables was evaluated for zero-inflation to determine if there was a need for specialised zero-inflation structure models. The observed proportion of zeros was calculated and compared to expected zero proportions under both the Poisson and the negative binomial distributions. Instances where the observed proportion of zeros exceeded the expected zero proportion by at least 10%, zero-inflation was considered present, indicating significant deviation from the standard count distribution, signifying the need for zero-inflation models.

3.2.2.2 Model Comparison and Selection

The variables of interest were all non-negative count variables; therefore, multiple generalised linear models (GLMs) were evaluated to identify the most appropriate distribution for each variable. Evaluated models included (1) Poisson GLM with log-link function, (2) Negative binomial GLM with log-link function, (3) Zero-inflated Poisson (ZIP), and (4) Zero-inflated Negative Binomial (ZINB).

Overdispersion was assessed using the ratio of residual deviance to residual degrees of freedom, with values above 1.5 deemed indicative of overdispersion, making negative binomial models appropriate (Zuur et al. 2009). When overdispersion was found present, negative

binomial GLMs were then fitted by maximum likelihood estimation using the MASS package (Ripley et al. 2013), which provides an estimate of the dispersion parameter (θ) that adjusts for extra-Poisson variation.

The model's performance was contrasted with Akaike's Information Criterion (AIC). The model with the smallest AIC was adopted as the optimum model best fitting each response variable, while $\Delta AIC > 2$ between models was considered meaningful evidence of model improvement (Burnham and Anderson 2002).

For cases when the zero-inflated model and the standard model shared similar AIC values ($\Delta AIC < 5$), Vuong tests were performed to test whether the inclusion of the zero-inflation term had significantly improved model fit (Vuong 1989). The Vuong test compares non-nested models by testing whether log-likelihood differences between them are significantly different from zero, providing a statistical basis for model selection.

3.2.2.3 Final models and inference

The model with the lowest AIC (best fit) for each of the response variables was selected for inference. The Wald z-test ($\alpha = 0.05$) was used to determine the statistical significance of predictor effects. The model coefficients are reported as log-scale estimates (β). For the negative binomial models, the exponential of coefficients (exp(β)) provided multiplicative effects on the expected counts, helping to interpret effect sizes. For example, exp (β)=0.1 indicated a 10% reduction in expected count compared to the reference category (*C. crystallinum*).

For visualisation, predicted counts were calculated across the observed plant size range for each host plant species using model predictions. The predicted values were transformed from the log scale to the original count scale for presentation purposes. The statistical packages employed included MASS (Ripley et al. 2013) for negative binomial models, pscl (Jackman

2020) for zero-inflated models, broom for tidying model output, and ggplot2 (Wickham 2016) for visualisation of the results.

3.3 Results

3.3.1 Best model fit

The AIC comparisons revealed that the negative binomial GLMs provided the best fit for all six response variables (Table 5). Although some variables had high proportions of zeros (54.3% - pupae, and 73.9% - adults), the negative binomial distribution adequately captured these zeros without the need for additional zero-inflation structure.

Table 5. Summary of model selection for *L. carinerostris* performance parameters. All models used a negative binomial GLM with a log-link function. Host plant species p-values indicate the overall significance of the host plant species effect ($\alpha = 0.05$)

Variable	AIC	Overdispersion ratio	% Zeros	Theta (θ)	P (Host plant species
Feeding holes	378.26	13.38	0.0	1.74	0.464
Oviposition holes	341.44	7.24	0.0	2.85	0.297
Larvae	316.62	14.68	10.9	8.45	0.001
Pupae	121.36	1.40	54.3	N/A	<0.001
Pre-emergent adults	82.80	1.07	73.9	N/A	<0.001
Exit holes	152.30	2.39	43.5	1.23	0.023

The mean counts of all response variables across all four *Cryophytum* host plant species are summarised in Figure 10. *Cryophytum barklyi* notably had more larval counts (30.33 ± 12.44) compared to the other host plant species (7.12-7.56). No pupae were recorded from *C. barklyi*

and *C. pellitum*. *Cryophytum crystallinum* exhibited the highest developmental success (pupae, adults, and exit holes)

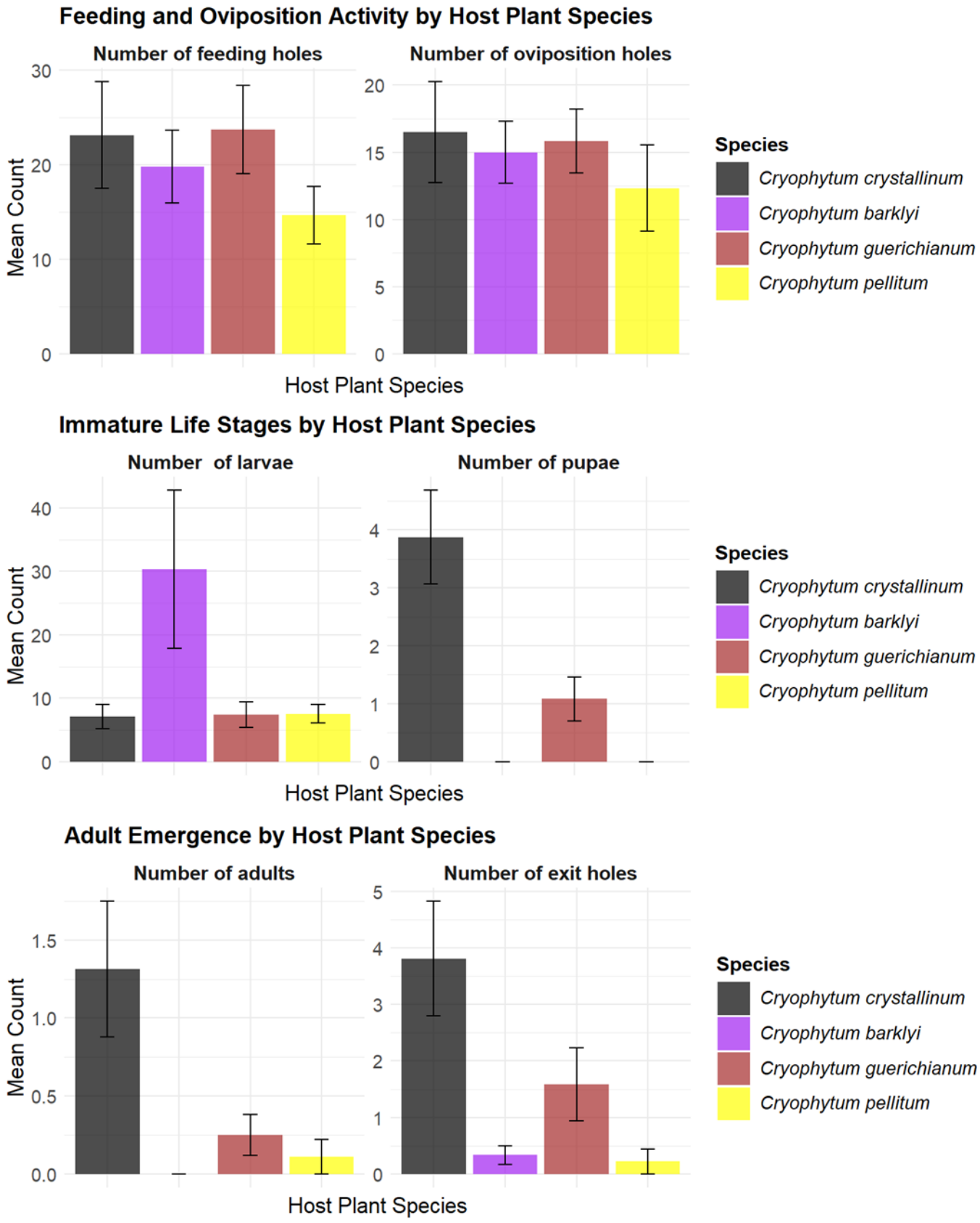

Figure 10. Descriptive summary plots of *L. carinerostris* performance between *C. barklyi* (purple), *C. crystallinum*

(black), *C. guerichianum* (brown), and C, pellitum (yellow). Bar plots represent the mean counts from each *Cryophytum* host plant species across all measured performance parameters.

3.3.2 External performance indicators

There was no statistically significant difference in the number of feeding holes across all *Cryophytum* host plant species. None of the *Cryophytum* host plant species had feeding hole counts that were significantly different from *C. crystallinum* (reference category) (Table 6 & Figure 11), suggesting that adult *L. carinerostris* demonstrates non-discriminatory feeding behaviour.

Table 6 Negative binomial GLM for number of feeding holes (Reference category: *C. crystallinum*, θ: 1.74 ± 0.38, AIC: 378.26)

Predictor	**β (log scale)**	**95% CI**	**z-value**	**p-value**	**exp(β)**
Intercept	2.58	(1.52, 3.64)	4.70	<0.001	13.20
Plant size	0.006	(-0.006, 0.018)	1.05	0.295	1.006
C. barklyi	0.10	(-0.39, 0.60)	0.26	0.792	1.11
C. guerichianum	-0.07	(-0.73, 0.59)	-0.22	0.823	0.93
C. pellitum	-0.17	(-1.00, 0.66)	-0.41	0.679	0.84

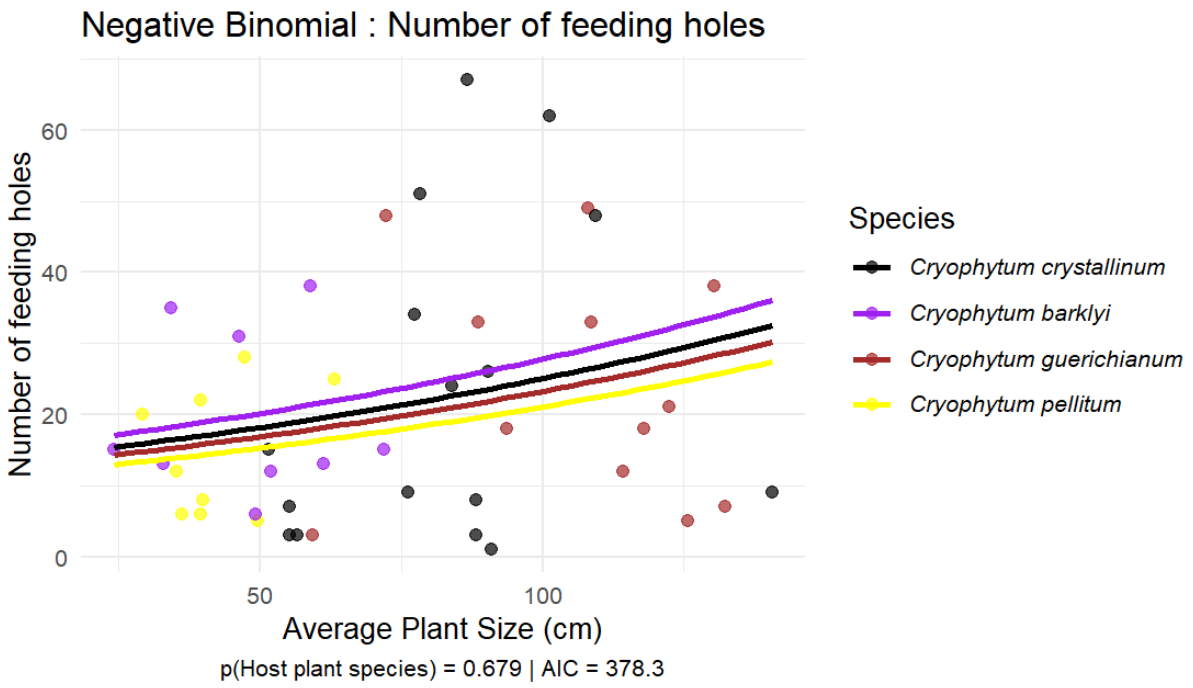

Figure 11. Comparative negative binomial GLM plot of the number of feeding holes created by *L. carinerostris* on the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. No significant host plant species effect was found (p = 0.464)

Similarly, oviposition hole counts did not significantly differ between the four *Cryophytum* host plant species. None of the oviposition holes on the *Cryophytum* host plant species differed significantly from those on *C. crystallinum* (Table 7 & Figure 12), which indicates that female *L. carinerostris* do not discriminate between the two host plant species when selecting oviposition sites.

Table 7. Negative binomial GLM for the number of oviposition holes (Reference category: *C. crystallinum*, θ: 2.85 ± 0.82, AIC: 341.44)

Predictor	**β (log scale)**	**95% CI**	**z-value**	**p-value**	**exp(β)**
Intercept	2.03	(1.11, 2.95)	4.34	<0.001	7.61
Plant size	0.009	(-0.001, 0.019)	1.70	0.089	1.009
C. barklyi	0.26	(-0.34, 0.85)	0.76	0.445	1.29

C. guerichianum	-0.19	(-0.75, 0.37)	-0.66	0.507	0.83
C. pellitum	0.08	(-0.55, 0.72)	0.24	0.814	1.09

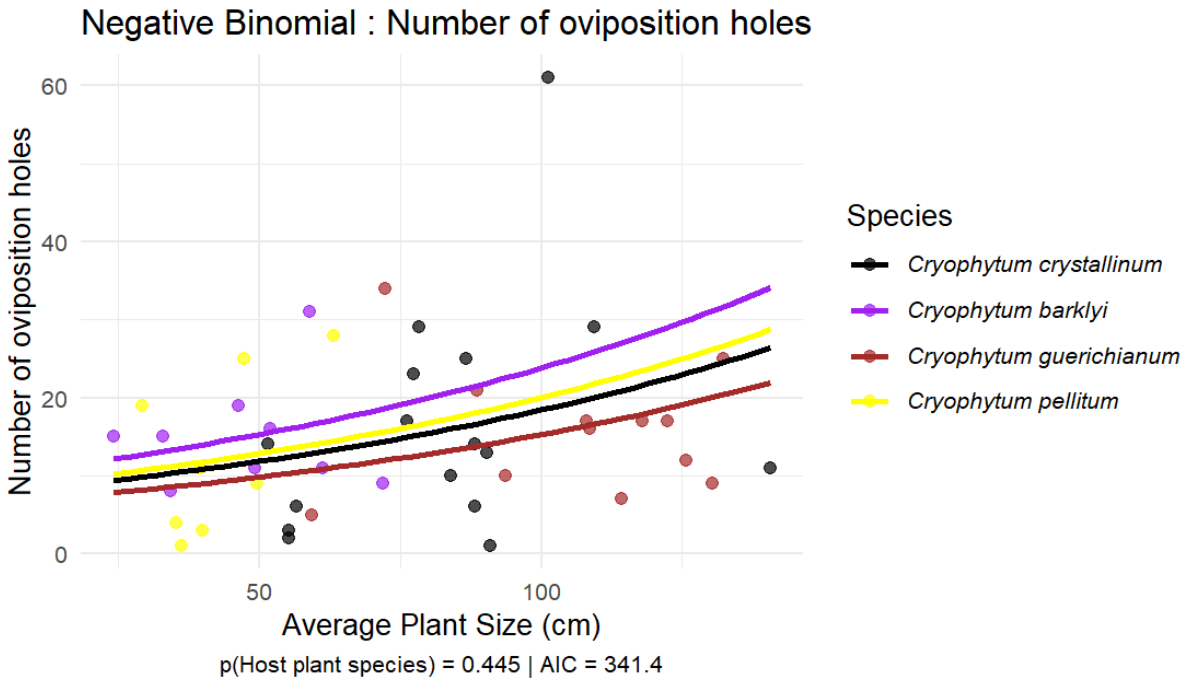

Figure 12. Comparative negative binomial GLM plot of the number of oviposition holes produced by *L. carinerostris* on the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. No significant host plant species effect was found (p = 0.297)

3.3.3 Internal performance indicators

Contrary to the aforementioned counts, pupal counts analysis revealed a statistically significant difference between *Cryophytum* host plant species. *Cryophytum barklyi* supported approximately 4.3 times more larvae than *C. crystallinum* (β = 1.78, 95% CI: 0.68 to 2.87, *p* = 0.001; Table 8). However, larval counts from *C. guerichianum* and *C. pellitum* did not significantly differ from *C. crystallinum* larval counts (Table 8 & Figure 13). *Cryophytum*

crystallinum supported significantly higher pupal densities within stems, while *C. guerichianum* showed minimal pupal development regardless of plant size.

Table 8. Negative binomial GLM for the number of larvae (Reference category: *C. crystallinum*, θ: 8.45 ± 1.23, AIC: 316.62)

Predictor	**β (log scale)**	**95% CI**	**z-value**	**p-value**	**exp(β)**
Intercept	1.24	(-0.35, 2.83)	1.57	0.117	3.45
Plant size	0.008	(-0.009, 0.025)	0.96	0.337	1.008
C. barklyi	1.78	0.68, 2.87)	3.19	0.001	5.93
C. guerichianum	-0.13	(-1.09, 0.82)	-0.28	0.778	0.88
C. pellitum	0.41	(-0.58, 1.40)	0.69	0.491	1.51

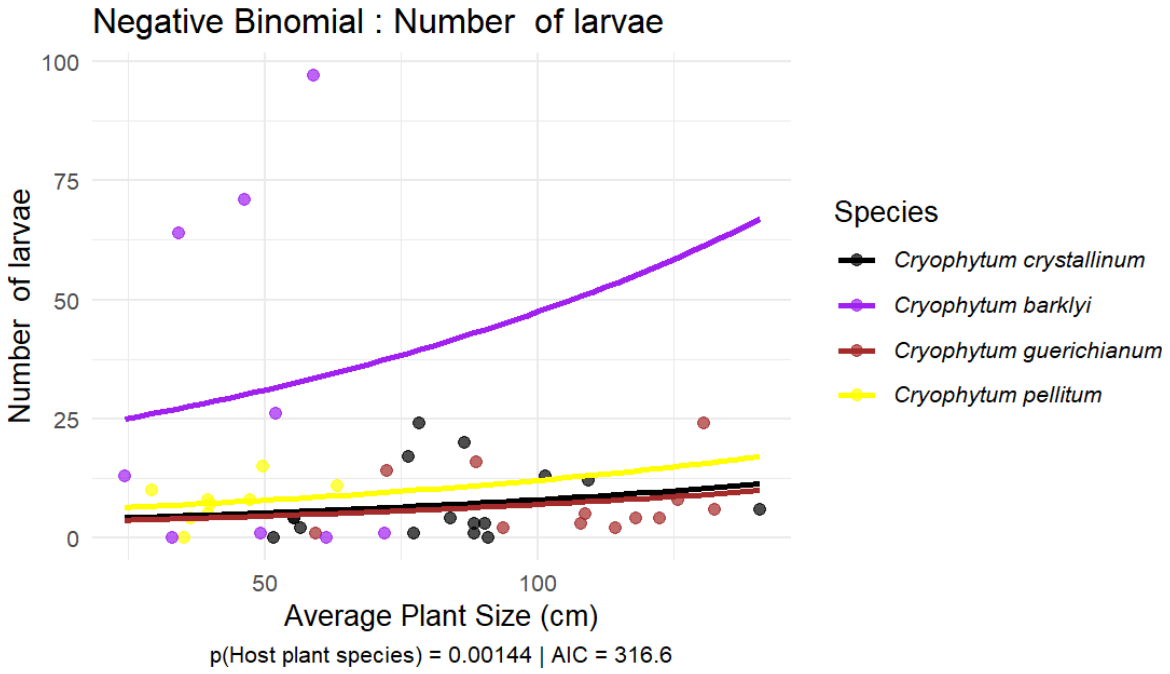

Figure 13. Comparative negative binomial GLM plot of the number of *L. carinerostris* larvae inside the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected (p = 0.00144)

The analysis revealed a statistically significant difference in the pupal densities amongst the four *Cryophytum* host plant species. *Cryophytum crystallinum* supported significantly higher pupal densities relative to all other host plant species. Both *C. barklyi* and *C. pellitum* had zero pupae across all sampled plants (Figures 13 & 14). *Cryophytum guerichianum* had significantly lower pupae (approximately 75% less pupal count) compared to *C. crystallinum* (β = -1.38, 95% CI: -2.12 to -0.64, p = 0.001; Table 9).

Table 9. Negative binomial GLM for the number of pupae (Reference category: *C. crystallinum*, AIC: 121.36)

Predictor	**β (log scale)**	**95% CI**	**z-value**	**p-value**	**exp(β)**
Intercept	0.54	(-91, 2.00)	0.73	0.463	1.72
Plant size	0.012	(-0.004, 0.028)	1.53	0.127	1.012
C. barklyi	-33.9	(-∞, -27.2)	<0.001	1.000	~0
C. guerichianum	-1.38	(-2.12, -0.64)	-3.66	<0.001	0.25
C. pellitum	-33.6	(-∞, -26.9)	<0.001	1.000	~0

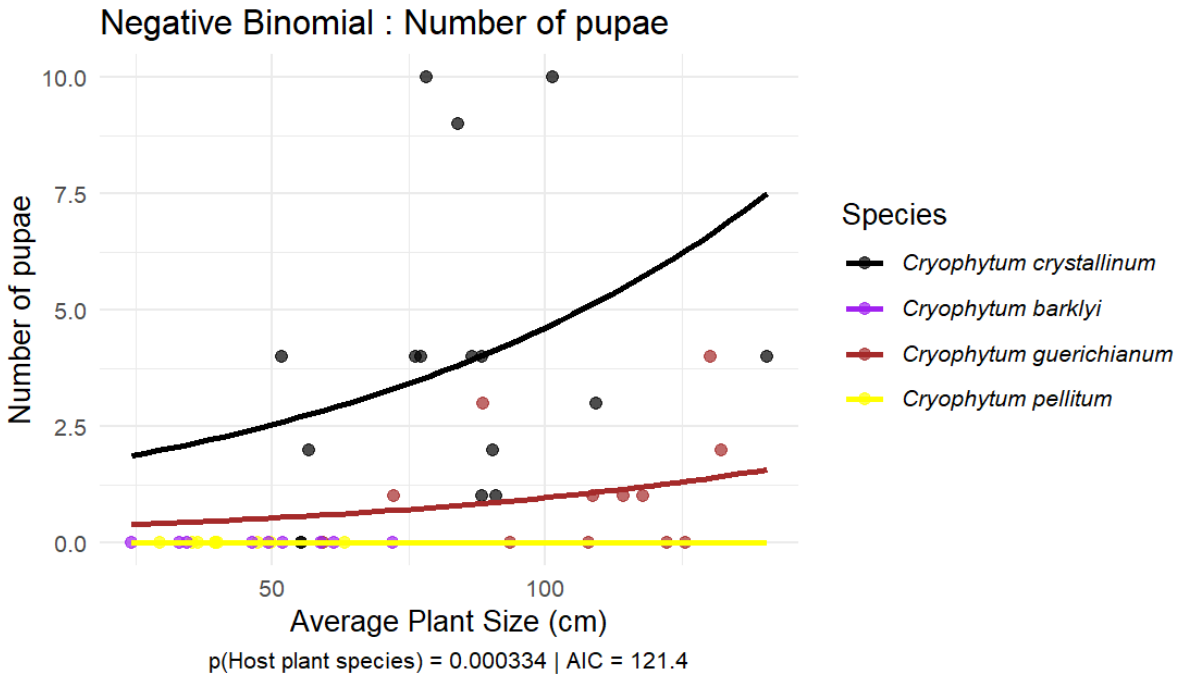

Figure 14. Comparative negative binomial GLM plot of the number of *L. carinerostris* pupae inside the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected (p = 1)

Similarly, *C. crystallinum* consistently had statistically significantly more pre-emergent adult counts than the other *Cryophytum* species. The data, therefore, revealed differential developmental success within the stems of the four host plant species, with *C. barklyi* and *C. pellitum* high levels of developmental failure (no evidence of weevils developing beyond the larval stage) of *L. carinerostris*, and lower but non-zero successful development on *C. guerichianum* (β = -1.92, 95% CI: -2.87 to -0.97, $p < 0.001$; Table 10 & Figure 15). This suggests that *C. crystallinum* provides superior internal conditions for complete *L. carinerostris* development.

Table 10. Negative binomial GLM for the number of pre-emergent adults (Reference category: *C. crystallinum*, AIC: 82.80)

Predictor	**β (log scale)**	**95% CI**	**z-value**	**p-value**	**exp(β)**
Intercept	-1.14	(-3.31, 1.03)	-1.03	0.304	0.32
Plant size	0.016	(-0.008, 0.040)	1.29	0.199	1.016
C. barklyi	-36.7	(-∞, -29.0)	<0.001	1.000	~0
C. guerichianum	-1.92	(-2.87, -0.97)	-3.94	<0.001	0.15
C. pellitum	-1.73	(-3.57, 0.12)	-1.84	0.066	0.18

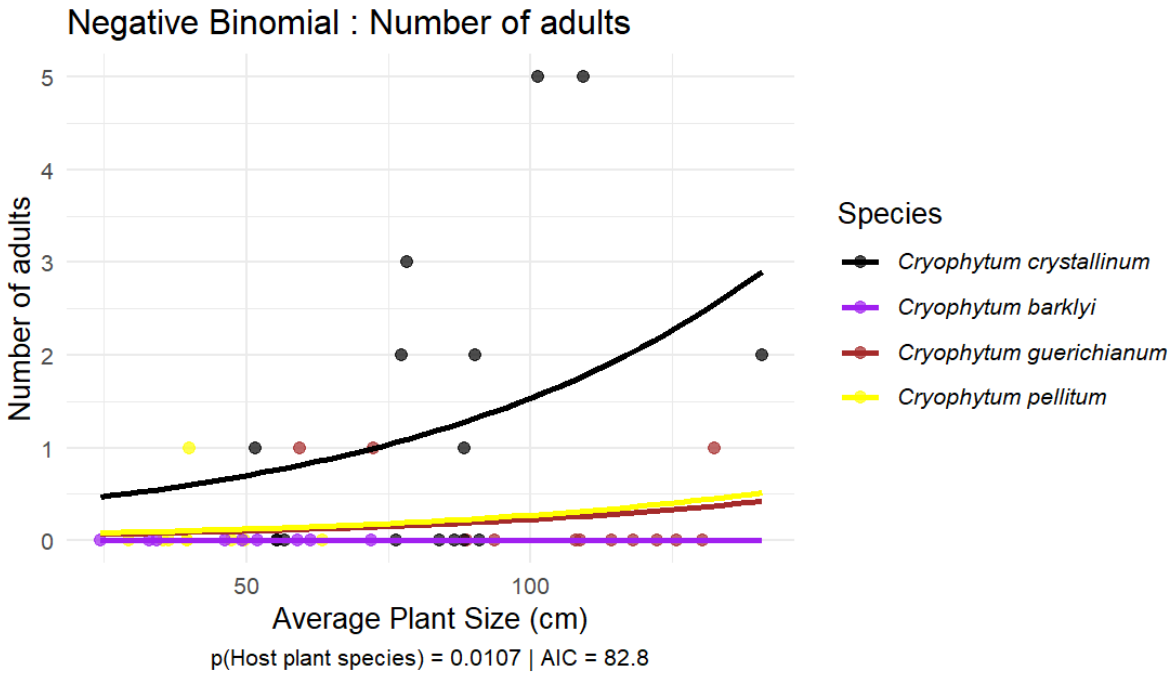

Figure 15. Comparative negative binomial GLM plot of the number of *L. carinerostris* pupae inside the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected (p = 1)

3.3.4 External Evidence of Successful Development

There was a statistically significant difference between the number of exit holes on the four *Cryophytum* host plant species (Table 11 & Figure 16), with *C. crystallinum* consistently showing higher exit hole counts across the plant size gradient, indicating higher successful *L. carinerostris* adult emergence from *C. crystallinum* than from the other three congeners.

Table 11 Negative binomial GLM for the number of exit holes (Reference category: *C. crystallinum*, θ: 1.23 ± 0.45, AIC: 152.30)

Predictor	**β (log scale)**	**95% CI**	**z-value**	**p-value**	**exp(β)**
Intercept	-34	(-1.97, 1.29)	-0.41	0.682	0.71
Plant size	0.020	(0.002, 0.038)	2.13	0.033	1.020
C. barklyi	-1.75	(-3.27, -0.24)	-2.28	0.023	0.17
C. guerichianum	-1.33	(-2.82, 0.15)	-1.76	0.079	0.26
C. pellitum	-2.07	(-3.89, -0.25)	-2.23	0.026	0.13

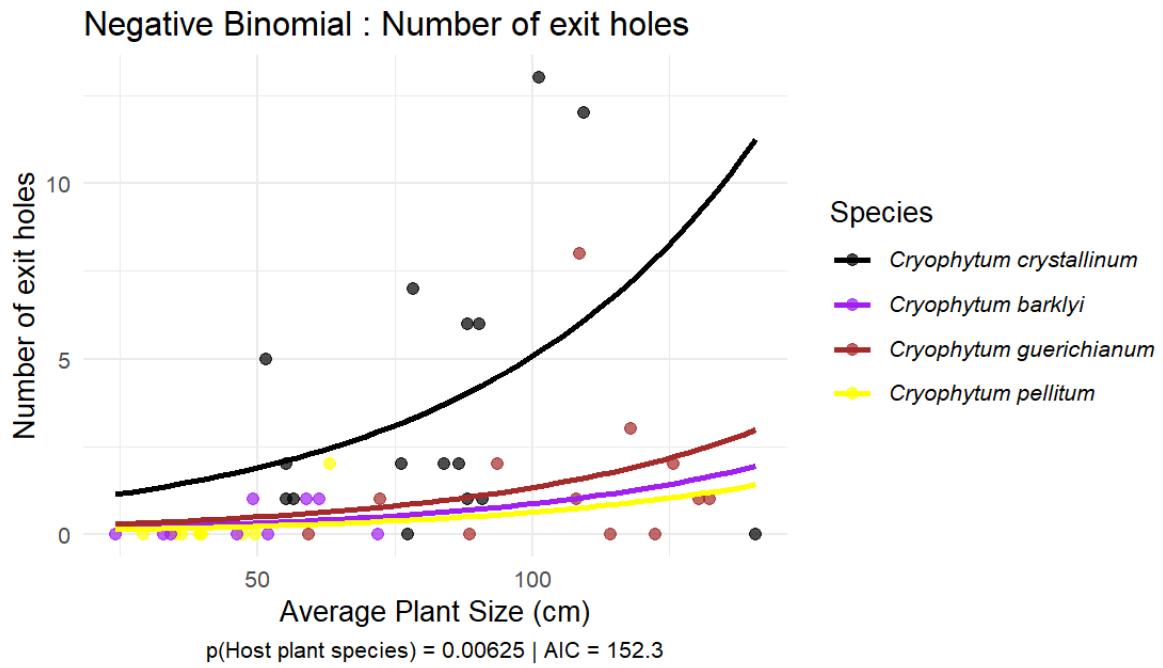

Figure 16. Comparative negative binomial GLM plot of the number of exit/emergence holes created by *L. carinerostris* on the stems of *C. barklyi* (purple), *C. crystallinum* (black), *C. guerichianum* (brown), and *C. pellitum* (yellow). The data points represent individual plant observations with fitted regression lines from the negative binomial GLM. Statistically significant host plant species effects were detected (p = 0.0228)

3.4 Discussion

3.4.1 Host Plant Species Specialisation and Performance Hierarchy

The findings of this study demonstrate that the performance of *L. carinerostris* varies considerably across all four *Cryophytum* host plant species. *Lixus carinerostris* exhibits differential survival and developmental success on *C. crystallinum*, *C. barklyi*, *C. guerichianum*, and *C. pellitum* despite these plant species being congeners. These findings are consistent with performance patterns documented from other oligophagous weevils like *R. conicus* on *Carduus* species and *B. orientis* on *Centaurea* species (Surles 1972, Maddox and Sobhian 1987). The observed weevil performance hierarchy is consistent with the broader pattern observed across the *Lixus* genus, where most *Lixus* species are oligophagous and typically specialise on phylogenetically related plant species, but with varying performance

levels among these related host plant species (Mangan et al. 2024, Volovnik 2024). In particular, *C. crystallinum* supported significantly higher pupal and pre-emergent adult densities compared to all the other *Cryophytum* host plant species, with *C. guerichianum* exhibiting intermediate yet reduced developmental success, and both *C. pellitum* and *C. barklyi* showing significantly lower adult emergence counts.

The results deviate from the preference-performance hypothesis (Renwick 1989, Gripenberg et al. 2010) in that they reveal an important decoupling between adult weevil feeding and oviposition behaviour versus offspring developmental success. While adult weevils exhibit similar feeding and oviposition behaviour across all four host plant species, pupal and pre-emergent adult counts revealed developmental constraints that differed significantly across the host plant species.

Most notably, *C. barklyi* showed significantly higher larval counts compared to the other three congeners, yet adult emergence was extremely low across all plant individuals that were sampled. This pattern could suggest elements of the evolutionary trap phenomenon reported in other phytophagous insects, which demonstrates the decoupling patterns between oviposition behaviour and offspring performance, with female insects ovipositing on host plant species that do not maximise offspring fitness and/or survival (Renwick 1989, Gripenberg et al. 2010, Menacer et al. 2021). Such observations have been documented from species like *P. napi oleracea* butterflies on *A. petiolata*, and *E. editha* on its different host plant species (Rausher 1982, Huang et al. 1994, Haribal and Renwick 2001).

The evolutionary trap phenomenon can arise from misleading environmental cues, plant chemistry, or ecological constraints, ultimately creating demographic sinks where reproductive investment does not always translate to successful offspring development and survival. However, it is unclear whether this pattern represents a true evolutionary trap phenomenon or

is a result of *L. carinerostris* females' inability to assess internal stem conditions before oviposition. One possible mechanistic explanation for the *C. barklyi*-associated weevil performance pattern might be the plant's physical structure. *Cryophytum barklyi* is relatively much more robust with harder and denser stems compared to the other *Cryophytum* host plant species (Gerbaulet 2017). The plant's structural robustness may cause larvae to become trapped inside hard stem tissues or failure to locate suitable pupation sites where they can successfully metamorphose. The high larval establishment, combined with low adult emergence, suggests that while *C. barklyi* stems provide suitable initial conditions for larval feeding and survival, the physical architecture of the stems more frequently prevents successful weevil development to the adult stage compared to other host plant species. Future research assessing *L. carinerostris* fitness consequences across multiple generations and spatial contexts, along with a detailed investigation of stem physical properties of host plant species and pupation site requirements, is necessary to verify the adaptive significance of the observed oviposition patterns.

3.4.2 Weevil adult behaviour and host plant external damage

The observed similarity in the number of feeding holes across all four *Cryophytum* host plant species suggests that *L. carinerostris* adults do not show a feeding preference for any of the four host plant species. This non-selective feeding behaviour suggests that the external morphological and chemical characteristics of all the *Cryophytum* host plant species present cues that are sufficiently similar for adult *L. carinerostris* to feed on them, likely reflecting their shared phylogenetic history (Thompson 2019). *Lixus carinerostris* feeding morphological traits appear to be equally suited to feeding on all host plant species, creating the characteristic external damage patterns without significant host plant species discrimination.

Similarly, the absence of a statistical difference in the number of oviposition holes among the four host plant species suggests that female *L. carinerostris* do not discriminate among these

congeners during oviposition. This behaviour suggests that the proximate cues guiding female oviposition decisions do not reliably predict the internal stem suitability for larval and pupal development as seen in some phytophagous insects (Renwick 1989, Gripenberg et al. 2010). Alternatively, *L. carinerostris* females may lack the sensory abilities to evaluate the internal stem differences through the external surface, or the costs of more discriminating oviposition behaviour may outweigh the benefits (Menacer et al. 2021).

3.4.3 Differential Host Suitability Mechanisms

The similar initial establishment of larvae across *C. crystallinum*, *C. guerichianum*, and *C. pellitum*, followed by significant divergence in pupal and adult success, indicates that the effects of host plant species mainly occur during the endophytic larval stage. This developmental stage-specific performance pattern is consistent with observations in *G. pusilla*, where early establishment success on different *Lythrum* species does not predict later developmental outcomes (Blossey et al. 1994). Host plant suitability is not only influenced by taxonomic relatedness, but also by stem internal physical structure, chemical composition, and intraspecific variation (Barbour et al. 2015, Rapo et al. 2019, Tielens and Gruner 2020).

The aforementioned factors influencing weevil performance parallel those documented in other stem-mining weevils. *Anthonomus grandis* is a species of weevils with varying performances across their host plant species (Scriber 1984). Several mechanisms may explain the variation in *L. carinerostris* performance across the four *Cryophytum* host plant species, though the current study cannot definitively differentiate them.

Chemical defence differences may represent another possible mechanism, where *C. guerichianum*, *C. barklyi*, and *C. pellitum* may have relatively higher concentrations of deterrent secondary metabolites in internal tissues that are not present in external tissues, rendering the external tissues accessible during feeding and oviposition. Additionally, there

may be significant differences in the physical habitat structures of the four *Cryophytum* species. *Lixus* species' endophytic lifestyle requires a suitable internal architecture for mining and pupation chamber construction. Differences in tissue organisation, stem density, and water content could significantly impact the survival of larvae and successful pupation.

The significantly higher number of exit holes on *C. crystallinum* than the other three congeners corroborates the internal performance indicators of pupal and pre-emergent adult counts, providing independent evidence that the observed difference in developmental success translates to adult emergence success.

3.4.4 Effects of host plant phenology on weevil performance

Cryophytum crystallinum is clearly *L. carinerostris*'s primary host plant species, evidenced by this host plant species supporting the highest developmental success and population recruitment. However, *C. crystallinum* is also the least apparent and has the shortest life span of the four *Cryophytum* host plant species. It is possible that *C guerichianum*, *C. barklyi*, and *C. pellitum* function as secondary host plant species, with *C. crystallinum* being preferred when it is available. Although the three plants support lower successful development of *L. carinerostris* compared to *C. crystallinum*¸ they are more apparent and have longer life spans, with *C. barklyi* and *C. guerichianum* being present throughout the year. *C. guerichianum* demonstrates intermediate performance that could contribute to *L. carinersostris* population persistence during periods when *C. crystallinum* is only present as seeds.

The phenological dynamics of these host plant species have significant implications for biocontrol. The higher performance of *L. carinerostris* on *C. crystallinum* suggests that the weevil could build large populations in invaded regions. However, if the plant phenology in the invaded regions varies from South Africa such that suitable *C. crystallinum* plants are absent for long periods of time, it could negatively impact weevil population persistence,

especially since *C. crystallinum* does not typically occur in sympatry with *C. guerichianum,* or any other *Cryophytum* in any invaded regions. However, *L. carinerostris* populations may be able to cope with such occurrences of host absence, as Louw (1993) reported that these weevils typically overwinter under the dead host plant matter.

3.4.5 Ecological and Evolutionary Implications

The external acceptance but different internal performance patterns of *L. carinerostris* has some important implications. These implications are consistent with theoretical predictions and empirical observations in other oligophagous insects. Studies on coevolutionary dynamics suggest that asymmetric performance patterns reflect differing degrees of historical association and physiological compatibility between phytophagous insects and their host plant species (Thompson 2019).

From a population dynamics standpoint, three of the four *Cryophytum* host plant species represent different types of habitats that receive oviposition effort but contribute relatively less to population recruitment and sustainability compared to *C. crystallinum*. *Cryophytum barklyi* appears to receive high oviposition efforts and initial larval establishment but contribute minimally to adult production, while *C. pellitum* demonstrates both low initial larval establishment and significantly reduced weevil developmental success, and *C. guerichianum* appears to function as a secondary host with reduced developmental success (but significantly higher than *C. pellitum* and *C. barklyi*). This could limit the effectiveness of these three plants as population reservoirs and may impact the spatial distribution of *L. carinerostris* populations where *C. crystallinum* occurs with one or more of the congeners in sympatry.

The significant performance differences also suggest that coevolutionary pressures may vary across the four host plant species. *L. carinerostris*'s preferential performance observed on *C.*

crystallinum may indicate a longer *L. carinerostris* - *C. crystallinum* coevolutionary history, resulting in better physiological compatibility.

3.4.6 Implications for Biological Control

The findings of this study support continued investigation of *L. carinerostris* as a potential biocontrol agent for *C. crystallinum*. The next essential steps include host specificity testing and impact assessment. *Lixus carinerostris* demonstrated high performance on the target IAPS, indicating that it could establish self-sustaining populations capable of providing effective long-term control of *C. crystallinum*. The fact that the target IAPS is evidently the primary host plant species is particularly encouraging for biocontrol prospects.

The fact that *C. crystallinum* occurs as the only *Cryophytum* species in regions like California and Mexico creates an ideal scenario. The absence of other *Cryophytum* host plant species eliminates concerns about potential non-target attacks on closely related plant species that may be of importance in these regions. This means that *L. carinerostris* populations will completely depend on *C. crystallinum* to sustain their populations, making plant phenology in invaded ranges a critical consideration for biocontrol success.

Lixus carinerostris's differential performance on the four host plant species has practical implications for the selection and rearing of biocontrol agents. If releasing weevils collected from the field, source population selection is important. *Cryophytum crystallinum*-associated populations may perform best on the target IAPS, especially since the nuclear ISSR marker data (Chapter 2) suggest some level of genetic differentiation between host plant species-associations. For laboratory biocontrol rearing, *C. crystallinum* clearly provides the best habitat for maintaining healthy and productive colonies.

3.5 Conclusion

One particularly important consideration for biocontrol efficacy is the phenology of *C. crystallinum* in the invaded regions relative to its native South African distribution. The presence of multiple *Cryophytum* species in South Africa may assist in sustaining *L. carinerostris* populations in the absence of *C. crystallinum*, or when the plant is still at an unsuitable developmental stage to be utilised as a host. The fact that *C. crystallinum* is the only *Cryophytum* species in California and Mexico eliminates the potential of this buffering effect. Before releasing *L. carinerostris,* studies must be conducted to characterise the seasonal availability of suitable *C. crystallinum* stems in the invaded regions to identify potential phenological mismatches that could limit population persistence over time. In the case that critical mismatches exist, it may warrant the need for strategic release timing and even supplemental releases to be incorporated into the biocontrol implementation planning.

This study has also provided baseline methodology for weevil establishment assessment post-release. Weevil establishment monitoring should be designed around the metrics that have shown to be informative in this study. While external stem damage may be a good indicator for weevil presence, it might not actually reveal how well-established and persistent *L. carinerostris* populations would be in the invaded regions, post-release. Instead, monitoring should emphasise internal developmental success indicators. These programs should track the ratio of pupae and adults to larvae across the sampled stems. This would provide early warnings/signs of developmental failures, potentially indicating unsuitable environmental conditions. The findings of this study further support the continued consideration of *L. carinerostris* as a potential biocontrol agent for *C. crystallinum*. Although this study provides valuable information about weevil performance in the field, future research in controlled laboratory studies across multiple weevil generations would be beneficial.

Chapter 4 — General Discussion

This thesis evaluated *L. carinerostris* as a potential biocontrol agent for *C. crystallinum* by addressing questions about the weevil's genetic diversity and structure, as well as host plant suitability, all of which are fundamental to the success of a biocontrol programme. The integrative approach of population genetics (Chapter 2) and performance assessment (Chapter 3) revealed patterns that are important for understanding both the safety and efficacy of *L. carinerostris* as a potential biocontrol agent.

4.1 Genetic Homogeneity Does Not Predict Performance Outcomes

This study discovered a disconnect between genetic structure and developmental performance across the four *Cryophytum* host plant species. While both the mtDNA COI and nuclear ISSR markers showed genetic homogeneity across *L. carinerostris,* with some differentiation in *C. pellitum*-associated weevils, the performance assessment revealed significant variation in *L. carinerostris's* developmental success. This mismatch demonstrates how host plant species suitability in this system may not be determined by the genetic adaptation of *L. carinerostris* populations to specific host plant species, but rather by intrinsic host plant species characteristics. This suggests that any weevil population collected from any of the four host plant species would exhibit similar performance hierarchy as documented in this study.

The nuclear ISSR analysis revealed genetic differentiation in *L. carinerostris* populations associated with *C. pellitum*, yet this genetic differentiation did not translate to better performance on this particular host plant species. Instead, *C. pellitum* demonstrated substantially low weevil developmental success. On the other hand, *L. carinerostris* populations associated with *C. crystallinum* demonstrated no unique genetic signature, yet the weevils demonstrated the highest developmental success on this host plant species. These findings directly contradict expectations under local adaptation scenarios, where genetic

differentiation typically correlates with adaptations to local conditions that should enhance performance (Kawecki and Ebert, 2004; Hufbauer et al.2005), instead, they suggest that *C. crystallinum* is the primary host plant species for *L. carinerostris*.

The weevil performance hierarchy observed in this study follows a clear phylogenetic pattern. *Cryophytum crystallinum* and *C. guerichianum* are the most closely related *Cryophytum* species, and these two species supported the highest weevil development success. In contrast, *C. pellitum* and *C. barklyi*, which represent more distantly related lineages within the genus, both demonstrated significantly low developmental success. This observation is well documented in oligophagous insects, where performance decreases with increasing phylogenetic distance from the primary host (centrifugal phylogenetic principle) (Briese 2003).

4.2 Implications for Biocontrol Safety and Efficacy

The results from both Chapters 2 and 3 have significant implications for evaluating *L. carinerostris* as a biocontrol agent. These implications can be separated into three main categories: Taxonomic clarity, Risk assessment, and Biocontrol effectiveness, all of which are discussed below.

4.2.1 Taxonomic clarity

The confirmation that *L. carinerostris* is a single species rather than a cryptic species complex eliminates the possibility of inadvertently releasing multiple species with different efficacies and host ranges. This risk has become a reality in other biocontrol programs, where cryptic species are being inadvertently released and exhibiting different host range patterns than expected (Smith et al. 2018). However, while the mtDNA COI shows complete genetic homogeneity, the genetic differentiation revealed by the nuclear ISSR analysis in *C. pellitum*-associated populations warrants the need for host range and efficacy testing of each host plant

species-associated population separately in a laboratory setting. Alternatively, laboratory experiments could focus on *C. crystallinum*-associated populations.

4.2.2 Risk assessment

The risk of non-target attacks is low in regions such as California and Mexico, where *C. crystallinum* is the only *Cryophytum* species. However, *L. carinerostris*'s oligophagous nature to the *Cryophytum* genus indicates that host specificity testing must extend beyond the *Cryophytum* genus to evaluate potential risk to other species of Aizoaceae. The performance assessment findings documented in this thesis provide a guide for predicting risk. Specifically, closely related genera with similar stem structure to *C. crystallinum* may need careful evaluation, while plants with substantially different structural characteristics may present lower risk even if they are phylogenetically closely related to *C. crystallinum*. This guide can help prioritise non-target species selection based on both phylogenetic relatedness and functional similarities in stem architecture. In host specificity testing of the four congeners in this study, it is expected that the greatest *L. carinerostris* performance would be observed from *C. crystallinum*, then *C. guerichianum*, followed by *C. pellitum*, and then finally *C. barklyi*, following the centrifugal phylogenetic pattern (Briese 2003)

4.2.3 Implications for effective control

The finding that *C. crystallinum* is *L. carinerostris*'s primary host plant species, supporting significantly higher weevil developmental success than its congeners, indicates positive prospects for biocontrol. This finding suggests that, if released, *L. carinerostris* populations will build up on the target IAPS (*C. crystallinum*) without being diluted across less suitable host plant species, especially in places like California and Mexico. However, the absence of alternative hosts in the invaded area presents both an advantage and a potential constraint. While the weevil populations cannot be diluted across multiple host species, they also lack the

phenological buffering provided by their secondary hosts in South Africa, where *C. barklyi* and *C. guerichianum* maintain year-round availability. If the phenology of *C. crystallinum* in invaded areas results in extended periods when suitable host plants are not available for oviposition and larval development, weevil populations may struggle to persist between growing seasons, potentially limiting long-term establishment. The observed adult weevil feeding and oviposition damage, combined with the high developmental success, suggests the potential for substantial population-level impact on *C. crystallinum* in invaded areas.

4.3 Source Population Selection

Beyond safety and efficacy considerations, this thesis also informs practical decision-making about population selection for mass rearing and release. The lack of isolation by distance and the absence of geographic population structure suggest that source populations can be collected from any locality within South Africa without major concern for local adaptation effects. However, the subtle genetic differentiation observed, especially in *C. pellitum*-associated populations, which may indicate early stages of divergence, warrants caution when considering these populations for biocontrol use. On the other hand, the genetic admixture among populations associated with *C. crystallinum*, *C. guerichianum*, and *C. barklyi* suggests that any population collected from any of these host plant species will likely perform well on the target IAPS. However, it is recommended that source population selection prioritise *L. carinerostris* populations collected from *C. crystallinum*, with *C. guerichianum*-associated populations serving as an alternative if needed.

Additionally, establishing colonies from multiple geographic localities across all three host plant species associations may provide insurance against unexpected local adaptation effects and maximise adaptive potential in the new environment by maintaining high genetic diversity. A meta-analysis by Hufbauer et al. (2015) showed that genetic diversity positively correlated with biocontrol agent establishment success across multiple taxa. This relationship likely

reflects the detrimental impact of founder effects, which reduce genetic diversity in introduced populations and can compromise the success of establishment (Dlugosch and Parker 2008b, Estoup et al. 2016). Other studies have also demonstrated how high genetic diversity in a biocontrol agent allows for the agent to cope better with environmental stresses and better adapt to new habitats, both of which are crucial for their survival and effectiveness after release (Chattington et al. 2025).

From a mass rearing standpoint, the performance analysis findings clearly show that *L. carinerostris* performs best on *C. crystallinum*; therefore, it would be best to use this host plant species for laboratory *L. carinerostris* rearing. This approach parallels successful mass rearing strategies in other biocontrol programs, such as the use of specific cactus species for the production of cochineal insects (*Dactylopius* spp.), where the selection of optimal host plants has been demonstrated to be critical for maintaining colony health and production efficiency (Paterson et al. 2021, Humphries et al. 2022). An attempt to use any of the other *Cryophytum* species would reduce the program's efficiency and potentially compromise colony fitness and would produce lower numbers for mass releases. The relatively high success rate on *C. crystallinum* should facilitate efficient mass rearing for field releases in invaded regions.

4.4 Prospects for Future Research

4.4.1 Host specificity testing framework and priority test species

Future studies should expand host specificity testing beyond the *Cryophytum* genus to other members of the Aizoaceae family that may occur in the invaded areas. The selection of non-target test plant species should follow the centrifugal phylogenetic method (Briese 2003), prioritising phylogenetically closely related species while taking into account ecological and biogeographical overlap with *C. crystallinum*. The different invaded regions present unique non-target risk profiles that may require tailored test plant lists.

For California, the non-target test plant list should prioritise *Sesuvium verrucosum* Raf. (Aizoacea), which is the only native in the family along this region's coastline (Vivrette and Muller 1977). This plant species shares the same halophytic coastal habitat as *C. crystallinum*, creating significant potential for spatial overlap with released *L. carinerostris* populations. Although *S. verrucosum* belongs to a different subfamily (Sesuvioidae) under Aizoaceae, thus distantly related to the *Cryophytum* genus (subfamily Mesembryanthemoidae) (Gerbaulet 2017), its ecological overlap with *C. crystallinum* warrants thorough testing.

Additionally, *Tetragonia tetragonoides* (Pall.) Kuntze (Aizoaceae), which has naturalised in the coastal regions of California (CAL IPC 2024), should be included in the host specificity programme. Although this plant originates from Australia and New Zealand, it now occupies similar habitats to *C. crystallinum* in California and is phylogenetically positioned within the Tetragonoideae subfamily of Aizoaceae. The presence of this plant in cultivation and as a food crop in California further warrants its inclusion in the host specificity testing of *L. carinerostris*.

The South African *Carpobrotus edulis* (L.) N.E.Br. (Aizoaceae) and *C. aequilaterus* (Haw.) N.E.Br. have established extensive populations along the coast of California. While both these plants are invasive, testing them is necessary to understand whether they could potentially serve as *L. carinerostris* reservoirs in the absence of *C. crystallinum*, just as *C. guerichianum* and *C. barklyi* do in South Africa.

In Mexico, several members of the Aizoaceae family share the coastal halophytic habitats with *C. crystallinum.* Priority test plant species should include some native *Sesuvium* and *Trianthema* (Aizoaceae) (Sandoval-Ortega et al. 2022), *Sesuvium portulacastrum* (L.) L. is especially important as it provides coastal stabilisation and may be used as a food and medicinal resource (Zhang et al. 2024b); therefore, non-target impacts on this plant species could have cascading ecological consequences. While *Trianthema* species typically occur more inland

compared to *C. crystallinum* (Gerbaulet 2017), there is still some potential for spatial overlap in transitional zones between coastal and inland saline areas, which warrants their inclusion in host specificity testing. *Trianthema portulacastrum* L. is particularly important due to its use in traditional medicine in some areas (Sukalingam et al. 2017), thereby necessitating its inclusion in host specificity testing as well. Similar to the case in California, though these plants are phylogenetically distantly related to *C. crystallinum* within Aizoaceae (Gerbaulet 2017), their ecological overlap with *C. crystallinum* elevates their priority for host specificity testing.

Aizoon hispanicum L. (Aizoacea) is native to the Mediterranean, North Africa, and the Middle East and occupies sandy plains, semi-arid areas, and halophytic habitats (Hartmann 2002), thus ecologically overlapping with invasive *C. crystallinum*. Compared to the *Sesuvium* and *Trianthema* genera, *Aizoon* is more phylogenetically closely related to the *Cryophytum* genus, thus elevating its priority for host specificity testing.

Similar to California, the coastal regions of the Mediterranean are invaded by some members of the *Caprobrotus* genus, particularly *C. edulis* and *C. acinaciformis* (L.) L. Bolus (Campoy et al. 2018). These species' phylogenetic proximity to *Cryophytum* and their wide distribution along the coastal Mediterranean region (Campoy et al. 2018) create potential for spatial overlap with released *L. carinerostris*. This scenario requires careful evaluation, as weevil establishment on the *Carpobrotus* species could represent either an unintended benefit (biocontrol of the two species) or a risk of unsuccessful biocontrol of *C. crystallinum* by diluting the weevil's effect.

Australia also has several native *Carpobrotus* species, with *C. rossii* (Haw.) Schwantes, *C. glaucescens* (Haw.) Schwantes, *C. modestus* (Haw.) Schwantes, and *C. virescens* (Haw.) Schwantes being the most widely distributed and ecologically important (Novoa et al. 2023). *Carpobrotus rossii* occupies the coastal dunes and rocky shores of the southern Australian

coastline, where it plays critical roles in dune stabilisation (Novoa et al. 2023). *Carpobrotus glaucescens* occupies the eastern Australian coastline and provides similar ecosystem services to the coastal dune systems (Gooden et al. 2020). The same potential benefits and risks mentioned about *Capobrotus* species in the Mediterranean also apply here.

The aforementioned species across all the different invaded regions represent the most phylogenetically related non-target plant species at risk. These species should form the core of the host specificity testing non-target plant species list, with particular emphasis on the native species of the different regions *C. crystallinum* has invaded. Additionally, employing the performance assessment methodology developed in this study, they can examine adult feeding and oviposition behaviour along with the weevil's development through all its life stages to identify its true reproductive host versus ecological trap hosts in a laboratory setting.

4.4.2 Mechanisms of differential host suitability

Future research could also investigate the cause behind the observed differential host suitability by examining the stem architecture and chemical composition of all four *Cryophytum* host plant species. Such studies could reveal specific compounds, differential physical stem architecture, or nutritional profiles that would explain why *C. crystallinum* appears to be a superior host for complete development, and informs predictions about *L. carinerostris* performance on other plants with similar phytochemistry to *C. crystallinum* (Berenbaum, 1981).

4.4.3 Impact assessment studies

Studies to quantify how the damage caused by *L. carinerostris* affects *C. crystallinum*'s fitness, seed production, and population dynamics would be valuable for making predictions about biocontrol efficacy by translating the observed damage and developmental success into population-level impacts (Kluge and Zachariades 2006). For instance, field studies on *Larinus*

latus examined how larval density affected seed destruction rates on *Onopordum* thistle flower heads, then used the findings to predict population-level impacts on *Onopordum* thistle seed production (Briese 2000a). Similarly, greenhouse experiments with two stem-mining weevils (*Ceutorhynchus alliariae* Brisout (Coleoptera: Curculionidae) and *C. roberti* Gyllenhal (Coleoptera: Curculionidae)) tested different weevil densities on potted plants (*A. petiolata*) to determine their impact on plant growth, flowering capacity, and seed production, providing valuable data for models predicting biocontrol effectiveness (Gerber et al. 2008). Therefore, pre-release impact studies for *L. carinerostris* should measure the relationship between weevil density and plant survival, and the effects of weevil density on plant growth and flowering. Data collected from such studies may inform biocontrol agent rearing efforts and release densities and ensure that the agent is suitably damaging to warrant release.

4.4.4 Native versus exotic population comparisons

Comparing *L. carinerostris*'s performance on *C. crystallinum* individuals from the invaded areas against ones from the native distribution could reveal whether the invasive populations have diverged from the native ones in the traits that affect weevil development. This is particularly important as evolutionary changes can occur in IAPS populations following introduction, potentially affecting biocontrol efficacy. For example, the common garden experiments that compared the impact of *Heteropederopsis bicallosicollis* Bechyne (Coleoptera: Chrysomelidae) and *Gadirtha inexacta* Walker (Lepidoptera: Nolidae) on native (from China) *Triadica sebifera* (L.) Small (Euphorbiaceae) and the introduced ones (from the USA) revealed that although the insects reached higher densities and performed better on the introduced populations, those plants also had relatively higher tolerance, so the net impact on performance was weaker than on native plants, despite higher herbivore loads (Wang et al. 2011). Similarly, a review of exotic versus native comparisons across 40 IAPS revealed that specialist herbivores typically perform better on exotic plant populations than native ones (Hinz

and Schwarzlaender 2004). Hinz and Schwarzlaender (2004) presented some compelling explanations for these preferential patterns. They stated that in exotic ranges, there is less selection for traits that aid in the defence against specialist herbivores and potentially more selection for those that aid in the defence against generalists. This was corroborated by comparative studies, which showed that specialists generally performed better on exotic populations and that those exotic populations were less well defended than native populations (Hinz and Schwarzlaender 2004). While this may be the case in most instances, high herbivore performance does not always translate to high impact on the IAPS, as seen in the *Triadica sebifea* study (Hinz and Schwarzlaender 2004). Therefore, when comparing the performance of *L. carinerostris* on native versus exotic *C. crystallinum*, it is recommended that some impact parameters on the plants be measured to inform predictions about biocontrol efficacy in the invaded range.

4.4.5 Climate compatibility and thermal biology assessment

Matching biocontrol agents and target climatic requirements is crucial for establishment success. Studies on climate matching and *L. carinerostris* thermal biology would assist in identifying the invaded regions that would best suit *L. carinerostris* establishment, thus helping predict how the distributions of both *C. crystallinum* and *L. carinerostris* might be affected under future climates, and guiding source population selection and release site prioritisation (Hopper et al. 1993, Bean et al. 2014a). Climate matching between native and invaded ranges is an important determinant of biocontrol success, as climatic incompatibility can limit agent establishment even when the target IAPS is abundant (Harms et al. 2021).

Thermal physiology studies should aim to determine *L. carinerostris*'s developmental thresholds through degree day modelling. Such studies could reveal thermal characteristics of *L. carinerostris* that would help predict how many generations this weevil could complete annually across different climatic zones in all the different invaded regions. Furthermore, the

critical thermal limits and lethal temperature thresholds should also be measured. These parameters will assist in predicting establishment potential and identifying potential climate mismatches with *C. crystallinum.* For example, the failure of some biocontrol agents to establish in certain regions has been attributed to thermal incompatibility, particularly the case of *E. catarinensis,* which failed to establish in some South African regions against *E. crassipes,* where the mean minimum temperatures fell below its minimum thermal critical limit (Byrne et al. 2004).

Ecological niche modelling could map climatic suitability of both *L. carinerostris* and *C. crystallinum* across invaded ranges, determining priority release sites where the highest biocontrol agent-target IAPS overlap is predicted (Minuti et al. 2022). Such models have been successfully used to predict establishment patterns in numerous biocontrol programs and can account for climate change scenarios to evaluate long-term persistence of both the biocontrol agent and target IAPS populations (Kriticos et al. 2021).

4.5 Conclusion

This thesis has demonstrated that *L. carinerostris* is a single oligophagous species with potentially high dispersal abilities and genetic homogeneity across its native distribution. *Cryophytum crystallinum*, the target IAPS, stood out as the potential biocontrol agent's primary host for complete developmental success. From a biocontrol standpoint, *L. carinerostris* shows significant promise for the management and control of *C. crystallinum* in invaded areas, especially in places like Mexico and California, where there are few close relatives of the target IAPS. *Lixus carinerostris*'s developmental success on the target IAPS, high potential damage, potentially high dispersal abilities, and lack of cryptic species concerns collectively support its consideration for classical biocontrol (Van Driesche and Hoddle 2009). However, further rigorous host specificity testing and impact assessment studies must still be conducted.

On a broader scale, this research contributes to the current understanding of how phylogenetic relatedness among host plant species influences biocontrol performance. The performance hierarchy observed in this study, where weevil developmental success decreases with phylogenetic distance from the primary host plant species, demonstrates the practical value of the centrifugal phylogenetic method in both biocontrol agent evaluation and non-target risk assessment (Briese 2003). These findings also suggest that host plant acceptance during oviposition does not reliably predict offspring performance.

While this thesis provides valuable baseline data, several important questions remain, particularly regarding host specificity beyond the *Cryophytum* genus and *L. carinerostris*'s potential impacts on *C. crystallinum* population dynamics. As the global biological invasions accelerate, the need for safe and effective biocontrol programs also grows. This thesis provides the fundamental genetic and ecological knowledge required for evidence-based decision-making regarding the use of *L. carinerostris* as a biocontrol agent for *C. crystallinum*. The methodology developed in this thesis, which combines population genetics and performance assessment, provides a template for future biocontrol agent evaluation studies, ensuring both safety and efficacy in biocontrol programs. By revealing that *L. carinerostris* is a single genetically uniform species with a strong developmental preference for *C. crystallinum*, this research has addressed important uncertainties that would otherwise have complicated biocontrol risk assessment and supports continued investigation of *L. carinerostris* as a potential biocontrol agent for the invasive *C. crystallinum.*

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Supplementary information

Table S1. Showing all species used in the phylogenetic analysis, along with their GenBank accession numbers and Geographic locality of collection

Species name	**GenBank Accession Number**	**Geographic Location**
Lixus angustatus	KU918695.1	Armenia: Goravan
	KM443634.1	Germany: Rhineland Palatinate, Saar-Nahe-Bergland, Bad Kreuznach, Langenlonsheim-Dorsheim, Weinberg Goldloch
Lixus angustus	MK891388.1	Slovakia: Nove Zamky
Lixus brevirostris	MK891562.1	Spain: Canary Islands, Tenerife
	KC783729.1	Spain
	KC783763.1	Spain

	MK891615.1	Spain: Canary Islands, La Palma
	KC783790.1	Spain
Lixus cinerascens	MW726810.1	Ukraine: Zaporozje, Melitopol
	MK891876.1_	Slovakia: Komarno
Lixus filiformis	KM440625.1	Slovenia: Severnoprimorska, Dinarski kras, Nova Gorica, Grgar, Ravnica
Lixus kraatzi	MW726815.1	Ukraine: Zaporozje
Lixus punctiventris	MW596722.1_	Spain
	MW596720.1	Italy
Lixus purpurariensis	MK347627.1	Spain: Canary Isl., Lanzarote
	MK347611.1	Spain: Canary Isl., Lanzarote
	MW520728.1	Spain: Fuerteventura, 2 km S Betancuria, strambed
Larinus sturnus	KU918625.1	Germany: Rheinland-Pfalz, Pfalz, Gruenstadt, Gemeindeberg
	KM446759.1	Germany: Rhineland Palatinate, Noerdliche Oberrheinebene, Gruenstadt, Gruenstadt, Gemeindeberg
	MK891941.1	Slovakia: Nove Zamky
	KJ964700.1	Finland: Savonia australis, Ristiina, Aartamaniemi
Larinus pollinis	MK891583.1	Poland
	MK891586.1	Czech Republic: Moravia mer.
	KM442715.1	Germany: Bavaria, Chiemgauer Alpen, Traunstein, Schleching-Ettenhausen, Achberg
Larinus obtusus	KM447748.1	Slovenia: Gorenjska, Poljane Sora Valley, Gorenja Vas?Poljane, Hotavlje, Surrounding
	MK892020.1	Austria: Kaernten
	KM449669.1	Slovenia: Gorenjska, Poljane Sora Valley, Gorenja Vas?Poljane, Hotavlje, Surrounding
	KM441957.1	Slovenia: Gorenjska, Poljane Sora Valley, Gorenja Vas?Poljane, Hotavlje, Surrounding
Larinus ursus	KC783875.1	Morocco
Pachycerus segnis	MW726753.1	Russia: Saratovskaya Oblast, Mokrous

	MK891933.1	Czech Republic: Moravia
Pachycerus madidus	MW726799.1	Russia: Buryatiya Republic, Ivolginsk
Cyphocleonus garajonay	MK892370.1	Spain: Canary Islands, La Gomera
	KC783777.1	Spain
Cyphocleonus sventeniusi	KC783760.1	Spain
Cyphocleonus dealbatus	MW726741.1	Armenia: Goravan

Table S2. *Lixus carinerostris* sample IDs along with the plant species name and geographic location they were collected. "✓" indicates if each sample was successfully used in the COI and/or ISSR analyses.

Samples	**COI**	**ISSR**	**Host plant species**	**Site**	**Lat**	**Long**
211	✓		*C. crystallinm*	Langebaan	-33.0931519653186	18.0333082738515 3
294	✓	✓	*C. guerichianum*	Elands Bay	-32.311222	18.343250
802	✓	✓	*C. barklyi*	Steinkopf	-29.212222	17.630556
805	✓	✓	*C. barklyi*	Steinkopf	-29.212223	17.630557
810	✓		*C. guerichianum*	McDougalls Bay	-29.2918089	16.8790139
820	✓		*C. guerichianum*	McDougalls Bay	-29.2918089	16.8790139
824	✓	✓	*C. barklyi*	Holgat	−28.897079	16.729594
825	✓	✓	*C. barklyi*	Holgat	−28.897079	16.729595
831	✓	✓	*C. barklyi*	Port Nolloth	-29.21325737903866	17.2993776215277 9
1A	✓		*C. barklyi*	N7	-30.80179970771393	18.1297172473008 5
1D	✓	✓	*C. barklyi*	N7	-30.80179970771393	18.1297172473008 5
1E	✓		*C. barklyi*	N7	-30.80179970771393	18.1297172473008 5
1F	✓		*C. barklyi*	N7	-30.80179970771393	18.1297172473008 5
1G	✓		*C. barklyi*	N7	-30.80179970771393	18.1297172473008 5
1H	✓	✓	*C. barklyi*	N7	-30.80179970771393	18.1297172473008 5

2A		✓	*C. guerichianum*	McDougalls Bay	-29.2918089	16.8790139
2B	✓		*C. guerichianum*	McDougalls Bay	-29.2918090	16.8790140
2D	✓	✓	*C. guerichianum*	McDougalls Bay	-29.2918091	16.8790141
3C	✓	✓	*C. guerichianum*	Port Nolloth	-29.147222	16.855000
7A	✓	✓	*C. guerichianum*	Langebaan	-33.0931519653186	18.0333082738515 3
7B	✓		*C. guerichianum*	Langebaan	-33.0931519653186	18.0333082738515 3
807i	✓	✓	*C. crystallinm*	Hondeklip Bay	- 30.28734065631627	17.2739575737593 7
807j	✓	✓	*C. crystallinm*	Hondeklip Bay	- 30.28734065631627	17.2739575737593 7
809i	✓	✓	*C. crystallinm*	Hondeklip Bay	- 30.28734065631627	17.2739575737593 7
810i	✓	✓	*C. guerichianum*	McDougalls Bay	-29.2918089	16.8790139
9B	✓		*C. pellitum*	Oudtshoorn	- 33.65378037841825	22.2005282564466 4
B3	✓		*C. guerichianum*	Baviaanskloof	-33.193639	23.930528
B4	✓	✓	*C. guerichianum*	Baviaanskloof	-33.193640	23.930529
B5	✓	✓	*C. guerichianum*	Baviaanskloof	-33.193641	23.930530
VCL2	✓		*C. pellitum*	Vanrhynsdorp	-31.526667	18.865556
CVL1	✓		*C. pellitum*	Vanrhynsdorp	-31.526668	18.865557
DC1A	✓	✓	*C. crystallinm*	Dwarskesbos	- 32.70454279360448	18.1987183832802 4
ECL1	✓	✓	*C. crystallinm*	Elands Bay	-32.311222	18.343250
EGL1	✓	✓	*C. guerichianum*	Elands Bay	-32.311223	18.343251
g1	✓		*C. guerichianum*	Robertson	-33.683056	19.594444
G2	✓		*C. guerichianum*	Robertson	-33.683057	19.594445
g3	✓	✓	*C. guerichianum*	Robertson	-33.683058	19.594446
g4	✓	✓	*C. guerichianum*	Robertson	-33.683059	19.594447
LC1	✓	✓	*C. crystallinm*	Leipoldtville	- 32.21966591384628	18.4800390080142 8
LC11	✓	✓	*C. crystallinm*	Leipoldtville	- 32.21966591384628	18.4800390080142 8

LC14	✓		*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
LC17	✓	✓	*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
LC2	✓	✓	*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
LC3	✓	✓	*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
LC4	✓		*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
LC8	✓		*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
LC9	✓	✓	*C. crystallinm*	Leipoldtville	-32.21966591384628	18.480039008014 8
OC2	✓		*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC3	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC4	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC5	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC6	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC7	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC8	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
OC9	✓	✓	*C. pellitum*	Oudtshoorn	-33.65378037841825	22.200528256446 4
PC1	✓	✓	*C. crystallinm*	Port Nolloth	-29.147222	16.855000
PC3	✓		*C. crystallinm*	Port Nolloth	-29.147223	16.855001
VCL1	✓	✓	*C. pellitum*	Vanrhynsdorp	-31.526667	18.865556
VCL2	✓		*C. pellitum*	Vanrhynsdorp	-31.526668	18.865557
VGL1	✓		*C. pellitum*	Vanrhynsdorp	-31.526669	18.865558

YCL1	✓		*C. crystallinm*	Yzerfontein	-33.34443942197688	18.172220966823588
YCL2	✓		*C. crystallinm*	Yzerfontein	-33.34443942197688	18.172220966823588
YCL3	✓		*C. crystallinm*	Yzerfontein	-33.34443942197688	18.172220966823588
YGL4		✓	*C. guerichianum*	Yzerfontein	-33.34443942197688	18.172220966823588
YGL5		✓	*C. guerichianum*	Yzerfontein	-33.34443942197688	18.172220966823588
YGL9	✓		*C. guerichianum*	Yzerfontein	-33.34443942197688	18.172220966823588

For any of the data and analysis R-codes used in this thesis, visit https://github.com/mukhawanajackeyjackson-dot/MSc-supplementary