Pasture mealybug (*Heliococcus summervillei*) host range, microbiome, and endosymbiont cophylogeny

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Abstract

The pasture mealybug *Heliococcus summervillei* Brookes (1978) (Hemiptera: Pseudococcidae) was recently confirmed as the primary causative factor in pasture dieback. Short-read sequencing of the bacterial 16S gene (V3-V4) has been used to generate a baseline microbial profile for *H. summervillei*, for which there is no equivalent in the current literature. Bacterial community composition and diversity in *H. summervillei* specimens from different host plants and geographic locations are described for the first time in this work. Neither host plant suitability nor geography appear to affect the bacterial microbiome, which is dominated by the primary endosymbiont, *Candidatus* Tremblaya phenacola.

H. summervillei has two known biotypes: an 'old' biotype associated with historical dieback outbreaks in the 1920s and 1930s, and a 'new' biotype that has been identified at all reported sites in the current outbreak (2015-present) and is spreading rapidly across eastern Australia. The only appreciable difference in bacterial diversity in the *H. summervillei* microbiome is associated with mealybug biotype, which in itself is attributed to sequence variation in the *T. phenacola* genome. *T. phenacola* associated with *H. summervillei* does not appear to match *T. phenacola* from any Phenacoccidae mealybugs published to date, which suggests the emergence of a new strain of the endosymbiont. We propose the designation "*Tremblaya phenacola* HSUM" in line with the naming conventions of published strains. More complete sequencing of both variants of *H. summervillei* and *T. phenacola* is recommended, as is further investigation into microbial functional diversity.

Long-term field trials are necessary to identify factors that may alter the impact of *H. summervillei* in real agricultural conditions, but mass testing of pasture varieties is a high-cost endeavour. Bioassays are proposed to help prioritise dieback-tolerant pasture varieties for long-term field trials. This research demonstrates proof of concept of a rapid laboratory-based bioassay for screening pasture varieties for susceptibility to dieback. This method uses the survival rate of *H. summervillei* on different grasses to infer their suitability as hosts for the mealybug; increased host suitability then suggests increased dieback susceptibility.

The association of a hypervirulent outbreak of pasture dieback with a new mealybug biotype, in conjunction with the emergence of a new strain of the endosymbiont, raises questions about the biology of *H. summervillei* and calls for revision of its biosecurity status. The baseline microbial profile and pasture screening method established in this thesis will yield more immediate results for the agricultural industry, primarily in pasture variety assessment for proactive management (e.g. resowing affected land) against future incursions.

Keywords

16S rRNA, biogeography, *Candidatus* Tremblaya phenacola, cophylogeny, endosymbiont, *Heliococcus summervillei*, host range, microbiome, pasture dieback, pasture mealybug

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Chapter 1: Introduction

This chapter describes the background and context of the research (section 1.1) and provides a literature review (section 1.2) with consideration for the real-world implications of the research problem (section 1.3). Further sections summarise the purpose (section 1.4), significance (section 1.5) and methodology (section 1.6) of the research. An outline of the subsequent thesis is also provided (section 1.7).

1.1 Background and Context

Pasture dieback is a complex condition associated with the pasture mealybug, *Heliococcus summervillei* Brookes (Brookes, 1978). It is characterised by premature and progressive death of pasture grasses, which subsequently reduces the productivity of affected pastures and has wide-ranging implications for the agricultural industry. Published records show that pasture dieback outbreaks associated with *H. summervillei* have occurred in Australia in 1926 (Summerville, 1928) and 1938 (Brookes, 1978); Pakistan in 1975 (Brookes, 1978); India in 1987 (Ghosh & Ghose, 1987); New Caledonia in 1998 (Brinon et al., 2004); Puerto Rico in 2019 (Hauxwell et al., 2022a-b); and Barbados in 2020 (Gibbs, 2020). More recent outbreaks of pasture dieback in Australia, first observed in the 1990s, were not conclusively associated with *H. summervillei* until recently (Hauxwell et al., 2022c-d).

Pasture dieback associated with *H. summervillei* has historical ties with the state of Queensland in eastern Australia, having affected paspalum grasses (*Paspalum dilatatum*) on the Sunshine Coast in 1926 (Summerville, 1928) and in Atherton in 1938 (Brookes, 1978). There is growing concern about a rapidly accelerating outbreak of the pasture mealybug and resulting dieback across Queensland and northern New South Wales. This outbreak began in the 1990s and has impacted approximately 400,000 hectares as of 2020 (Meat & Livestock Australia (MLA), 2021a). Early occurrences of the current dieback outbreak in buffel grasses (*Cenchrus*) near Moura in 1993 were also reported to be associated with a mealybug, but the species was not identified (Graham & Conway, 1998). The Queensland grazing industry has lost an estimated AU$2 billion in productivity based on reported dieback cases between 2015 and 2021 (AgForce, 2021), though local graziers believe losses are in the order of AU$3-4 billion (Halter, 2023). Further losses are expected as the outbreak area moves south into New South Wales.

H. summervillei, commonly known as the pasture mealybug, was recently confirmed as the primary causative factor in pasture dieback (Hauxwell et al., 2022c-d). *H. summervillei* has two known biotypes (i.e. variant phenotypes with similar or identical genotypes) found at pasture dieback sites across Australia. The first biotype was discovered at the site of the first recorded outbreak of pasture dieback in Australia in 1926 (Summerville, 1928), hereafter referred to as the 'old' biotype. The second biotype is associated with the current outbreak and has been identified at all reported pasture dieback sites (Schutze et al., 2019), hereafter referred to as the 'new' biotype. The current pasture dieback outbreak is more widespread and severe than past outbreaks, affecting at least 26 known grass varieties (Hauxwell et al., 2022e) and spreading almost four times as fast (MLA, 2021b). The association of a new pasture dieback outbreak with a new mealybug biotype raises questions about the biology and biosecurity status of *H. summervillei*.

In Australia, biosecurity risk analyses consider all mealybug species as a collective due to their similar biological characteristics and thus presumably similar manners of distribution (Department of Agriculture, Fisheries and Forestry (DAFF), 2020). The former Department of Agriculture and Water Resources (DAWR) (2019, p39-44) estimates that mealybugs have a moderate overall likelihood of establishment and spread, and that the potential economic and environmental consequences of their establishment are low in magnitude and scale. However, this generalised risk estimate is challenged by the rapid spread of the new *H. summervillei* biotype across eastern Australia and the high severity of the pasture dieback associated with it (Hauxwell et al., 2022d-e). These characteristics suggest that *H. summervillei* poses a more significant threat to Australian biosecurity, beyond the published risk estimates for mealybugs as a group. Furthermore, recent outbreaks of pasture dieback in Puerto Rico (Hauxwell et al., 2022b) and Barbados (Gibbs, 2020) have been confirmed to be attributed to the new *H. summervillei* biotype (Hauxwell et al., 2022b), which suggests that *H. summervillei* should in fact be considered an international biosecurity threat.

H. summervillei is a soft scale insect that uses piercing-sucking mouthparts to penetrate plant tissue and feed on phloem sap directly from the host plant vascular system. Low numbers of *H. summervillei* can induce significant and rapid onset of pasture dieback symptoms (Hauxwell et al., 2022c). Mealybugs have limited capacity to deplete plant sap due to their small size (<5mm), and these symptoms are therefore unlikely to be caused by mealybug feeding alone. Feeding by the solenopsis mealybug *(Phenacoccus solenopsis)* is understood to disrupt plant defence signalling pathways mediated by jasmonic acid (JA) and salicylic acid (SA) (Zhang et al., 2011, 2015), and recent work suggests that salivary bacteria

play a key role in this response (Zhao et al., 2023). *H. summervillei* feeding is suspected to disrupt plant immune responses in a similar manner (Hauxwell et al., 2022d-e).

JA and SA signalling pathways are mutually antagonistic, with the induction of one leading to suppression of the other (Hou & Tsuda, 2022). JA pathways are responsible for inducing defence mechanisms against necrotrophic pathogens, while SA pathways induce defence against biotrophs/hemi-biotrophs (Li et al., 2019). Sufficient signal interference of either phytohormone would leave the host plant susceptible to the associated pathogen type. Transcriptomics on American buffel grass (*C. ciliaris* var. USA*)* suggests that *H. summervillei* feeding suppresses the JA pathway and activates the SA pathway, thus rendering the host plant susceptible to secondary infection (Hauxwell et al., 2022; Munro & Hauxwell, 2023). Secondary infection by necrotrophic fungi, particularly *Fusarium* spp., corresponds with the symptoms seen in pasture dieback associated with *H. summervillei*, and multiple species and strains of *Fusarium* are typically isolated from affected pastures (Hauxwell et al., 2022d).

Historical management strategies rely on preventative measures (i.e. farm biosecurity) to minimise the spread of pasture dieback (Buck et al., 2022; MLA, 2021c), but these practices have limited efficacy on the affected areas themselves. Biological and chemical controls are shown to be effective for targeting foliar-feeding mealybugs (Hauxwell et al., 2022a). However, adult females disperse in late summer and do not feed on leaf, and overwintering populations also disperse from foliage – they persist underground, around the roots, and in dense thatch (Hauxwell et al., 2022b). These behaviours decrease the effectiveness of insecticide application and increase the likelihood of reinfestation. For longer-term management, a promising strategy is to regenerate affected areas by re-sowing with grass varieties that are resistant or tolerant to pasture dieback (Buck et al., 2022; MLA, 2021c). Recent work shows that the new *H. summervillei* biotype has a very wide host range in the field (Hauxwell et al., 2022b; MLA, 2023). Differences in host suitability can be detected in short-term laboratory and screenhouse assays (Hauxwell et al., 2022d), which may be used as an initial screen for host range suitability prior to long-term and high-cost field testing.

Insects, and particularly hemipterans, are typically associated with complex microbial communities that significantly influence various aspects of their biology (Gurung, 2019). For example, gut microbiota can provide essential amino acids the host insect cannot synthesise themselves (Schmidt & Engel, 2021), and bacteriocyte-dwelling microbes can produce toxins as a chemical defence against predators (Van Arnam et al., 2018). Insect-associated microbes appear to play a larger role in insect-plant interactions than previously thought (Coolen et al., 2022). Bacterial symbionts of other mealybugs are associated with the

suppression of host plant defence responses (Zhao et al., 2023), increasing the potential susceptibility of the host plant to secondary infection. Further investigation is required to understand the composition and potential role of the microbiome (i.e. all the microorganisms that live on and within the insect host) in the interactions of *H. summervillei* with host grasses in pasture dieback.

Within the *H. summervillei* microbiome, the primary endosymbiont *Candidatus* Tremblaya phenacola (Proteobacteria: Betaproteobacteria) is of particular interest. It is responsible for synthesising critical amino acids and vitamins to compensate for the nutritional deficiencies of the sap-based mealybug diet and is therefore essential for their survival (McCutcheon & von Dohlen, 2011). Studying diversity in the bacterial communities of *H. summervillei* and in *T. phenacola* at a sequence level may provide insight into the role of bacteria in the severity of effects of the pasture mealybug on susceptible grass varieties.

For species with low genetic diversity like *H. summervillei*, standard population genetic analyses with microsatellites or barcodes have limited resolving power (Ma et al., 2020). A cophylogenetic approach that also investigates the primary endosymbiont *T. phenacola* may therefore be useful for resolving *H. summervillei* systematics. This has been demonstrated in a multi-genome approach to tracking the Russian wheat aphid (*Diurapis noxia*) using its primary endosymbiont (*Buchnera aphidicola*) (Zhang et al., 2014). Cophylogenetic analyses could exploit the long evolutionary history between mealybugs and *Ca.* Tremblaya that is shaped more by their genetic interdependence than by biogeography or plant host range (Hardy et al., 2008), and could thus facilitate the identification and differentiation of endosymbiont biotypes.

This thesis will investigate the following: rapid bioassays for screening relative susceptibility and suitability of pasture grasses as hosts for *H. summervillei*; bacterial communities associated with *H. summervillei* on different grass hosts and across the geographic range of the current pasture dieback outbreak in Australia; diversity in the *H. summervillei* primary endosymbiont *T. phenacola* across a wide geographic range; and potential cophylogeny between *H. summervillei* and *T. phenacola*.

1.2 Literature Review

This chapter presents literature reviews on the following topics: pasture dieback and its current status in Australia (section 1.2.1); the biology and host range of the pasture mealybug (section 1.2.2); and the bacterial communities associated with mealybugs, including their primary endosymbionts (section 1.2.3). Sections 1.3-1.5 then highlight implications of the literature and develop the conceptual framework for the study.

1.2.1 Pasture dieback

The term ‘dieback’ describes numerous conditions characterised by progressive plant death and encompasses a range of causal agents across a range of plant types. ‘Pasture dieback’ is a particular condition currently affecting grass pastures across eastern Australia, primarily in Queensland and New South Wales. For many Australian graziers, pasture dieback is regarded as a mysterious and devastating condition (Courtney, 2020; Nason, 2018; Nugent, 2019). However, recent research has established that the causal agent of the current pasture dieback outbreak is the pasture mealybug, *Heliococcus summervillei* Brookes (Hauxwell et al., 2022a-e).

Mealybugs are known to affect several important grasses in Australia. In Queensland, the sugarcane mealybug *Saccharicoccus sacchari* and the rhodesgrass mealybug *Antonina graminis* are frequent pests of their eponymous hosts (Hauxwell, 2018; Samson, Sallam & Chandler, 2019). The tuttle mealybug *Brevennia rehi* has been identified on native grasses in Far North Queensland (Grimshaw & Donaldson, 2007), but is usually found on rice in the Northern Territory (Ben-Dov, 2008; Williams, 1985). The ryegrass mealybug *Phenacoccus graminicola* is mostly reported on barley and other cereal crops in South Australia (Department of Primary Industries and Regions, 2023). Neighbouring countries cite two mealybug species as pests of various pasture grasses, both referred to as the pasture mealybug in their corresponding reports: *Balanococcus poae* in New Zealand (Charles et al., 2009) and *Heliococcus summervillei* in New Caledonia (Brinon et al., 2004).

Distribution

In Australia, pasture dieback caused by the mealybug later classified as *H. summervillei* was first reported around the Cooroy district of South East Queensland in 1926 (Summerville, 1928; Brookes, 1978), but subsequent observations were sporadic. Pasture dieback and *H.*

summervillei were not observed again until 1938 in the Atherton Tablelands of Far North Queensland (Brookes, 1978). *H. summervillei* was next observed the 1990s, when dieback associated with an unidentified mealybug was reported in the Dawson and Callide Valleys of Central Queensland (Graham & Conway, 1998). From 2015, a rapid and wide geographic spread of dieback and the unidentified mealybug was reported across Central Queensland and outwards into South East and Far North Queensland (MLA, 2021b-c). By 2020, this outbreak had spread into northern New South Wales (MLA, 2021b-c).

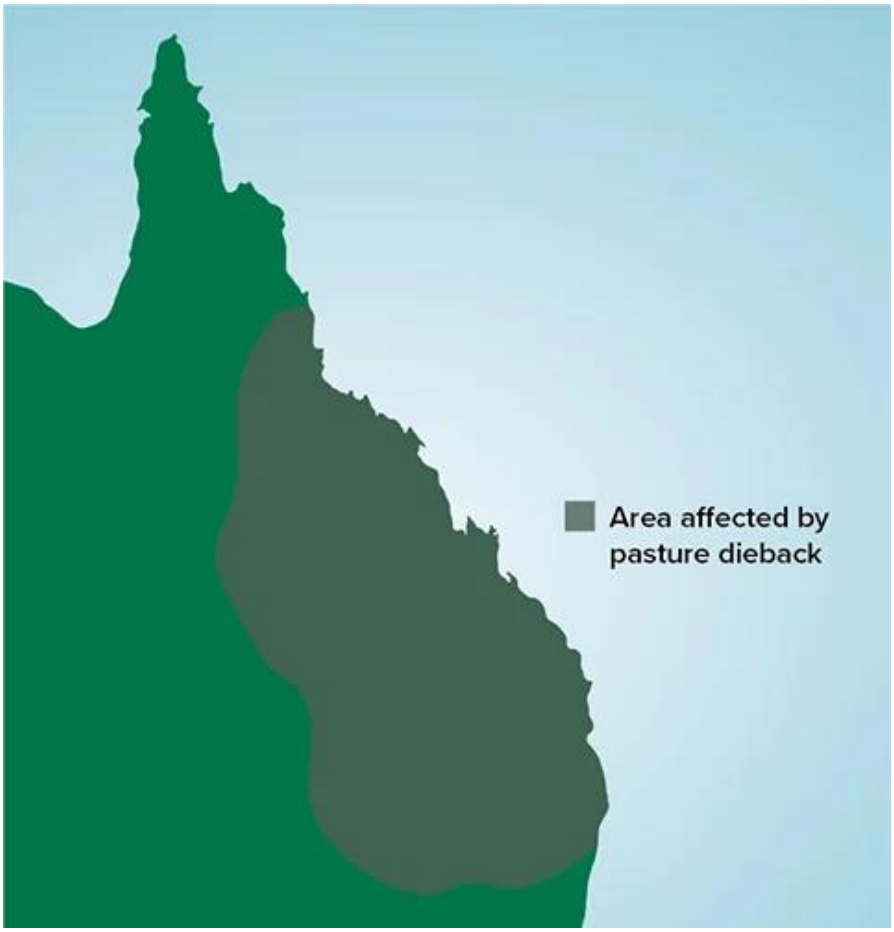

Figure 1. Approximate geographic range of the current pasture dieback outbreak (2015 to present) across eastern Australia based on reported cases (Photo: Meat & Livestock Australia).

A conservative estimate of 400,000 hectares of land across eastern Australia is impacted by pasture dieback (MLA, 2021a) (Figure 1), at least 200,000 of which is productive grazing land (Buck, 2019). Note that these figures likely underestimate the actual area affected, as not all cases are reported nor identified. There are well over 16,000 agricultural businesses in Queensland (Department of Agriculture and Fisheries (DAF), 2018) and these conclusions are based on reports of pasture dieback from approximately 120 landholders. Anecdotal

evidence suggests that some landowners are reluctant to report cases of pasture dieback due to concerns about biosecurity and/or land devaluation (Buck, 2017).

H. summervillei is also an internationally recognised pest, with published records of outbreaks on sugarcane in Pakistan in 1975 (Brookes, 1978); on rice in India in 1987 (Ghosh & Ghose, 1987); on sedge and grasses in New Caledonia in 1998 (Brinon et al., 2004); and on pasture grasses in Barbados in 2020 (Gibbs, 2020). Outbreaks have also been reported on unconfirmed host plant/s in Puerto Rico in 2019 (Hauxwell et al., 2022b).

Causation

Research into causal agents of 'pasture dieback' since the 1990s has focused on pests and pathogens found at dieback-affected sites. The literature reports a range of suspected agents: mealybugs, ground pearls, nematodes, fungi, and viruses (Applied Horticultural Research (AHR), 2019; Bransgrove, 2017; Buck et al., 2022; Hauxwell et al., 2022a-e; Mercer et al., 2008; Rogers, 2017; Schutze et al., 2019; Summerville, 1928; Thomson et al., 2020). Recent work, however, has confirmed that *H. summervillei* Brookes is the primary causal agent of the current pasture dieback outbreak in Australia (Hauxwell et al., 2022c-e).

Symptoms

An early symptom of pasture dieback in most grasses is leaf yellowing, followed by purple-red streaking or blocking along the leaf margins (Hauxwell et al., 2022a-c) (Figure 2a). These changes typically affect the oldest leaves first, beginning at the leaf tip and moving down the leaf blade (Baker et al., 2021; Hauxwell, 2018; MLA, 2021b-c). Dieback-associated discolouration can be hard to differentiate from other conditions like drought, grazing stress, or nutrient deficiency and related opportunistic diseases (MLA, 2021c), which can make diagnosis difficult. As the condition progresses, plant growth slows or stops, and premature senescence may occur (Baker et al., 2021). The grass may eventually die, especially with warm, wet weather in late summer. Such conditions facilitate secondary infections (e.g. by *Fusarium* spp.) wherein the grass becomes fragile and characteristically grey, with loss of root structure (Hauxwell et al., 2022c). The death of pasture grasses allows the spread of broadleaf weeds and unpalatable grasses, thereby reducing overall pasture quality and productivity (Baker et al., 2021; MLA, 2021c).

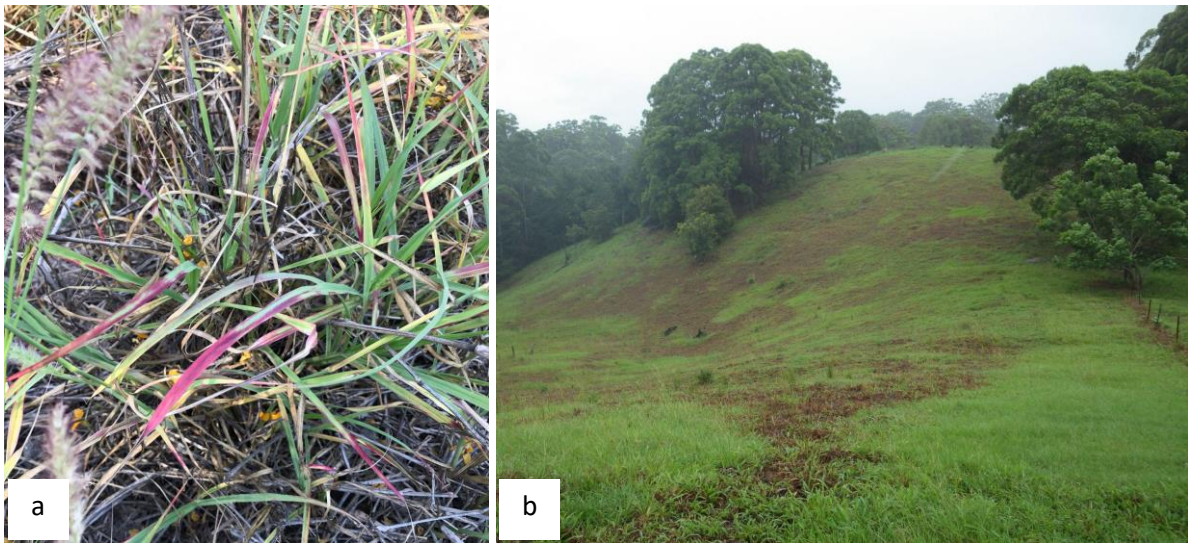

Figure 2. (a) Yellowing and purple-red streaking in dieback-affected American buffel grass (*Cenchrus ciliaris* var. USA). (b) Large patches of dieback on an affected hillside. (Photos: C. Hauxwell, QUT)

The early symptoms and presence of mealybugs were frequently overlooked in initial reports of pasture dieback. Graziers would often only observe what appeared to be the grey, ashy 'death' of grass and how affected areas increased rapidly in size, from square metre patches to paddocks of several hundred hectares within weeks (Figure 2b). The rapid spread of dieback is especially likely following rainfall in the growing season, when secondary infection of mealybug-infested grasses leads to the rapid death of the grass, and this often spreads in the direction of prevailing winds (Baker et al., 2021; Hauxwell et al., 2022a, 2022c; Hauxwell, 2018; MLA, 2021b-c) - i.e. conditions that facilitate mealybug dispersal.

Management

Management strategies to date rely on farm biosecurity, initially to prevent the spread of 'unknown' causal agents and more recently to prevent spread of *H. summervillei* (Buck et al., 2022; Hauxwell 2018, MLA, 2021a-c). Key biosecurity measures include monitoring for symptoms; limiting livestock access to affected areas; washing down vehicles/equipment/personnel/etc. that enter the property, or when moving between affected and unaffected areas; and planting windbreaks, especially if recurrent outbreaks occur in upwind areas (Baker et al., 2021; MLA, 2021a-c). Where mealybug populations are established, management options include slashing and grazing to reduce dense layers of grass where mealybugs thrive (Hauxwell et al., 2022a-d; Hauxwell, 2018; MLA 2021a-c). For longer-term management, re-sowing affected pastures with less susceptible pasture varieties and/or forage crops is an attractive option, as it can reduce the severity of dieback while still providing livestock with high quality feed (Buck, 2022; Hauxwell et al., 2022a-e; Hauxwell,

2018; MLA, 2021a-c). Recent work has shown significant differences in susceptibility between grass varieties, with corresponding differences in the development and survival of mealybugs (Hauxwell et al., 2022e). This suggests that replanting with varieties more tolerant or less suited as hosts of the mealybug is a promising strategy for proactive recovery.

Dicotyledonous pasture species are not suitable hosts for *H. summervillei* and are not affected by pasture dieback (Buck et al., 2022; Department of Primary Industries (DPI), c2021; MLA, 2021c; Whitton et al., 2022), and broadleaf forage crops such as brassicas, legumes and herbs are thus recommended options for re-sowing in dieback-affected pastures (DPI, c2021; Hauxwell et al., 2022d-e; MLA, 2021c). Grasses infested with *H. summervillei* are reportedly unpalatable to livestock (AHR, 2019; Baker & Boschma, 2020; Buchanan, 2018) and forage crops can compensate by providing high quality livestock feed (DPI, c2021). In the event of pasture death, forage crops can also help to prevent erosion by maintaining ground cover and preserve agricultural productivity by competing with broadleaf weeds, which are also resistant to pasture dieback (DPI, c2021; Hauxwell et al., 2022e).

The brief duration of the New Caledonia pasture dieback outbreak associated with *H. summervillei* was attributed to control by natural enemies (Brinon et al., 2004). Natural enemies of *H. summervillei* include the predators *Cryptolaemus montrouzieri* and *Mallada signatus. C. montrouzieri* is a lady beetle that exhibits predatory behaviour against various mealybug species and is widely used as a biocontrol agent (Gunawardana & Hemachandra, 2020) (Figure 3a). *M. signatus* is a green lacewing species that is commonly used in biological pest control as a generalist predator (Manners & Duff, 2015) (Figure 3b). Parasitoids of *H. summervillei* include the chalcid wasp *Callipteroma sexguttata* (Baker et al., 2020; Hauxwell, 2018) (Figure 3c) and two new parasitoid wasp species of the genera *Parectromoidella* and *Yamatsuiola* (Hymenoptera: Encyrtidae) (Hauxwell et al., 2022d) (Figure 3d-f). However, all observed parasitoids of *H. summervillei* in Australia have low parasitism rates and do not provide meaningful levels of biological control.

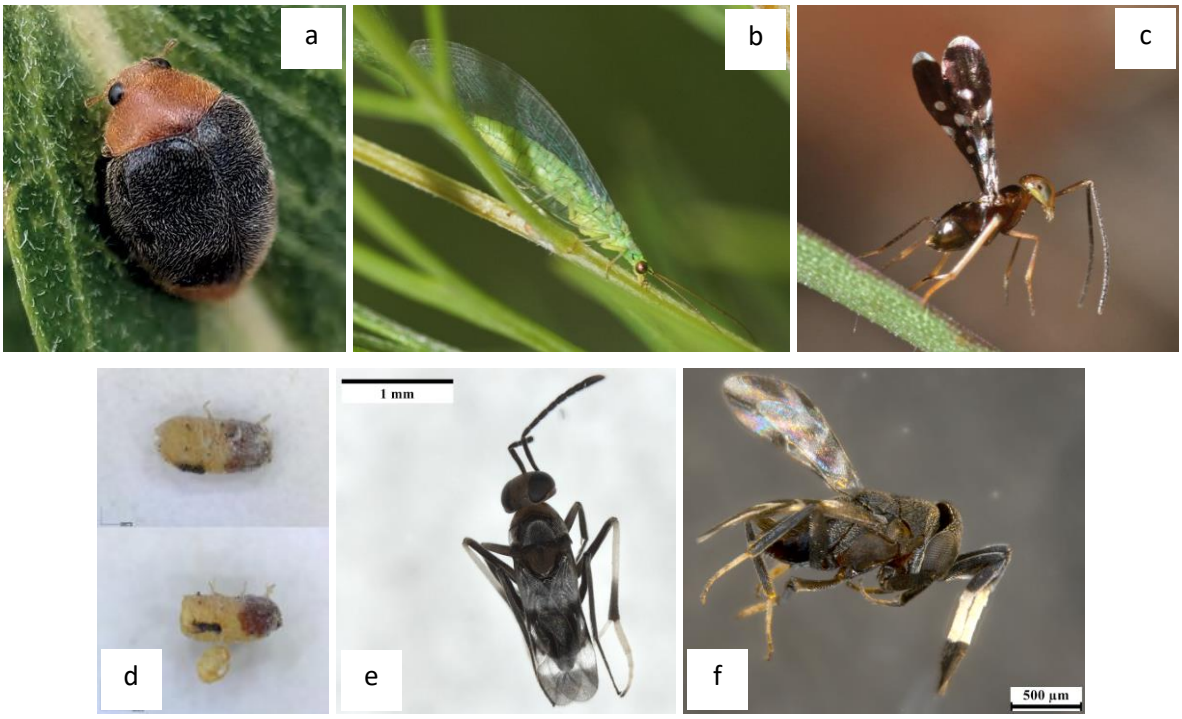

Figure 3. Natural enemies and parasitoids of *H. summervillei*. (a) *Cryptolaemus montrouzieri* (Photo: N. Diplock). (b) *Mallada signatus* (Photo: L. Woodmore). (c) *Callipteroma sexguttata* (Photo: L. Sanders). (d) Mummified *H. summervillei* host before (top) and after (bottom) parasitoid wasp emergence (Photos: E. Bryans, QUT). (e) *Parectromoidella* sp. (Photo: E. Bryans, QUT). (f) *Yasumatsuiola orientalis* (Photo: E. Bryans, QUT).

The systemic insecticides Movento® (spirotetramat) and Confidor® (imidacloprid) are effective against *H. summervillei* and may be used under permit (emergency and minor use permit, respectively) in Queensland and New South Wales (Hauxwell et al., 2022c). Chemical controls can be effective against small incursions where mealybug populations are building rather than established, but they are not recommended for widespread application (Hauxwell et al., 2022d-e). It is also important to note that contact insecticides may not provide sufficient control of mealybugs. Their protective wax coating and tendency to persist deep in the soil profile (Hauxwell, 2018; Manners & Duff, 2015) can put them beyond the reach of surface sprayed chemicals, and any surviving mealybugs can then go on to re-establish populations. Further drawbacks of chemical use include the negative impacts on beneficial insect populations; high cost of insecticide application; and risk of chemical residues persisting in grazing livestock meat (Hauxwell, 2018; MLA, 2021).

1.2.2 Pasture mealybug, *Heliococcus summervillei*

General mealybug morphology

Mealybugs (Hemiptera: Pseudococcidae) are a diverse group of phloem-feeding scale insects (>1000 species) that target an increasingly broad range of plants (>300 genera). This includes several economically important commercial crops like cereal, citrus, coffee and cotton (Zarkani et al., 2021; Finch et al., 2020; Wang et al., 2020a; Tong et al., 2019; Wei et al., 2019). Like all hemipterans, mealybugs possess specialised piercing-sucking mouthparts in which the mandibles and maxillae are modified to form a tubular piercing structure known as a stylet (Jockusch & Fisher, 2021). Variations in the ultrastructure of these mouthparts thought to reflect adaptations in feeding behaviour (Alliaume et al., 2018). For example, variation in the length and/or number of serrations of the distal stylets may represent adaptations to feeding on different plant types (e.g. herbaceous vs. woody) or plant structures (e.g. leaf vs. stem), as seen in other hemipterans (Brozek et al., 2015; Wang et al., 2015). Several mealybug species are highly polyphagous (Subramanian et al., 2021) and have the potential for widespread invasion, with considerable consequences for global agriculture and food security. Although mealybugs are a known biosecurity risk in Australia, current risk estimates indicate only a moderate overall likelihood of establishment and spread given the strict verification measures for Australian commercial imports (DAWR, 2019).

Mealybugs of both sexes are flat and oval-shaped for much of their life cycle, from birth to the third/fourth instar stage. Sexual dimorphism only becomes unambiguous at adulthood: females remain neotenous (juvenile-like) (Figure 4a), while males metamorphose into a macropterous (winged) form (Figure 4b) (Bain et al., 2021; Vea et al., 2016). Taxonomic assessment of adult male mealybugs has proven challenging. Field sampling is difficult due to their small size (both body length and wingspan are typically 1.0mm or less) and cryptic nature, and laboratory colonies are notoriously difficult to maintain due to the handling trauma associated with frequent transfers to new host plants when previous hosts become overwhelmed (O'Hearn & Walsh, 2018; Johnson & Giliomee, 2013; Waterworth et al., 2011). Mealybug taxonomy is therefore largely based on the morphological characteristics of adult females (Bahder et al., 2015).

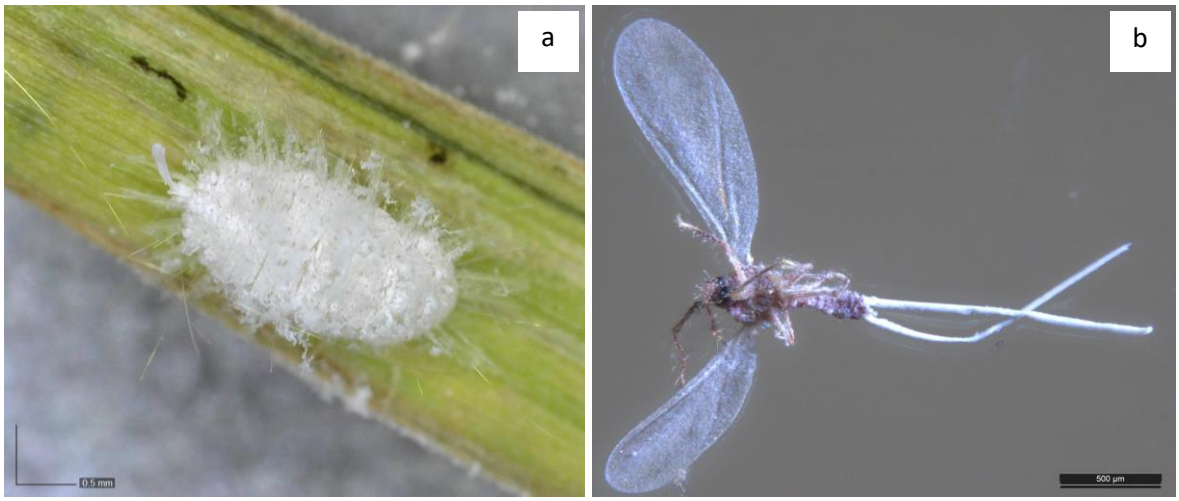

Figure 4. (a) Neotenous live adult female *H. summervillei*, showcasing the powdery white filamentous wax coating that mealybugs are named for (Photo: E. Bryans, QUT). (b) Macropterous dead adult male *H. summervillei*, demonstrating the extreme sexual dimorphism in mealybugs (Photo: G. Dickson, QUT).

Mealybug taxonomy is made more challenging by varying reproductive behaviours between species. Many species demonstrate primarily sexual reproduction but also possess the capacity for facultative parthenogenesis in the event of fluctuating or insufficient male populations (Sompalaym et al., 2016). The latter may disqualify the use of males in species identification as it can create inconsistencies in subsequent phylogenetic studies.

Older juveniles and adult females secrete a powdery white filamentous wax as a protective barrier against water loss and exposure to toxic substances (Tong et al., 2022). This wax coating imparts a ‘mealy’ appearance for which mealybugs are named (Figure 4). Given that adult females are morphologically similar across many mealybug species, even more so between closely related species, identification to species level is difficult without specialist knowledge and microscopic examination (da Silva et al., 2013). Common morphological characters used in mealybug species identification pertain to the antennae (number of segments), dermal pores and ducts (location/shape/size/number), claw structure (simple vs. developed) and auxiliary setae (presence/absence) (Figure 5) (Mani & Shivaraju, 2016a).

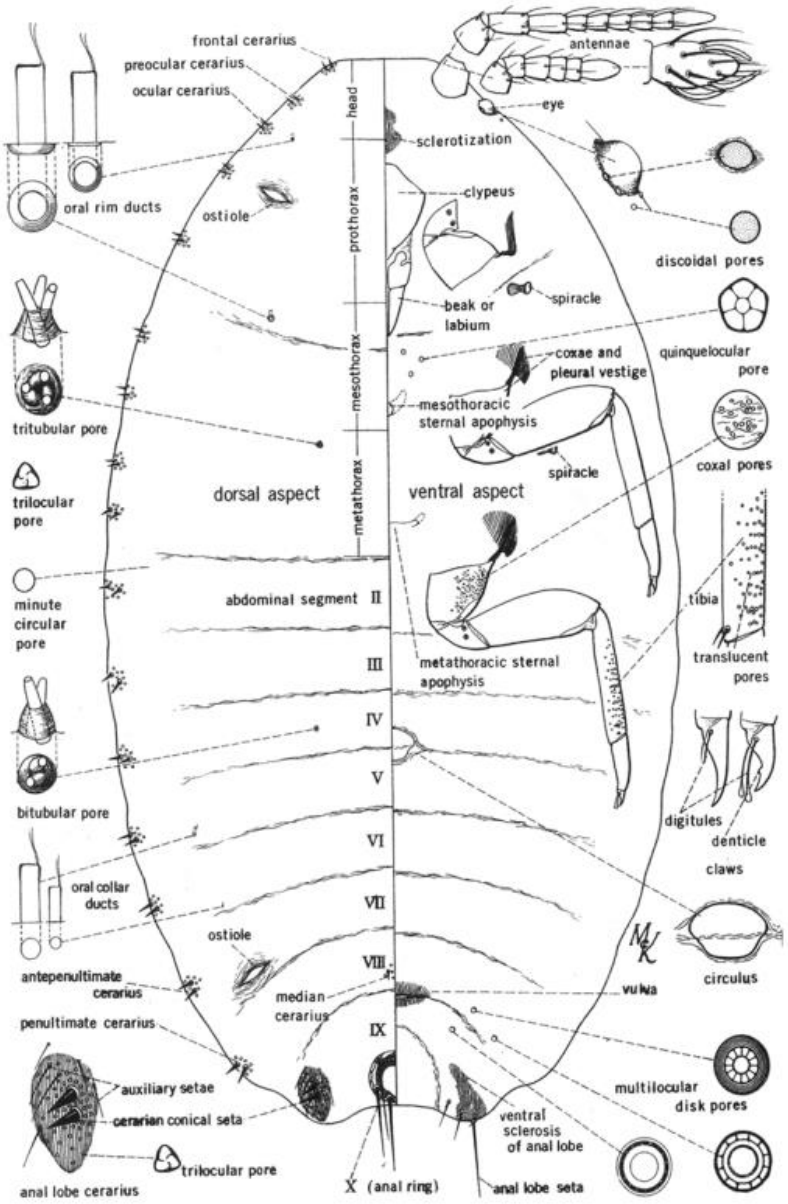

Figure 5. Generalised scientific illustration of a mealybug, highlighting morphological characters that are commonly seen on the dorsal (left) and ventral (right) surfaces (Illustration: D. J. Williams).

Species identification of *H. summervillei*

The following morphological description refers to key characters in the adult female holotype and paratypes of *H. summervillei* Brookes found in Queensland in 1926 (Brookes, 1978; Summerville, 1928), as well as mealybugs sampled from the current (2015-present) pasture dieback outbreak across eastern Australia that have been identified conclusively as *H. summervillei* Brookes (Schutze et al., 2019; Hauxwell et al., 2022d-e) (Figure 6). Adult females of this species have an elongated oval body shape (approx. 2.5-3.5mm long and 1.0-2.0mm wide) and conspicuous nine-segmented antennae (approx. 0.5-1.5mm long). The

legs are long and slender (approx. 0.4mm from trochanter to femur and 0.5mm from tibia to tarsus) and the tibiae feature clusters of pores that become more numerous distally. The claws have fine digitules (finger-like structures) that are longer than the claw itself, and a prominent denticle (tooth-like structure) on the plantar surface. The dorsal ostioles (paired, slit-like apertures) lack setae (hair-like structures) and pores on the upper lip, but feature lanceolate (lance-shaped) setae and quinquelocular (five-lobed) pores on the lower lip. The posterior cerarii (mealybug-specific structures consisting of pores and setae) are distinctly developed, with setae emerging from sclerotised bases and quinquelocular pores set in protuberances. Body setae are sparse and fine, and more numerous on the dorsal surface, where they occur on the abdomen in segmented rows.

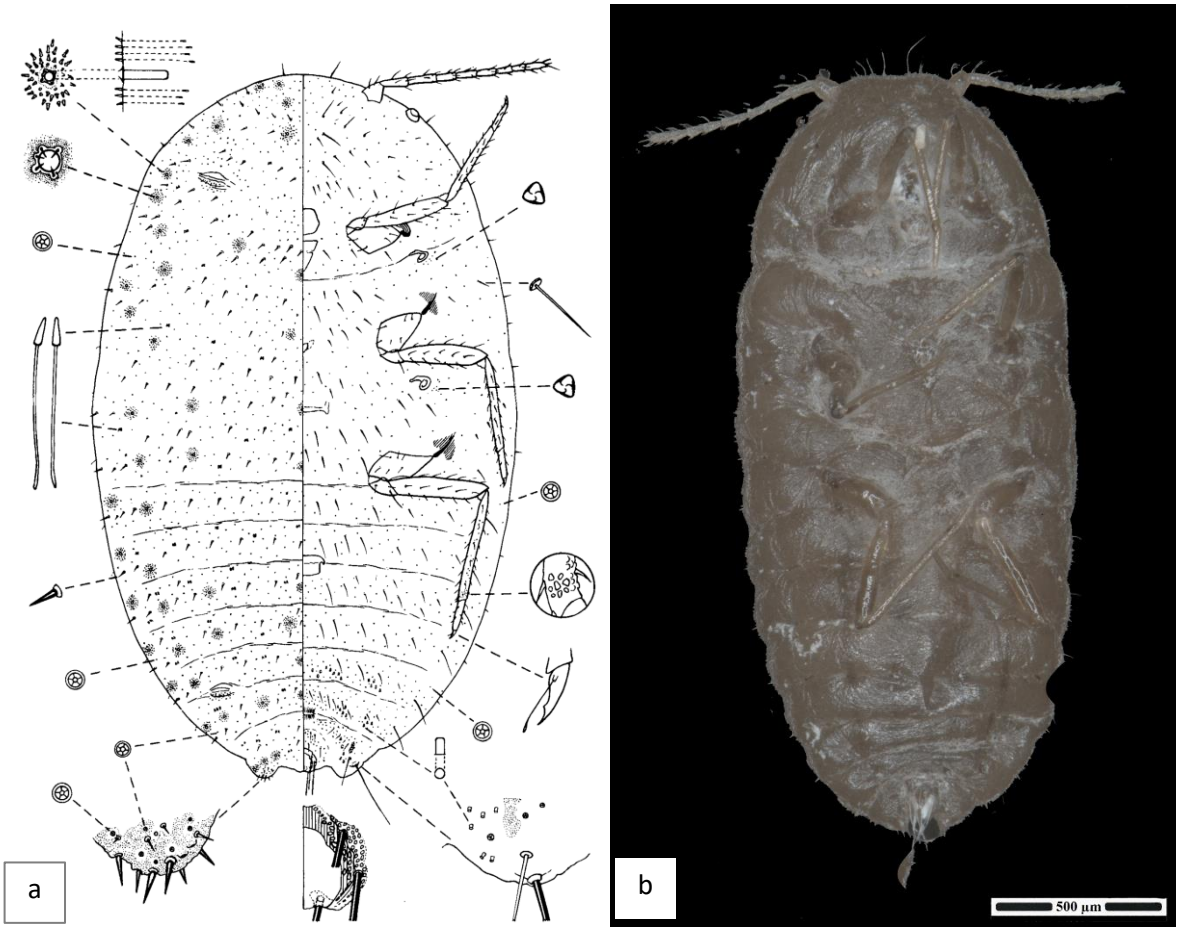

Figure 6. (a) Scientific illustration of the ventral surface of an adult female individual of the species *H. summervillei* Brookes, with key characteristics derived from specimens collected in Queensland and Pakistan (Illustration: H. M. Brookes). (b) Light microscopy image of the ventral side of an adult female *H. summervillei* specimen collected in Queensland from the current (2015-present) pasture dieback outbreak (Photo: G. Dickson, QUT).

Life history

Like many mealybug species, *H. summervillei* is capable of both sexual and asexual reproduction, a decision likely driven by environmental factors (Hauxwell et al., 2022a, 2022d; Summerville, 1928). For example, under laboratory conditions in which adult males failed to emerge, Summerville (1928) observed adult females producing a second generation by parthenogenesis. It is not yet known how many generations can continue to reproduce asexually but based on the enigmatic nature of adult male mealybugs, facultative parthenogenetic reproduction may be a necessary strategy for species survival (Galis & van Alphen, 2019). Asexual reproduction has not been observed in any subsequent laboratory-based experiments on *H. summervillei*. However, field experiments have reported unmated adult females (distinguished by their white colour, as females turn pink when mated) closely associated with neonates in the soil during winter (Hauxwell et al., 2022d) (Figure 7). This suggests a possible parthenogenetic life stage during winter under field conditions.

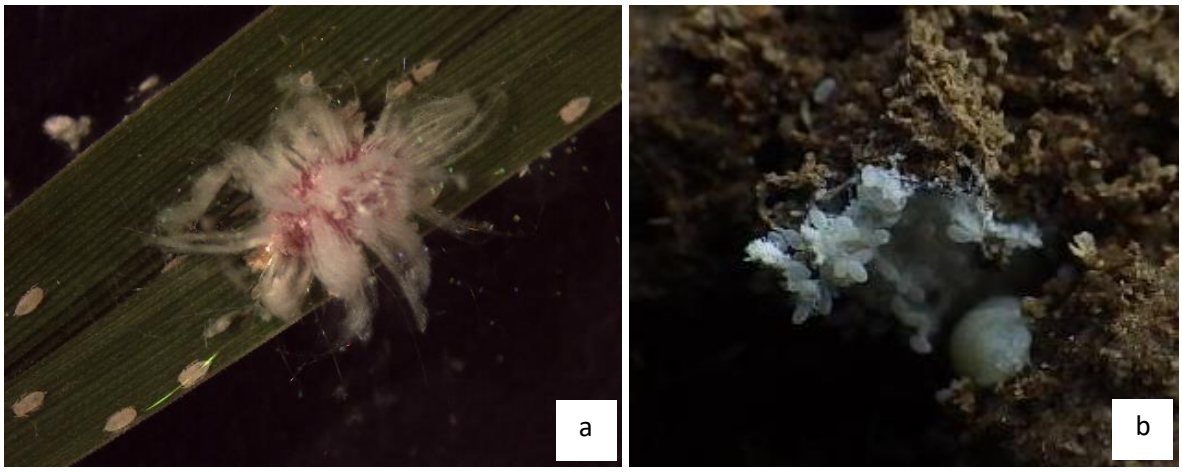

Figure 7. (a) Mated (pink) adult female *H. summervillei* surrounded by first instars on leaf. Females only turn pink when mated; colouration here therefore indicates that sexual reproduction has occurred. (b) Unmated (white) adult female *H. summervillei* surrounded by first instars in soil, a possible indicator of parthenogenetic reproduction. (Photos: C. Hauxwell, QUT).

Summerville (1928) documented that each female can give birth to about 250 offspring, with the first production of young occurring at around 70 days. More recent work reports production of about 100 young on average, at around 40 days (Hauxwell et al., 2022d). Temperature may be a contributing factor; other mealybug species demonstrate shortened development duration with higher temperatures (Prasad et al., 2012; Amarasekare et al., 2008; Chong et al., 2003). The effect of temperature on development time is not yet confirmed in *H. summervillei*, as historical works do not report the relevant parameters.

Host range

H. summervillei feeds exclusively on grasses (Poaceae) and sedges (Cyperaceae). Known host species are listed in Table 1.

Table 1. Grasses and sedges that are known hosts of *H. summervillei*. This information is based on records from the scale insect literature database ScaleNet (Garcia Morales et al., 2016) and Meat & Livestock Australia technical reports (Hauxwell et al., 2022e).

Plant Species	Common Name	Published Records
Axonopus compressus	Broad leaf carpet grass	Brinon et al., 2004
Bothriochloa bladhii	Australian bluestem	Hauxwell et al., 2022e
Bothriochloa insculpta (cv. Bisset)	Creeping bluegrass	Hauxwell et al., 2022e
Brachiaria/Urochloa decumbens (cv. Basilisk)	Signal grass	Hauxwell et al., 2022e; Mille et al., 2016
Brachiaria ruziziensis	Congo grass	Brinon et al., 2004
Cenchrus clandestinus	Kikuyu grass	Hauxwell et al., 2022e
Cenchrus ciliaris (cv. American, Biloela, Gayndah)	Buffel grass	Hauxwell et al., 2022e
Chloris gayana (cv. Callide)	Rhodes grass	Brinon et al., 2004; Hauxwell et al., 2022e
Cyperus rotundus	Nut grass	Hauxwell et al., 2022e
Dichanthium sericeum	Queensland bluegrass	Hauxwell et al., 2022e
Digitaria didactyla	Queensland blue couch	Hauxwell et al., 2022e
Digitaria eriantha	Pangola grass	Hauxwell et al., 2022e
Digitaria milanjiana	Milanje finger grass	Brinon et al., 2004
Eleocharis sp.	Spike sedge	Brinon et al., 2004
Heteropogon contortus	Black spear grass	Hauxwell et al., 2022e
Lolium rigidum	Annual ryegrass	Hauxwell et al., 2022e
Megathyrsus maximus	Guinea grass	Hauxwell et al., 2022e
Melinis minutiflora	Molasses grass	Hauxwell et al., 2022e
Melinis repens	Natal grass	Hauxwell et al., 2022e
Ochthochloa compressa	Wire grass	Brinon et al., 2004
Oryza sativa	Rice	Ben-Dov, 1994; Ghosh & Ghose, 1987; Varshney, 1992
Panicum maximum (cv. Green, Gatton)	Panic grass	Brinon et al., 2004; Hauxwell et al., 2022e
Paspalum dilatatum	Dallis grass	Ben-Dov, 1994; Hauxwell et al., 2022e; Summerville, 1928; Williams, 1985
Paspalum mandiocanum	Broad-leaved paspalum	Hauxwell et al., 2022e
Paspalum notatum	Bahia grass	Hauxwell et al., 2022e
Saccharum officinarum	Sugarcane	Ben-Dov, 1994; Brookes, 1978
Setaria spacelata (cv. Splendida)	Giant setaria	Hauxwell et al., 2022e
Stenotaphrum secundatum	Buffalo turf	Hauxwell et al., 2022e
Themeda triandra	Kangaroo grass	Hauxwell et al., 2022e
Urochloa mosambicensis (cv. Nixon)	Sabi grass	Brinon et al., 2004
Urochloa oligotricha	Signal grass	Hauxwell et al., 2022e

In Australia, pasture dieback was first observed in buffel grasses (*Cenchrus ciliaris*) (Graham & Conway, 1998; Makiela & Harrower, 2008) and initially referred to as 'buffel grass dieback' or 'buffel ill-thrift' (Buck, 2017; Makiela & Harrower, 2008). However, the condition was later seen in bluegrass and paspalum species, bringing a shift in terminology to the broader 'pasture dieback' (Baker et al., 2020; Buck, 2017; Hauxwell 2018). Several economically important grasses are now known to be hosts of *H. summervillei* resulting in dieback, including panic grass (*Panicum maximum*), Rhodes grass (*Chloris gayana*) and sugarcane (*Saccharum officinarum*) (Baker et al., 2020; Buck, 2017; Hauxwell et al., 2022a; Schutze et al., 2019). Meat & Livestock Australia (2023) recently released a guide on the relative tolerance and susceptibility of grass varieties to pasture dieback caused by *H. summervillei*. Among the most highly susceptible varieties are creeping bluegrass (*Bothriochloa insculpta* cv. Bisset), buffel grass (*Cenchrus ciliaris* cv. American and Gayndah), broad-leaved paspalum (*Paspalum mandicorum*), Kikuyu grass (*Cenchrus clandestinus*), Sabi grass (*Urochloa mosambicensis* cv. Nixon), Pangola grass *(Digitaria eriantha*) and Queensland bluegrass (*Dichanthium sericeum*) (MLA, 2023).

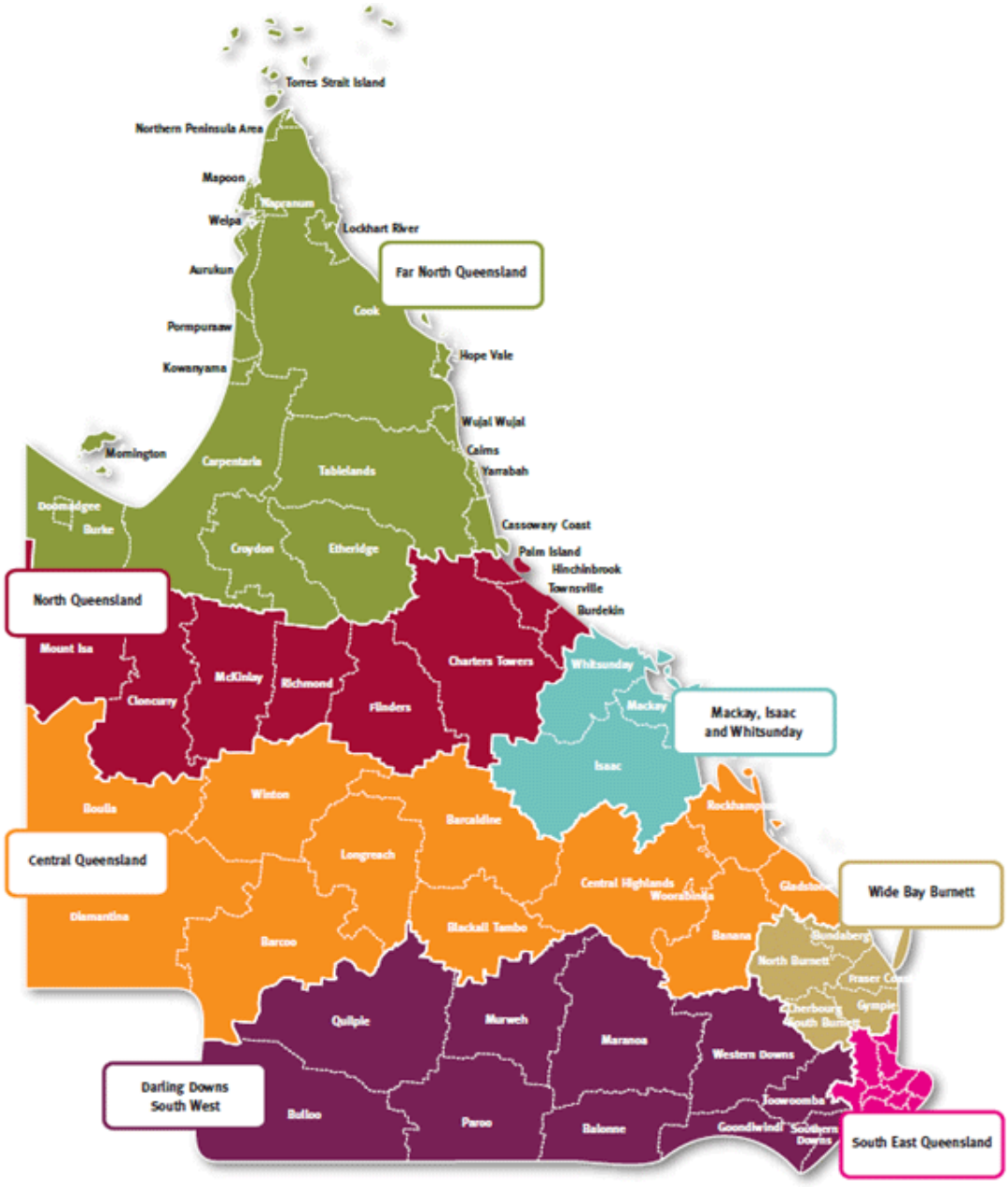

Figure 8. Regions of Queensland, Australia. The state is divided into regions for administrative and statistical purposes, to account for its large size (1.85 million square kilometres) and decentralised population (<50% located in the capital city). (Photo: Queensland Government)

In Queensland, the predominant affected grass appears to vary by region (Buck, 2017) (Figure 8): buffel grass across Central Queensland; creeping bluegrass across Wide Bay Burnett and South East Queensland; Pangola grass across Mackay Isaac Whitsunday; and signal grass (*Brachiaria/Urochloa decumbens*) across Far North Queensland.

Damage to host plants

Phloem sap is a key component of the plant vascular system, responsible for transporting sugars and amino acids (among other important metabolites) from source to sink (Hijaz & Killiny, 2014). As mealybugs feed on phloem sap of the host plant, they excrete a sugar-rich waste product known as honeydew, on which sooty moulds (i.e. saprobic ascomycetes) will typically grow (Windbiel-Rojas & Messenger-Sikes, 2020). Secondary infection by sooty moulds typically only causes cosmetic damage to the host plant (Nelson, 2008). However, heavy or prolonged mealybug infestation may encourage mould growth over a significant surface area, indirectly leading to reduced photosynthetic ability and stunted development in the host plant (Mani & Shivaraju, 2016b; Subramanian et al., 2021).

Mealybug feeding likely causes more damage through the dysregulation of plant defence systems than by removal of phloem sap or growth of sooty moulds. Solenopsis mealybug attacks on cotton (Zhang et al., 2011) and tomato (Zhang et al., 2015) have been shown to suppress plant-induced defences by disrupting the expression of phytohormones that mediate plant defence signalling pathways, jasmonic acid (JA) and salicylic acid (SA). JA pathways are activated in response to necrotrophic pathogens and involve the induction of chemical and/or physical defences to keep host plant cells alive. Conversely, SA pathways are activated to induce localised cell death, which is beneficial against biotrophic pathogens as it opposes their requirement for living host tissue. SA pathways are typically activated in response to piercing-sucking insects, whereas chewing insects trigger a JA-based response (Mason, 2020).

Transcriptome analysis on American buffel grass suggests that *H. summervillei* feeding disrupts defence signalling pathways in a similar manner to the solenopsis mealybug, repressing JA pathways and activating SA expression (Hauxwell et al., 2022d; Munro & Hauxwell, 2023). Concurrent JA repression and SA activation would render the host plant susceptible to necrotrophic and hemibiotrophic pathogens. Multiple species and strains of the broadly pathogenic fungal genus *Fusarium* have been identified in association with the roots and soil of dieback affected pastures (Hauxwell et al., 2022e). Secondary infection by necrotrophs like *Fusarium* spp. corresponds with the symptoms seen in pasture dieback associated with *H. summervillei* (Hauxwell et al., 2022c-d) - e.g. leaf yellowing, stunted growth, eventual plant death.

1.2.3 Mealybug bacterial symbionts

Endosymbiosis in insects

Symbiotic associations with microbes are ubiquitous in nature, and virtually all higher-level organisms have acquired microbial symbionts over the course of their existence (Drew et al., 2021). Time and time again throughout their 479-million-year evolutionary history (Misof et al., 2014), insects have established symbioses with microbes to acquire traits that provide a fitness advantage (McCutcheon et al., 2019). The acquisition of novel, beneficial traits is a major driving force in evolution. It facilitates expansion into ecological niches that may have been previously inaccessible, and subsequently catalyses ecological diversification (Cornwallis et al., 2022).

Microbial symbionts can confer a wide range of host-beneficial traits, owing to their extensive metabolic diversity. These include molecular defences against pathogens and predators (Van Arnam et al., 2018), resistance to abiotic stresses (Renoz et al., 2019), and increased fecundity and reproductive success (Wang et al., 2020b). The most common microbial symbioses provide the insect host with nutritional benefits, typically by synthesising nutrients that are deficient or absent in the host diet, or by enabling the digestion of alternative or recalcitrant substrates for use as a food source (Gupta & Nair, 2020; Sudakaran et al., 2017). The acquisition of diverse microbial symbionts has thereby allowed insects to adapt to an extensive range of environmental conditions and survive on a wide variety of nutritionally incomplete diets (Cornwallis et al., 2022; McCutcheon et al., 2019; Rosenblueth et al., 2017; Sudakaran et al., 2017).

Microbiomes and metabarcoding

Recent work on the solenopsis mealybug indicates that salivary bacteria play a role in modulating inducible plant defences. By eliminating and selectively reinoculating salivary bacteria in *P. solenopsis*, Zhao et al. (2023) identified that phloem ingestion and overall mealybug survival is enhanced when Enterobacteriaceae or *Stenotrophomonas* are present. Subsequent cotton plant infestation showed that feeding by Enterobacteriaceae- or *Stenotrophomonas*-inoculated mealybugs results in decreased expression of JA-responsive genes (i.e. anti-herbivore defences) and increased expression of SA-responsive genes (Zhao et al., 2023). This corresponds with plant defence modulation strategies seen in other insects.

For example, Chung et al. (2013) demonstrates that the Colorado potato beetle (*Leptinotarsa lycopersicum*) uses bacterial symbionts to exploit the mutual antagonism between JA and SA signalling. Secretion of specific oral bacteria into plant wounds was shown to trigger SA-regulated defences, which interfere with induction of JA-regulated defences that are the appropriate response to herbivory (Chung et al., 2013). Induced plant defences were suppressed when at least one of three bacteria, *Stenotrophomonas*, *Pseudomonas* or *Enterobacter* was present (Chung et al., 2013). Similarly, the bacterial symbiont *Hamiltonella defensa* in silverleaf whitefly (*Bemisia tabaci*) saliva is shown to downregulate JA responses and upregulate SA responses, thus suppressing anti-herbivore plant defences to the benefit of the insect herbivore (Su et al., 2015).

Exploitation of JA/SA antagonism is not limited to salivary bacteria. Bacteria present in fall armyworm (*Spodoptera frugiperda*) gut regurgitant have been shown to reduce the anti-herbivore response in tomato plants through modulation of JA-mediated defences (Acevedo et al., 2017). This was attributed to the presence of bacteria from five different genera of the family Enterobacteriaceae, of which *Pantoea ananatis* was the only isolate identified to species level (Acevedo et al., 2017). Such findings imply that examination of the whole microbiome is important for identifying taxa that play a role in modulating plant defence responses, as in the exploitation of JA/SA signalling in the above insect systems.

As discussed in section 1.2.2, the suppression of JA-regulated defences and activation of SA-regulated pathways would leave the host plant susceptible to necrotrophic and hemibiotrophic pathogens. *H. summervillei* feeding is shown to elicit this response in American buffel grass (Hauxwell et al., 2022d; Munro & Hauxwell, 2023), which suggests that mealybug attack renders the host plant vulnerable to secondary infection by necrotrophs like the ubiquitous environmental pathogen *Fusarium.* Indeed, *Fusarium* spp. are abundant in plant and rhizospheric material from dieback-affected sites across the current outbreak in eastern Australia (Hauxwell et al., 2022d). Pasture dieback and *Fusarium* infection elicit concordant symptoms like leaf yellowing and eventual death of the plant (Hauxwell et al., 2022c-d).

Characterisation of the whole microbiome of *H. summervillei* may yield novel data that could inform further research on the roles of mealybug-associated microbes in grass-mealybug interaction and plant response. This project will use metabarcoding methods to profile the bacterial communities in the *H. summervillei* microbiome and establish baseline data on this species. This data will be used to investigate whether there is a relationship between *H. summervillei* microbiota and host plant range and pathology. It can be used to identify taxa

that may be involved in modulating JA/SA signalling or other plant defences, as seen in *P. solenopsis* (Zhao et al., 2023), *L. lycopersicum* (Chung et al., 2013), *B. tabaci*. (Su et al., 2015) and *S. frugiperda* (Acevedo et al., 2017). Metabarcodes or short-read sequences for this project will be generated based on the bacterial 16S rRNA V3-V4 region, which is highly conserved between bacterial species (Martinez-Porchas et al., 2017). 16S metabarcoding, especially the V3-V4 variable region, is demonstrated to be sufficient for specifies identification and phylogenetics in insect systematics (Lin et al., 2019).

Mealybugs and *Candidatus* Tremblaya

Dietary intake of the nine essential amino acids is a growth limiting factor for living organisms. Insufficient levels of any one essential amino acid constrains the ability to synthesise proteins and thus limits the rate of growth (Moriyama & Fukatsu, 2022). Plant phloem sap is a rich source of carbon due to its high carbohydrate content, but a poor source of nitrogen thanks to low ratios of essential:non-essential amino acids. This ratio is typically around 1:4 to 1:20 for phloem – substantially lower than the 1:1 ratio seen in most animal proteins (Douglas, 2006).

Despite this, insects of the order Hemiptera have evolved the capacity to utilise phloem sap as the sole food source throughout their lifecycle (Jing et al., 2016). Phloem-feeding is a conserved trait exclusive to hemipterans (Bennett & Moran, 2013), made possible through the acquisition of symbionts that can supplement limiting nutrients (Sudakaran et al., 2017). For instance, to satisfy the nutritional deficiencies of their phloem-based diet, mealybugs (Hemiptera: Pseudococcidae) maintain endosymbioses with a betaproteobacterial genus known as *Candidatus* Tremblaya (hereafter referred to as *Ca.* Tremblaya) that can perform critical amino acid and vitamin biosynthesis (McCutcheon & von Dohlen, 2011). Mealybugs are unusual in harbouring betaproteobacterial endosymbionts; the endosymbionts of related insect species are largely of gammaproteobacterial origin (Gatehouse et al., 2012; O'Fallon, 2007).

Endosymbiosis involves a free-living organism undergoing adaptations to transition from an extracellular to an intracellular environment and to maintain their existence within the host (Rafiqi et al., 2022). These evolutionary pressures typically lead to directional selection for defences against the host immune response and relaxed selection for traits that no longer serve an essential function (Kinjo et al., 2021). For example, traits required for extracellular survival are generally lost, given the relative safety of an intracellular environment (Keeling &

McCutcheon, 2018). This ultimately results in loss of the ability to exist as a free-living organism (Alarcon et al., 2022).

In mealybugs, these endosymbionts reside in specialised cells (bacteriocytes) within a specialised organ (bacteriome), located centrally in the body cavity (Garber et al., 2021; Gatehouse et al., 2012). This system is thought to have evolved as a means of stabilising symbiotic associations and is found in many other insects (Alarcon et al., 2022; Ferrarini et al., 2022; Rafiqui et al., 2022). Physical compartmentalisation serves a purpose for both host and symbiont. The host reaps nutritional benefits from the symbiont (Rafiqui et al., 2022) and is protected from harmful effects the symbiont may cause (Alarcon et al., 2022), and the symbiont is protected from the host immune system (Ferrarini et al., 2022).

Ca. Tremblaya is the primary endosymbiont found in the mealybug subfamilies Pseudococcinae and Phenacoccinae. Two species are described in the literature: *Ca.* Tremblaya princeps (*T. princeps* hereafter) in subfamily Pseudococcinae (Gatehouse et al., 2012; Husnik & McCutcheon, 2016; Koga et al., 2013; Kono et al., 2008; Lin et al., 2019; Lopez-Madrigal et al., 2015; Szabo et al., 2017; Thao et al., 2002; von Dohlen et al., 2001) and *Ca.* Tremblaya phenacola (*T. phenacola* hereafter) in subfamily Phenacoccinae (Gil et al., 2018; Gruwell et al., 2010; Husnik et al., 2013; Koga et al., 2013; Lin et al., 2019; Michalik et al., 2019a). *Ca.* Tremblaya is a monophyletic lineage that shares congruent phylogenies with its mealybug hosts (Lin et al., 2019; Szabo et al., 2017) (Figure 9). This pattern suggests that their association originated from a single endosymbiosis event before mealybugs split into subfamilies and was followed by long-term co-speciation (Baumann & Baumann, 2005; Gruwell et al., 2010; Lopez-Madrigal et al., 2015; Szabo et al., 2017).

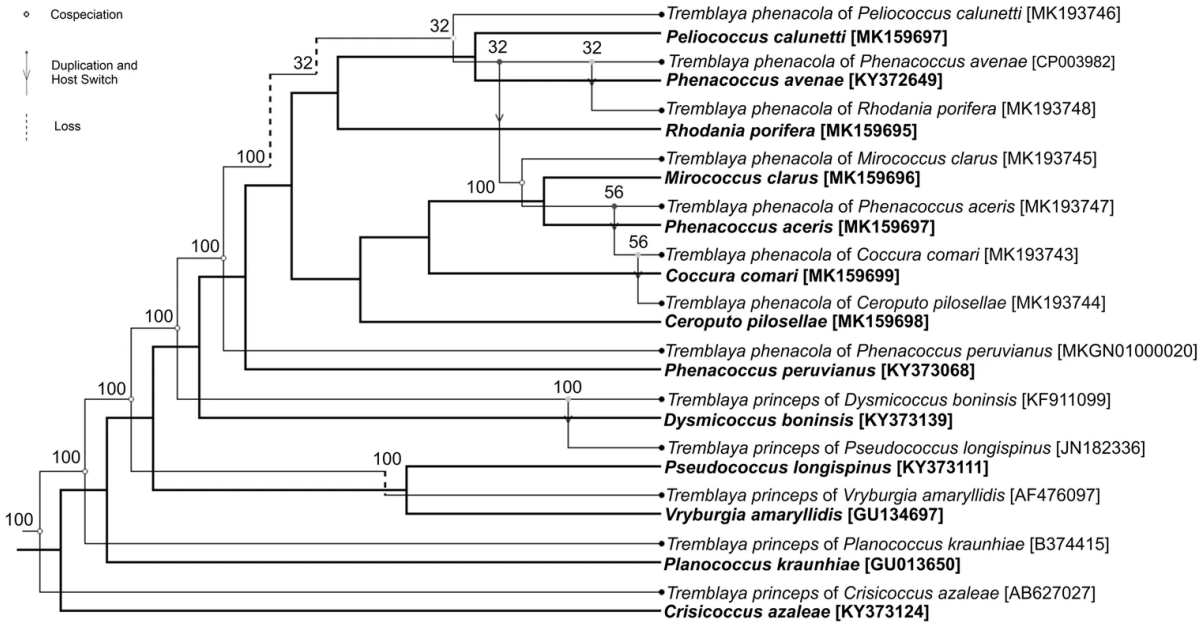

Figure 9. Cophylogeny showing the congruent evolution between various *Ca.* Tremblaya species (thin black lines) and their mealybug hosts (bold black lines). Potential cospeciation, duplication and host-switching events are also noted. (Image: Michalik et al., 2019a)

These primary endosymbionts are passed between generations through vertical maternal transmission (Gatehouse et al., 2012; Lopez-Madrigal & Gil, 2017). In male mealybugs, however, the endosymbiont system is understood to degenerate progressively (Kono et al., 2008). This is likely because adult males have no nutritional requirements (they have no mouthparts and exist only to fertilise females) and thus no need for nutritional symbionts.

Tremblaya princeps vs. *Tremblaya phenacola*

Ca. Tremblaya has an unusual history with endosymbiosis, beyond its acquisition by ancient mealybugs. Phylogenetic analyses based on 16S-23S ribosomal RNA suggest that, in some *Ca.* Tremblaya lineages, the precursor betaproteobacteria were recurrently infected by different gammaproteobacteria (Gatehouse et al., 2012; Garber et al., 2021; Husnik & McCutcheon, 2016; Koga et al., 2013). This gave rise to an unusual multi-partner endosymbiosis: secondary endosymbionts (gammaproteobacteria) nested within the primary endosymbiont (betaproteobacteria) harboured by the host insect (mealybug) (Figure 10).

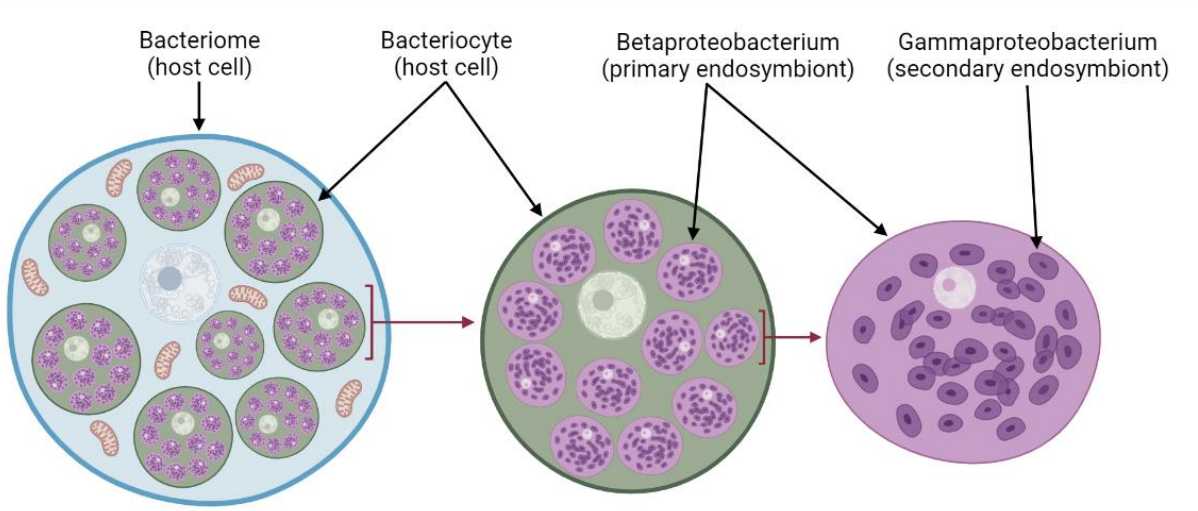

Figure 10. Simplified diagram showing the multi-partner endosymbiosis seen in Pseudococcinae mealybugs. Secondary endosymbionts (gammaproteobacteria) are nested within the cytoplasm of the primary endosymbionts (betaproteobacteria). This nested system is then contained in bacteriocytes in the bacteriome of the host insect. In contrast, Phenacoccinae mealybugs maintain a single-partner endosymbiosis, harbouring only primary endosymbionts in their bacteriocytes/bacteriomes. (Created with BioRender.com)

This multi-partner system has only been identified in Pseudococcinae mealybugs harbouring *T. princeps* as the primary endosymbiont. The *T. princeps* genome is a near-perfect subset of the *T. phenacola* genome (Husnik et al., 2013), only lacking many of the genes involved in translation (Lopez-Madrigal et al., 2015). The loss of translation-related genes is noteworthy, as many other bacterial symbiont genomes, although reduced, retain these genes (McCutcheon & Moran, 2012). This indicates that *T. princeps* has undergone further reductive genome evolution, beyond that involved in establishing endosymbiosis.

The acquisition of additional endosymbionts is the likely catalyst for this extreme genome degeneration. Secondary endosymbionts that also fulfil the host nutritional needs would allow for relaxed selection and eventual loss of redundant genes in the primary endosymbiont (Husnik & McCutcheon, 2016; Szabo et al., 2017). This is seen in the nested three-way symbiosis in citrus mealybugs (*Planococcus citri*), where many of the metabolic genes missing in the associated *T. princeps* genome are present in the secondary endosymbiont genome, *Moranella endobia* (McCutcheon & von Dohlen, 2011; Douglas, 2016). Nested endosymbiosis appears to be a relatively recent development in mealybugs; the gammaproteobacterial endosymbionts associated with *T. princeps* have reduced genomes compared to their closest non-endosymbiotic relatives, but do not yet show significant genome reduction consistent with long-term endosymbiosis (Garber et al., 2021).

In contrast, the Phenacoccidae maintain single-partner symbioses with *T. phenacola* as the primary endosymbiont (Douglas, 2016; Gruwell et al., 2010; Michalik et al., 2019a). Genetic screening of endosymbiotic systems from Pseudococcidae and Phenacoccidae, specifically genes involved in essential amino acid biosynthesis, show that *T. phenacola* alone can provide the same nutritional functions as the *T. princeps* endosymbiotic consortium (Husnik et al., 2013; Lopez-Madrigal et al., 2014; Michalik et al., 2019a). Further analysis has revealed the presence of 80 gammaproteobacterial genes within the *T. phenacola* genome (accounting for 46% of the genome), identified by their characteristic GC content and patterns of biased codon usage (Gil et al., 2018). Consequently, *T. phenacola* is thought to have undergone cellular fusion followed by genomic fusion with a gammaproteobacterium at some point in its lineage (Gil et al., 2018; Lopez-Madrigal & Gil, 2017).

It is posited that the *T. phenacola* genome represents more than just a series of horizontal gene transfers, but rather a whole-genome fusion phenomenon between betaproteobacteria and gammaproteobacteria, resulting in a unique chimeric structure (Gil et al., 2018; Lopez-Madrigal & Gil, 2017). While its current form is significantly different from the endosymbiotic system seen in Pseudococcidae, it has not been determined whether Phenacoccidae maintained a similar nested consortium before the proposed cellular/genomic fusion event. *T. phenacola* has been examined in only a handful of Phenacoccidae mealybugs to date (Husnik et al., 2013; Gil et al., 2017; Lopez-Madrigal et al., 2014; Michalik et al., 2019a) and would benefit from further study.

Cophylogeny in insect systematics

Effective monitoring of pest species requires an understanding of their population genetics. Analyses with polymorphic microsatellite markers and barcode sequences such as COI (cytochrome c oxidase 1), 18S and 28S can have limited resolving power for parthenogenetic species like *H. summervillei* due to low levels of genetic diversity. Obligate bacterial endosymbionts can potentially be used to support the analysis of insect lineages (Choi & Lee, 2022; Rafiqi et al., 2022). The rapid generation time of endosymbionts relative to their hosts gives rise to faster mutation rates and provides genetic variation that can be utilised in population genetic analyses. Insect monitoring can therefore be enhanced by incorporating cophylogenetic analyses that target alternative genetic markers in endosymbiont genomes in addition to markers in the host insect. This is a promising approach for monitoring mealybugs, whose evolutionary history has been shaped more by their relationship with *Ca.* Tremblaya than by historical biogeography or host range (Hardy et al., 2008). Zhang et al. (2014) demonstrates this approach using three genes from the primary endosymbiont

(*Buchnera aphidicola*) of the parthenogenetic Russian wheat aphid (*Diuraphis noxia*), which provided sufficient resolution to track global invasion of the host insect.

Bacterial symbionts could be used to enhance population genetic analyses for *H. summervillei* and improve resolution of insect systematics and lineage. The primary endosymbiont, *T. phenacola*, is a key target. In characterising the bacterial communities in *H. summervillei* microbiome, this project will generate short reads or metabarcode sequences based on the bacterial 16S rRNA V3-V4 region. *T. phenacola* reads from this work will be used for a preliminary investigation into the validity of endosymbiont markers in *H. summervillei* population genetics. Further study would require more complete sequencing of *T. phenacola*. Only two complete genomes have been published: *T. phenacola* PAVE from *Phenacoccus avenae* (Husnik et al., 2013) and *T. phenacola* PPER from *Phenacoccus peruvianus* (Gil et al., 2018).

Metabarcode sequence processing

Metabarcode analysis with next-generation sequencing (NGS) data presents various challenges. NGS data often involves millions to billions of sequencing reads and requires several gigabytes of RAM to store and analyse (Callahan et al., 2017). This is compounded by the fact that sequencing errors can be introduced at various stages of the conventional NGS workflow and may significantly skew the results of downstream analyses if unaccounted for (Ma et al., 2019). Furthermore, taxonomic assignment of 16S reads is complicated by the nature of bacterial taxonomy, which remains incomplete due to the rapid advancement of NGS technologies and the exponential rate at which new taxa are discovered (Ferraz Helene et al., 2022).

A common approach to preparing NGS data for analysis is to cluster highly similar reads, often at the arbitrarily selected threshold of 97% nucleotide similarity, into representative sequences. These are referred to as ‘operational taxonomic units’ (OTUs) and can be used to assign taxonomy (Nguyen et al., 2016). This approach assumes that sequences with greater nucleotide similarity represent more phylogenetically similar taxa and can reduce the computational resources necessary for NGS analysis (Callahan et al., 2017; Nguyen et al., 2016). However, the means of generating OTUs and assigning taxonomies are not infallible. For example, a lax similarity threshold can cause ambiguous sequences to be arbitrarily assigned to OTUs and obscure the true taxonomic diversity, and unidentified sequencing errors can generate spurious OTUs that overinflate diversity estimates (Tarlinton, 2021).

An alternative approach to NGS data preparation is to incorporate sequencing error profiles for run-specific error-correction, which enables resolution into exact sequences known as ‘amplicon sequence variants’ (ASVs) (Jeske & Gallert, 2022). ASVs are intended to correspond to real biological sequences (Tarlinton, 2021) and can identify sequence variation at the level of a single nucleotide (Callahan et al., 2017; Chiarello et al., 2022). However, it should be noted that ASV-based methods have limited ability to deal with undefined bases (‘N’) and non-overlapping reads, which are less of a problem in OTU-based methods (Jeske & Gallert, 2022). For this project, single-nucleotide resolution may be required to differentiate between *T. phenacola* sequence variants; ASV-based methods have therefore been selected over OTU-based methods for raw sequence processing.

1.3 Literature Implications

Phenacoccinae mealybugs are largely overlooked in the literature compared to their Pseudococcinae relatives, and this includes the species in question. Despite the billions of dollars of damage and the potential global threat to pasture production, research dedicated to *H. summervillei* is lacking. Few publications on the biology of *H. summervillei* exist beyond the initial observations recorded by Summerville in 1928 and the species description by Brookes in 1978. Most reports on the current outbreak of pasture dieback only mention mealybugs as a potential causative factor (AHR, 2019; Baker et al., 2020; Buck, 2017; DPI 2021), despite early research that proposed *H. summervillei* as the primary cause (Hauxwell, 2018) and recent work that now confirms this (Hauxwell et al., 2022c).

The majority of *H. summervillei* specimens associated with the current (2015-present) and rapidly spreading outbreak of pasture dieback have minor, but consistent, morphological characteristics that are distinct from the holotype and paratypes identified by Summerville (1928) and Brookes (1978). Mealybugs in the current outbreak differ by the absence of translucent nodules on the hind tibia (Schutze et al., 2019; Biosecurity Queensland, 2022). Their prevalence suggests that the current pasture dieback outbreak may result from an incursion of a new variant of *H. summervillei*.

There is a clear gap in the literature on the biology of *H. summervillei,* the taxonomy and systematics of the variant in the current outbreak of pasture dieback, and mechanisms by which the mealybug contributes to the severity of impacts on the plant host and virulence in the current outbreak.

1.4 Purposes

The purposes of this research are threefold:

- Provide proof of concept for a rapid screening method to determine the suitability of pasture grasses as hosts for *H. summervillei* and thus evaluate their potential tolerance or resistance to dieback.
- Establish baseline data on the bacterial communities associated with *H. summervillei* in the current pasture dieback outbreak and examine how (or if) the microbiome of the mealybug changes between host plants of differing susceptibility and over its geographical range.
- Explore diversity in the new variant of *H. summervillei* and identify differences from the 1926 *H. summervillei* biotype using a systematics approach that considers insect and endosymbiont sequencing data.

This work will generate novel information with practical applications in pasture variety selection for proactive, long-term mealybug management. It will also support analysis of the diversity and systematics of the new *H. summervillei* variant through short-read sequencing of associated bacterial microbiota. This bacterial metabarcoding data will also enable preliminary investigation into the use of endosymbiont genes, like that from the *H. summervillei* primary endosymbiont *T. phenacola*, as molecular markers for monitoring potential insect pest incursions.

1.5 Significance and Scope

Sowing, or re-sowing, pastures with more tolerant grass varieties is a recommended option for proactive and long-term mealybug management (Hauxwell et al., 2022a-b, 2022d-e; MLA, 2021a-c). Host range testing for susceptibility to pasture dieback is therefore important for Australian livestock industries, but such variety trials typically require extensive and costly field trials lasting several years at multiple locations using a limited number of varieties. Previous work to test grasses for dieback tolerance has failed because accurate evaluation requires application of the causal agent, *H. summervillei*, which was not yet confirmed when the trials were run (Peck et al., 2022; Silcock, 2020). Glasshouse assays can provide some indication of susceptibility by infestation over a few generations of the mealybug, but variance in these assays is high (Hauxwell et al., 2022c); they are also dependent on favourable seasonal conditions, labour intensive, and require several months to conduct. A

rapid, statistically powerful screen that tests pasture grasses for their suitability as hosts for *H. summervillei* could reduce the cost of field tests by focusing on varieties with lower relative susceptibility to the mealybug. Screening will provide early results to support selection of more tolerant varieties by pastoralists seeking a solution to dieback.

This research will also address the lack of data available on the microbiome of the invasive agricultural pest, *H. summervillei*, which is severely underrepresented in the literature compared to other mealybug species like *P. citri* and *P. solenopsis*. Using targeted next generation sequencing to establish baseline data on the composition of bacterial communities associated with *H. summervillei*, with particular focus on diversity and systematics in *T. phenacola*, will help to close this gap in the knowledge. In addition to contributing to the literature on Phenacoccinae and *Ca*. Tremblaya, this work will constitute preliminary investigation into the use of endosymbiont-based systematics to identify and monitor insect pest incursions.

1.6 Methodology

This project employs an experimental approach with plant/insect survival bioassays, microbial bioinformatics, and molecular systematics. Short-term plant/insect survival bioassays were used to monitor mealybug survival on different grass varieties over time, relative to a standard variety, to generate quantitative data towards development of a rapid, statistically-powerful screening assay that can determine the suitability of grasses as hosts for *H. summervillei*. Metabarcode sequencing and bioinformatics were used to establish baseline data on the diversity of bacterial communities in *H. summervillei* specimens that were reared on different grass varieties grown in screenhouses, and *H. summervillei* specimens collected from dieback-affected field sites across Queensland and New South Wales. These methods generated qualitative (e.g. genus-level identification) and quantitative (e.g. abundance) data on bacterial communities present in the current mealybug population. Barcode sequences from the primary endosymbiont *T. phenacola* were extracted from these datasets and used alongside short-read sequences from the host insect *H. summervillei* in cophylogenetic analysis of this host/symbiont system.

All laboratory-based work was conducted at QUT Gardens Point campus in the M5 and R1 laboratories. Plants were reared at the Redlands Research Station and insects at the Samford Ecological Research Facility (SERF). Statistical analyses were performed in

RStudio-2022.07.2 (RStudio Team, 2020) using R-4.2.2 with Rtools42 for Windows (R Core Team, 2022); relevant R packages are cited in the methods section of each chapter.

1.7 Thesis Outline

Chapter 1 of this thesis evaluates the literature on pasture dieback associated with the pasture mealybug, *H. summervillei*; the biology and host range of *H. summervillei*; and the bacterial communities and primary endosymbionts associated with mealybugs. It highlights the implications of the literature and gaps in the current knowledge, which form the conceptual framework for this study. Each subsequent chapter covers the aims, methods, results and discussion on the given topic.

Chapter 2 describes a novel and rapid laboratory-based bioassay for screening relative susceptibility to pasture dieback in different grass varieties (relative to a standard variety), based on the survival rate of *H. summervillei* on the host plant. **Chapter 3** establishes baseline data on the composition of the bacterial communities associated with *H. summervillei* on different grass varieties using targeted next generation 'metabarcode' sequencing of the V3-V4 region of the bacterial 16S rRNA gene. **Chapter 4** determines the diversity in bacterial communities associated with the mealybug across the geographic range of the known *H. summervillei* population using samples collected from dieback-affected sites across Queensland and New South Wales. Data were generated using 16S metabarcode sequencing (as in chapter 3) and enable further investigation into the patterns between *H. summervillei* microbial diversity and host plant suitability and pathology across the current hypervirulent outbreak of pasture dieback.

Chapter 5 explores the diversity and differences in new *H. summervillei* variants in the field by isolating short V3-V4 region 16S sequence reads from the NGS data that correspond to the primary endosymbiont *T. phenacola*, and collating this with 28S sequencing data from *H. summervillei* for systematic analyses. This approach constitutes preliminary investigation into the use of relatively variable endosymbiont molecular markers for monitoring insect pests that are difficult to track with standard population genetic analyses due to low genetic diversity. **Chapter 6** provides a summary and discussion of the results presented in previous chapters and highlights key areas of future research.

Chapter 2: Preliminary pasture screening for *H. summervillei* host range testing

2.1 Aims

The majority of knowledge on grass varieties that support damaging populations of *H. summervillei* in Australia is based on field observations (Hauxwell, 2018; MLA, 2021c) and landowner reports (Buck, 2017; MLA, 2021c). Previous attempts to test susceptibility and tolerance to pasture dieback have failed (Peck et al., 2022; Silcock, 2020) due to a lack of understanding of the cause of dieback, i.e. the pasture mealybug *H. summervillei* (Hauxwell et al., 2022c). Host range testing is necessary to identify resistant or tolerant grass varieties and offer options with which to sow/re-sow affected pastures, as per the recommended strategies for proactive and long-term mealybug management (Hauxwell et al., 2022a-b, 2022d-e; MLA, 2021a-c, 2023). This is especially pertinent given the rapid spread of *H. summervillei* across eastern Australia (MLA, 2021b), into highly productive grazing land through Queensland and northern New South Wales (Figure 1).

Long-term pasture trials (2+ years) are necessary to identify field-based factors (soil nutrition, soil microbes, environmental stress, etc.) that may alter dieback susceptibility and the impact of *H. summervillei* in real agricultural conditions. For example, phosphorus is an essential plant nutrient involved in a wide range of cellular processes and is required at all developmental stages (Malhotra et al., 2018). However, it is notoriously deficient in Australian soils (Kooyman et al., 2017), which are geologically ancient and have lost many soluble nutrients over time due to extensive weathering (Eldridge et al., 2018). Phosphorus deficiency reduces pasture growth and resilience (Malhotra et al., 2018), potentially making host plants more susceptible to pasture mealybug and dieback.

Another factor that may influence the severity of mealybug impact is soil fungal community composition, particularly the presence and abundance of pathogenic fungi like *Fusarium* and beneficial endophytes like *Trichoderma* and *Penicillium*. Various studies demonstrate the role of *Penicillium* species in suppressing *Fusarium* species (Miao et al., 2019; Win et al., 2021; Zhao et al., 2021), many of which have the capacity to become plant pathogens (Dinolfo et

al., 2017) that suppress innate immune pathways (Nag et al., 2022). These findings suggest that the balance between beneficial and pathogenic soil fungi may play a role in host plant defence and thus susceptibility to *H. summervillei* attack and pasture dieback (Hauxwell et al., 2022e). Similarly, environmental stressors like drought or waterlogging are known to modulate plant hormone signalling and the expression of plant defence genes (Kim et al., 2021) and are therefore likely to also influence how dieback presents or progresses in the field.

Large-scale field trials to test pasture varieties are ideally conducted over several years and at multiple locations (Hort Innovation, AHR & RM Consulting Group, 2019; Reid, 2005) but require significant investments in time and money to establish and conduct, and may be limited to a few varieties due to the cost and time required to generate results. Smaller-scale shadehouse or glasshouse trials can reduce these costs (Hauxwell et al., 2022e; Inspector-General of Biosecurity, 2022; Kubiriba et al., 2001) but are seasonally dependent, labour intensive, and still take several months. More rapid methods of screening for host suitability are needed to identify varieties for field testing and give graziers options to reduce the impacts on their businesses in the face of the rapid spread of *H. summervillei* in eastern Australia. Furthermore, field trial data is largely categorical (e.g. variety A vs. variety B, affected vs. unaffected) and can therefore only be analysed through an analysis of variance (ANOVA). In contrast, the bioassay method we propose uses proportion of deaths over time, which can be analysed by regression, a more powerful statistical analysis (Faraway, 2002), to generate a median survival time with confidence intervals. This approach is similar to median lethal dose bioassays commonly used in pesticide screening (Kaur & Goyal, 2019; Kiljanek et al., 2017).

Field reports and screenhouse assays suggest that American buffel (*Cenchrus ciliaris* var. USA) and Gatton panic (*Panicum maximum* var. Gatton) grass are relatively susceptible to pasture dieback compared to Mekong brizantha (*Brachiaria brizantha* var. Mekong), which exhibits greater tolerance to mealybug attack (Buck, 2017; Hauxwell, 2018; MLA, 2021c). Life history studies of early instar mealybugs feeding on American buffel and Callide rhodes grass (*Chloris gayana* var. Callide) resulted in poor survival and more rapid death on the less susceptible Callide rhodes (Hauxwell et al., 2022e). This chapter will build on these life history studies using three pasture grasses (American buffel as a standard, Gatton panic and Mekong brizantha as test species) as a model for the development and proof of concept for a rapid 2-3 week laboratory-based test for relative suitability as a host for *H. summervillei*. These results can then be used to inform and focus conventional field trials. Preliminary

results using an early version of this method can be seen in MLA technical report B.PAS.0006 (Hauxwell et al., 2022e).

Second instars were used for these bioassays as first instars are very small and prone to handling death. Second instars are easier to handle with little damage, and their early developmental stage would still allow for sufficient on-plant development time to observe differences in survival on the different varieties. Strategies employed to minimise handling deaths included using glass vials for sample collection, as plastic specimen tubes can generate static electricity that has proportionately more influence on small insects like mealybugs (Edwards, 1960); collecting only actively moving mealybugs, as inactive mealybugs may be feeding and dislodging them could damage the mouthparts; and transferring mealybugs to/from leaves by gently brushing with a fine wet paint brush to avoid piercing the soft tissue.

The results of this chapter will have practical applications for Australian agriculture. The development of a rapid and statistically robust assessment for pasture varieties will benefit those seeking a solution to dieback, particularly pastoralists in the Australian livestock industry, which is in the top five largest beef exporters in the world (MLA, 2022). The outcomes of susceptibility screening will help support the selection of grasses that are more tolerant to *H. summervillei*, thus facilitating long-term pasture productivity.

2.2 Methods

2.2.1 Bioassays

Three pasture grass varieties commonly used in Queensland cattle grazing were used as a model system to test a rapid bioassay method: American buffel (*Cenchrus ciliaris* var. USA), Gatton panic (*Panicum maximum* var. Gatton), and Mekong brizantha (*Brachiaria brizantha* var. Mekong). American buffel was included as a standard for comparison of susceptibility and as the host plant on which the mealybugs were reared. Host plants were grown in sterilised potting mix in screenhouses at Redlands Research Station. Plants were grown for two months to allow the leaves to be developmentally robust enough to support insect cages.

Insect cages were fabricated and modified for the bioassays in this project. In development of these cages, key requirements were identified as follows: transparent walls to enable visual mealybug assessments without opening the cage; a complete seal around the

perimeter of the cage to prevent mealybugs from escaping; soft edges along the seal to accommodate live grass leaves without damaging their vasculature; and ventilation to prevent the build-up of condensation. The final cage design can be seen in Figure 11.

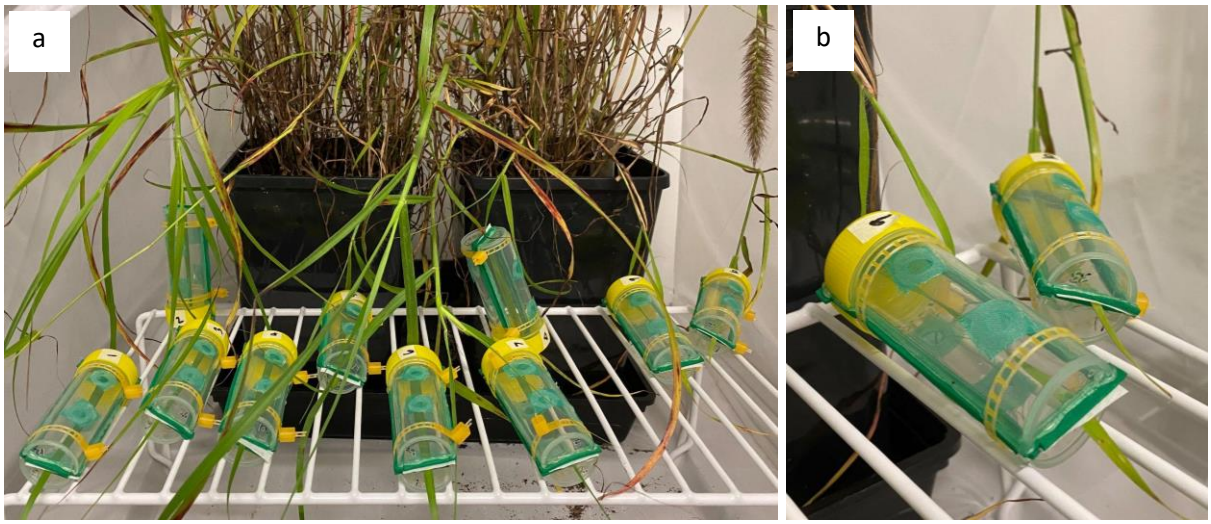

Figure 11. Insect cages designed and fabricated for the bioassays conducted in this project. The cages contain *H. summervillei* on American buffel grass. (Photos: Y. Hernandez-Europa, QUT)

Second instar *H. summervillei* specimens were collected from a culture on American buffel grass maintained in screenhouses at the QUT Samford Ecological Research Facility. 10 mealybugs were placed in each cage and 10 cages were used on each grass variety, giving a total of 100 mealybugs per variety. To minimise mechanical stress on the host plant, an elevated wire rack was used to support the weight of the cages (Figure 11). Host plants were maintained in separate insect rearing tents with twice weekly watering in a temperature-controlled room at 26°C (± 5°C) and 60% humidity, simulating conditions that pasture grasses would typically be exposed to in Queensland and northern New South Wales.

Mealybugs were observed daily, and the number of live mealybugs recorded until there were at least two consecutive time points with no additional mealybug deaths. After this point, it was assumed that the mealybugs had ‘survived’ the assay and further deaths would not occur in a practicable observation period. Survivorship of the remaining live mealybugs was accounted for (i.e. right censored) in subsequent survival analyses (Schober, 2018).

2.2.2 Survival analysis

Median survival time was determined using the R statistical software (v4.2.2; R Core Team, 2022) in RStudio (v2022.07.2; RStudio Team, 2020), with right censoring to account for individuals that did not die during the observation period. Time-to-death analyses and visualisations were performed using the R packages 'survival' (v3.5-5; Therneau et al., 2023) and 'ggsurvfit' (v0.3.0; Sjoberg et al., 2023).

Kaplan-Meier curves (Equation 1) were generated to graphically represent population survival for each variety. Log-rank tests were used to conduct significance tests according to variety. Cox regression (Equation 2) was used to quantify the relative risk of mealybug death between varieties, presented here as hazard ratios (HR). These represent the probability of death in one group relative to another group; a HR greater than one therefore indicates an increased risk of death relative to the specified group.

Equation 1. Equation for the Kaplan-Meier curve, a conditional probability of survival, as calculated in the R package 'survival' (Therneau et al., 2023). Survival at a certain time point (S_{t+1}) is a product of the cumulative survival probabilities prior to that time (S_t) and the proportion of individuals who are still alive (N_t) - i.e. minus deaths (D_t) - at that time (N_t-D_t/N_t). Graphically, this is represented as a step function that drops with each death.

$$S_{t+1} = S_t \times \frac{N_t - D_t}{N_t}$$

Equation 2. The Cox regression model, as calculated in the R package 'survival' (Therneau et al., 2023). Cox regression is a multivariate statistical model that models the transition rate between states (e.g. alive to dead) and is commonly used in survival analysis. The hazard ratio between two groups of interest at a certain time point (h_t) is a product of the underlying baseline hazard rate (h_0) and the exponential (e) of the regression coefficients (b) and predictor variables (x).

$$h_t = h_0 \times e^{b_1 x_1 + b_2 x_2 + \cdots + b_i x_i}$$

The impact of potential handling death (as opposed to death by treatment exposure, i.e. feeding on the host plant) was evaluated with a competing risks analysis, a type of survival analysis that aims to estimate the probability of an event (treatment death) in the presence of competing events (handling death). This was performed using the 'tidycmprsk' package (v0.2.0; Sjoberg & Fei, 2022).

2.3 Results

Time-to-death analysis based on Kaplan-Meier survival curves (Figure 12) indicates a statistically significant difference in overall survival probability according to grass variety (log-rank test, p=0.02).

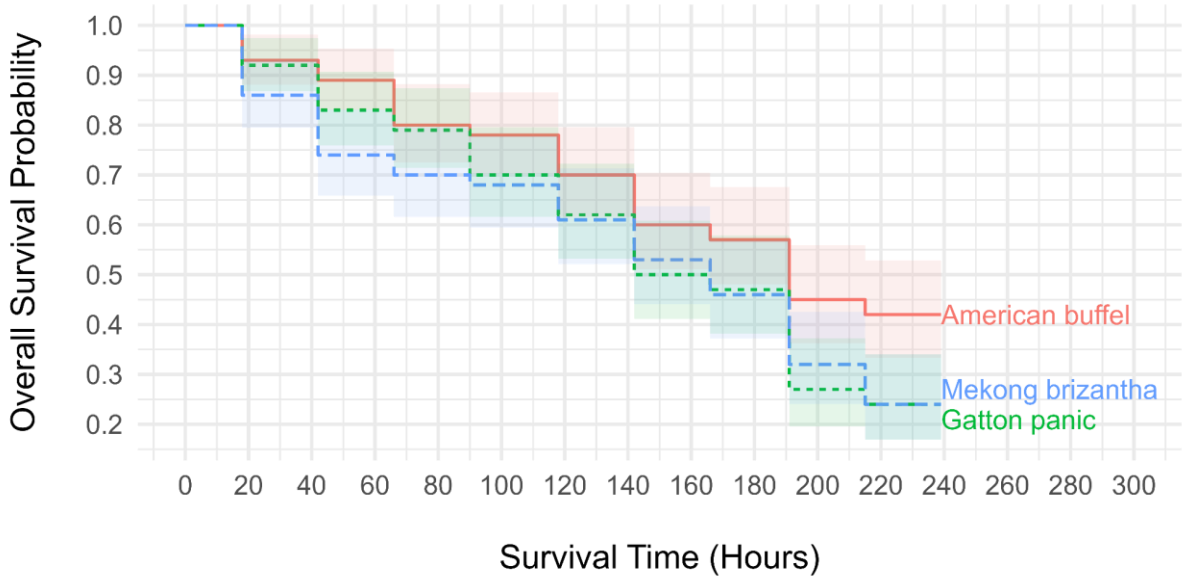

Figure 12. Kaplan-Meier curves representing *H. summervillei* population survival on different grasses, which demonstrate a statistically significant difference in overall survival probability according to grass variety (log-rank test, p=0.02). Further significance testing was performed relative to American buffel (median survival time of 191 hours [166, NA]) and shows that mealybugs have a lower relative probability of survival on Mekong brizantha (median survival time of 166 hours [142, 191]; hazard ratio of 1.57 [1.11, 2.20], p=0.010) and Gatton panic (median survival time of 154 hours [142, 191]; hazard ratio of 1.55 [1.10, 2.18], p=0.012).

Median survival time per variety is positively correlated with effect size between varieties (Table 2). Mealybugs demonstrated a median survival time of 191 hours [166, NA] on American buffel, greater than that of Gatton panic (154 hours [142, 191]) and Mekong brizantha (166 hours [142, 191]). Correspondingly, when compared to mealybugs on American buffel, mealybugs on Gatton panic (HR=1.55) and Mekong brizantha (HR=1.57) are estimated to have a significantly higher risk of death (Cox regression, p=0.013). These results suggest that a relationship exists between grass variety and mealybug survival on alternate hosts.

Table 2. Results of survival analyses using data from bioassays with *H. summervillei* on live pasture grass varieties. Kaplan-Meier survival curves (see Figure 12) have been used to estimate median survival time in hours. Cox regression has been used to quantify effect size in the form of hazard ratios, which represent the probability of death in one group relative to another group (a hazard ratio greater than 1 therefore indicates an increased relative risk of death); the hazard ratios here have been calculated relative to American buffel. 95% confidence intervals are shown for both median survival time and effect size.

Factors			Median survival time		Effect size		
Grass variety	**Sample size**	**Number of deaths**	**Time in hours**	**95% CI**	**Hazard ratio**	**95% CI**	**p-value**
American buffel	100	58	191	166, NA*	NA**	NA**	NA**
Gatton panic	100	76	154	142, 191	1.55	1.10, 2.18	0.012
Mekong brizantha	100	76	166	142, 191	1.57	1.11, 2.20	0.010

*A finite upper confidence limit cannot be calculated for American buffel due to too few individuals (n=58) experiencing the event (i.e. death) during the observation period

**Effect size is calculated with respect to American buffel

There appears to be no meaningful difference in hazard ratios between grass varieties when handling death is regarded as a competing risk to treatment death (Gray's test, p=0.068). The results of competing risks analysis are similar to those of standard survival analysis: when compared to mealybugs on American buffel, mealybugs on Gatton panic (HR=1.62, 95% CI [1.12, 2.32], p=0.010) and Mekong brizantha (HR=1.48, 95% CI [1.02, 2.15], p=0.037) are estimated to have a significantly higher risk of death (competing risks regression, p=0.022).

2.4 Discussion

2.4.1 Bioassay results support field and screenhouse observations

The bioassays show statistically significant differences in mealybug survival time and predicted dieback susceptibility between grass varieties compared to American buffel (Figure 12). American buffel demonstrates the greatest suitability as a host for *H. summervillei* (i.e. longest mealybug survival time) (Table 2), suggesting higher susceptibility to pasture dieback than Gatton panic or Mekong brizantha. These results are supported by field observations and screenhouse trials (Hauxwell et al., 2022c-e), which report greater suitability as a host for *H. summervillei* and greater severity of dieback symptoms in *C. ciliaris* (buffel) varieties compared to *P. maximum* (panic) and *B. brizantha* (brachiaria) varieties.

2.4.2 Host suitability correlated with phenotypic traits

Screenhouse trials indicate an apparent correlation between varieties with thick stems and tillers and reduced susceptibility to dieback (Hauxwell et al., 2022e). This pattern persists in the rapid bioassays, with the thick-stemmed (>10mm) Mekong brizantha demonstrating lower relative susceptibility to dieback than the thin-stemmed Gatton panic (5-10mm) or American buffel (<5mm). These findings suggest that mealybug survival may be influenced by phenotypic traits of the host plant (see Table 3 for morphological descriptions of the pasture grasses in this study).

Table 3. Morphological descriptions of the pasture grasses in this study. Descriptions have been compiled from the Register of Australian Herbage Plant Cultivars (Commonwealth Scientific and Industrial Research Organisation (CSIRO), 1990), plant identification keys (Cook, 2007; Cox, 2008; Johnson & Lloyd, 2008) and animal feed datasheets (Heuze et al., 2016a-b; Heuze & Tran, 2020).

Grass variety	***Cenchrus ciliaris* var. USA ("American buffel")**	***Panicum maximum* var. Gatton ("Gatton panic")**	***Brachiaria brizantha* var. Mekong ("Mekong brizantha")**
Height	Medium (30-150cm)	Medium (50-150cm)	Short (60-70cm)
Stem	Herbaceous, slender (<5mm), extensively branched	Herbaceous, slender (5-10mm), moderately branched	Herbaceous, thick (>10mm), non-branching
Leaf shape	Linear (i.e. elongated), flat, tapered to a fine point	Linear, prominent midrib, rough margins, tapered	Triangular, thick, widens before tapering to a point
Leaf size (at maturity)	Moderate length (30cm), thin (5-10mm)	Long (40-100cm), moderate width (<15mm)	Short (<30cm), wide (>25mm)
Leaf stiffness	Extremely lax	Moderately lax	Moderately erect
Trichomes	Mostly hairless but short hairs (<2mm) may be present around leaf base or stem nodes	Leaves are generally hairy but this can vary somewhat, short bristles on leaf sheaths	Soft hairs on upper and lower leaf surfaces, fine bristles around leaf base and covering leaf sheaths
Growth habit	Typically starts erect or decumbent but becomes sprawling, dense, tussocky	Mostly erect to slightly decumbent, tussocky	Erect, dense, tussocky

Indeed, certain plant traits appear to better accommodate mealybug physical characteristics and/or facilitate mealybug behaviour. For instance, mealybugs have somewhat delicate piercing-sucking mouthparts (see section 1.2.2 for details on mealybug morphology) that may make it difficult to penetrate and feed on host plants with tougher or thicker leaves, like Mekong brizantha. This suggests that plant traits that confer structural robustness (e.g thick

stem, thick leaves) contraindicate mealybug attack and may therefore impart some resistance to dieback. Leaf lamina thickness has been shown to be negatively correlated with sucking insect population size in other plant/pest systems, like green jassid on cotton (Khalil et al., 2017) and two-spotted spider mites on melon (Xu et al., 2019).

Other physical plant defences include trichomes, epidermal structures that can vary from soft hairs to sharp bristles and may be widely distributed on the surface of the plant (Liu et al., 2017). Trichome presence and density are understood to negatively affect insect dispersal and feeding, as they mechanically interfere with insect locomotion and access to the leaf epidermis proper (Belete, 2018). Mealybugs are a soft-bodied scale and can be easily damaged by physical defences like trichomes, which would limit their capacity to move and settle for feeding (da Silva-Torres et al., 2013). This suggests that increased plant pubescence may deter mealybug establishment and thus decrease susceptibility to pasture dieback.

Screenhouse trials with Gatton panic and Green panic (both varieties of *P. maximum*) further support this hypothesis: these varieties are genetically similar due to facultative apomixis (i.e. asexual reproduction) but demonstrate significantly different susceptibility to *H. summervillei* (Hauxwell et al., 2022e). This may be attributed to phenotypic differences between the varieties, with Gatton panic (broader leaves with denser, short bristles) featuring more effective physical mechanisms of resistance than Green panic (shorter leaves with sparser, long hairs) (Moore, 2018). The results of the rapid bioassays support this hypothesis, with the glabrous variety (American buffel) demonstrating greater suitability as a host than the pubescent varieties (Gatton panic and Mekong brizantha) (section 2.3).

H. summervillei exhibits strong negative phototactic behaviour (i.e. avoids light), observed in both field and laboratory conditions. Grass varieties with lax leaves that droop down and/or sprawling growth habits that put leaves in a more decumbent position offer more shade, possibly making them a more attractive host than erect varieties. This again corresponds with the results of the rapid bioassays (section 2.3) and early screenhouse trials (Hauxwell et al., 2022c, 2022e). The lax-leafed, sprawling American buffel is more susceptible to *H. summervillei* attack than the erect-leafed, non-sprawling Mekong brizantha; the moderately lax-leafed and slightly decumbent Gatton panic falls somewhere between the two extremes.

The characteristics of the less suitable host plants may also reduce susceptibility to severe dieback symptoms and subsequent death of the grass. Gatton panic and Mekong brizantha exhibit phenotypic traits that are less favourable for the dispersal and feeding of *H.*

summervillei compared to American buffel (Table 3). The wider stems, thicker leaf laminae, higher trichome density, more erect leaf orientation and more erect growth habits of the Mekong brizantha and Gatton panic varieties may confer decreased susceptibility to the symptoms of dieback and death of the grass by reducing the opportunity for *H. summervillei* to establish.

The above observations on the effect of plant phenotype on host suitability require further validation beyond the scope of this study. Future experiments should involve formal assessment of phenotypic traits (for example: stem thickness, leaf thickness, trichome density, trichome length, leaf orientation, growth habit) with a larger sample size to enable robust statistical analysis on the influence of plant phenotype on susceptibility to mealybug.

Mealybugs used in this experiment were reared on American buffel grass, and thus might be expected to be less well-adapted to alternate plant hosts. The assay method requires further testing on a range of plant varieties, including those more susceptible to the mealybug, to determine a range of survival times (relative to American buffel as the standard) to confirm the validity of the test.

2.4.3 Rapid bioassays for mass pasture dieback screening

Technical reports by Hauxwell et al. (2022c, 2022d, 2022e) provide a baseline for the length of conventional plant screening for pasture dieback (Table 4). Field trials were performed over a 2-year period between March 2020 and May 2022 (Hauxwell et al., 2022d, p. 45-50), with screenhouse pot plant trials conducted concurrently over a 1-year period between December 2020 and January 2021 (Hauxwell et al., 2022c, p. 20-22). Both methods have provided valuable longitudinal data on the impact of season on mealybug populations and recovery of grasses. These reports also detail life history studies on *H. summervillei* and exploratory survival time assays that form the basis of the rapid bioassays presented in this thesis. Laboratory life history studies followed *H. summervillei* neonates from birth to death over a 5-month period, demonstrating a maximum observed lifespan of approximately 30 days in males and up to 145 days in females (Hauxwell et al., 2022d, p. 26-27). Survival time assays were conducted over 10-14 days in the laboratory and shown to determine sensitive and statistically robust differences in susceptibility between grass varieties (Hauxwell et al., 2022e, p. 24-26).

Table 4. Comparison of experimental approaches to *H. summervillei* monitoring and pasture dieback screening. Data are based on field trials, screenhouse trials and life history studies conducted by Hauxwell et al. (2022c, 2022d, 2022e) and rapid bioassays presented in this thesis.

Experiment	**Field trial**	**Screenhouse trial**	**Life history study**	**Rapid bioassay**
Time required	Years	Months	Months	Weeks
Labour intensity	High	Moderate	High	Low
Data produced	Categorical (e.g. variety A vs. variety B, affected vs. unaffected)	Categorical	Numerical (e.g. number dead, proportion of deaths over time)	Numerical and categorical
Analysis	Analysis of variance	Analysis of variance	Regression	Regression, analysis of variance
Advantages	Allows long-term investigation of biological and physiological responses in a natural environment	Allows investigation of biological and physiological responses in a semi-natural environment, with some control over environmental parameters; can be studied long-term	Captures full life history; controlled environment allows for clearer understanding of cause and effect; not seasonally dependent	Controlled environment allows for clearer understanding of direct cause and effect; not seasonally dependent; can generate results in a short timeframe
Limitations	Costly to establish and maintain; no control over environmental parameters; seasonally dependent; requires long-term monitoring to generate data	Costly to establish and maintain, but less so than field trials; seasonally dependent; requires long-term monitoring to generate data	Intensive labour costs (e.g. continuous monitoring); biological and physiological responses may be altered in a controlled environment	Cannot capture full life history; biological and physiological responses may be altered in a controlled environment; does not facilitate long-term study

Long-term field trials are undoubtedly important for assessing plant disease presentation and progression in real-world conditions. However, in the case of screening for pasture dieback susceptibility, it would quickly become a costly endeavour to perform mass testing of all relevant grass varieties. With this in mind, we have demonstrated proof of concept of a rapid

laboratory-based bioassay that has generated similar results to previous screenhouse trials but in a shorter timeframe and can identify pasture varieties that are less susceptible hosts for *H. summervillei* for inclusion in field trials. Although these rapid bioassays are limited in that they cannot capture the full insect life history and the laboratory environment may alter insect biological/physiological responses, they offer a valuable means of focusing the scope of future field trials, thereby reducing associated costs and prioritising practical results for the agricultural industry.

This bioassay would ideally be further tested before use in pasture screening. A key limitation of the experimental approach here is the potential impact of insect adaptation to host plants. Mealybugs used in this experiment were reared on American buffel, one of the subject species, and thus might be expected to be less well-adapted to alternate hosts. Differentially host-adapted insects were unable to be reared for each pasture species in this project due to time and resource constraints. Reciprocal transplant trials could be conducted to validate the findings presented in this chapter. Such trials should involve testing host-adapted mealybugs on the corresponding plant hosts as well as a common host (i.e. plant species that the mealybugs have not been introduced to) to examine whether local adaptation has an effect.

Nonetheless, for commercial screening purposes, comparison with a standard like American buffel may be more practical. The bioassay method requires further testing on a range of plant hosts, including grass varieties reported to be equally or more susceptible to the mealybug than American buffel (the standard used here), such as annual ryegrass (*Lolium multiflorum*). Testing across varieties of the same species with known differences in suitability as hosts for the mealybug, such as American/Biloela/Gayndah buffel (Hauxwell et al., 2022e), is also recommended.

Chapter 3: Composition and diversity of bacterial communities in *H. summervillei* on different host plants

3.1 Aims

This chapter aims to establish baseline data on the composition and diversity of bacterial communities in *H. summervillei*, identify potential core bacteria, and examine whether differences in host plant suitability affect the mealybug microbiome. This was achieved using targeted next generation sequencing of the V3-V4 region of the bacterial 16S gene from total DNA extracted from adult female *H. summervillei* mealybugs adapted to various pasture grasses. This research has generated novel data on microbiome diversity and community composition in *H. summervillei*, for which there is no equivalent in the current literature.

This chapter also explores whether the mealybug microbiome contributes to the apparent dysregulation of plant immune systems, as per the pathology of pasture dieback caused by *H. summervillei* attack (section 1.2.1). *P. solenopsis* mealybug feeding has been shown to disrupt JA/SA signalling pathways involved in plant defences (Zhang et al., 2011, 2015) and recent work suggests that *H. summervillei* operates in a similar manner. Transcriptomics on the effect of *H. summervillei* feeding on gene expression in American buffel grass suggests that mealybug feeding suppresses the anti-herbivore plant defence response mediated by JA (Hauxwell et al., 2022d; Munro & Hauxwell, 2023). Differential expression analysis illustrates that *H. summervillei* feeding results in significant induction of SA biosynthesis, SA-responsive and SA-dependent genes, as well as a repressor of JA-related responses (Hauxwell et al., 2022d; Munro & Hauxwell, 2023). Upregulation of SA-related genes would disrupt the antagonistic JA/SA crosstalk that modulates induced defence responses and effectively suppress JA-based defences that would be beneficial against mealybug attack (Hauxwell et al., 2022d).

The mechanisms by which JA signalling is suppressed and/or SA signalling is promoted in *H. summervillei* have not been confirmed. These effects may be caused directly by *H. summervillei*, or through endosymbionts like *T. phenacola* or other associated microbiota. Recent work suggests that specific endosymbionts in *P. solenopsis* mealybug saliva play a

role in modulating herbivore-induced plant defences (Zhao et al., 2023) and thus facilitate mealybug establishment and survival on the host plant. The presence of salivary Enterobacteriaceae and *Stenotrophomonas* resulted in decreased expression of JA-responsive genes in the host (with corresponding increase in expression of SA-responsive genes) and enhanced phloem ingestion in the solenopsis mealybug (Zhao et al., 2023). When *P. solenopsis* was treated with antibiotics to remove these salivary endosymbionts, feeding was shown to trigger JA-responsive gene expression in the host and reduce the duration and volume of phloem ingestion in the mealybug (Zhao et al., 2023). Identifying core bacteria in the *H. summervillei* microbiome may provide insight into whether plant defence is regulated at the level of the microbiome as in *P. solenopsis*.

As discussed in the previous chapter, host plant suitability for *H. summervillei* appears to be partially influenced by phenotypic traits like leaf lamina thickness and the presence of trichomes (section 2.4.2). Further work is required to understand the factors that influence host selection and susceptibility in *H. summervillei*. Determining the composition and diversity of bacterial communities in *H. summervillei* on different host plants may shed light on whether the mealybug microbiome plays a role in host selection, or vice versa.

3.2 Methods

3.2.1 Sample collection

White adult female mealybugs were collected from American buffel, Callide rhodes, Gatton panic, and sugarcane (*Saccharum officinarum*) grass varieties grown in screenhouses at the Samford Ecological Research Facility, as per MLA technical report B.PAS.0006 (Hauxwell et al., 2022e). Mealybugs were cultured on host plants for six (6) weeks to allow for microbiome adaptation or adjustment. Three (3) adult females were collected from each of three (3) plants of each plant variety, for a total of nine (9) mealybugs per variety. Samples were stored in 70% ethanol at -20°C until processed.

3.2.2 Pretreatment

Mealybugs were pretreated to remove wax from the body surface and minimise inhibitory effects during DNA extraction, as described by Wang et al. (2019) in their comparative analysis of DNA extraction methods for mealybugs. Specimens were removed from ethanol

storage and soaked in trichloromethane for 30 minutes, transferred to ultrapure water and soaked for a further six (6) hours, then air-dried on sterile filter paper.

3.2.3 DNA extraction

DNA extraction was performed using the DNeasy Blood & Tissue kit (QIAGEN, 2022), following manufacturer instructions with minor modification to the lysis incubation step, which was extended from four (4) hours to eight (8) hours to achieve sufficient DNA yield. Purified DNA was stored at -20°C.

3.2.4 Sequencing

PCR amplification and sequencing were performed by the Australian Genome Research Facility (AGRF) using their 16S (V3-V4) microbial diversity profiling service. PCR amplicons were generated using the primer pair 341F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGGTATCTAAT 3') and conditions outlined in Table 5. Thermocycling was completed with an Applied Biosystems Veriti 384-well thermal cycler using Platinum SuperFi II PCR Master Mix (Invitrogen, Australia). Expected amplicon size was approximately 464bp.

Table 5. PCR cycling parameters used in the 16S (V3-V4) microbial diversity profiling service by the Australian Genome Research Facility (Melbourne, Australia).

Cycles	Pre-Denaturation	Denaturation	Annealing	Extension	Final Extension
30	98°C for 30 sec	98°C for 10 sec	60°C for 10 sec	72°C for 30 sec	72°C for 5 min

The primary PCR amplicons were cleaned using magnetic beads and visualised on 2% SYBR E-Gel (Thermo Fisher). A secondary PCR to index the amplicons was performed with the same master mix and cleaned again using magnetic beads. The resulting amplicons were quantified by fluorometry (Promega Quantifluor) and normalised. The equimolar pool was cleaned a final time using magnetic beads to concentrate the pool and then measured using High Sensitivity D1000 Tape on an Agilent 2200 TapeStation. The pool was diluted to 5nM; molarity was confirmed using a Qubit High Sensitivity dsDNA assay (Thermo Fisher). This was followed by sequencing on an Illumina MiSeq (San Diego, CA, USA) with a V3 600-cycle reagent kit (2 x 300bp, paired-end reads).

3.2.5 Microbiome analysis

Sequence processing and preliminary data analyses were performed using QIIME2 (Quantitative Insights Into Microbial Ecology) (Bolyen et al., 2019) with the following plugins: ‘q2-demux’ for demultiplexing; ‘q2-dada2’ for denoising; ‘q2-feature-classifier’ for taxonomic classification; ‘q2-phylogeny’ for phylogenetic reconstruction; and ‘q2-diversity’ for the computation of diversity metrics.

Raw reads were inspected via interactive quality plots produced in QIIME2 (Estaki et al., 2020). Reads were trimmed to remove 341F/806R primer sequences (17bp for forward reads, 20bp for reverse reads) and where sequence quality scores dropped below 20 (Q20). Subsequent quality filtering of trimmed reads was performed with a maximum expected errors (maxEE) threshold of 2. Paired forward and reverse reads were merged to obtain the full denoised sequences. Chimeric sequences were removed through a de novo chimera filtering algorithm used by dada2 (Callahan et al., 2016).

SILVA SSU Ref NR 99 v138.1 (SILVA small subunit rRNA non-redundant reference database with 99% identity criterion to remove highly similar sequences) (Glockner et al., 2017; Robeson et al., 2020) was used as the reference database for taxonomic assignment. BLAST+ local alignment was performed between the query and reference sequences. The top 10 hits (taxonomic assignments) for each query sequence were identified by filtering for 80% identity and 80% query cover. Being the first investigation into the microbiome of this species, a relatively relaxed (<95%) percent identity threshold was necessary to capture similar sequences for consensus taxonomy and other phylogenetic analysis. For each query sequence, a consensus taxonomy was assigned if 50% of hits matched the top hit. The QIIME2 ‘core-metrics-phylogenetic' pipeline was used to compute diversity metrics (Shannon index, observed features) and distance matrices (UniFrac, weighted and unweighted) for downstream analysis. A sampling depth of 10,000 was selected based on the minimum sequence count across non-control samples.

End products of the QIIME2 workflow are an ASV table, which records the number of times each ASV is observed in each sample; a representative sequences table, which provides the sequence for each ASV; Newick-formatted phylogenetic tree files; and vectors for various biodiversity metrics, as detailed above. Decontamination and microbiome analyses were performed on these products with the R statistical software (v4.2.2; R Core Team, 2022) in RStudio (v2022.07.2; RStudio Team, 2020) using the ‘decontam’ (Davis et al., 2018) and ‘phyloseq’ (McMurdie et al., 2013) packages, respectively. Multiple sequence alignment was

performed in the Clustal Omega program (Madeira et al., 2022) and visualised in R using the 'ggmsa' package (Zhou et al., 2023). Phylogenetic reconstruction and tree annotation were performed using the 'ggtree' (Yu et al., 2017) package.

3.3 Results

3.3.1 Overall bacterial diversity

Overall bacterial diversity was low. 16 bacterial species were identified across the bacterial microbiomes of the entire cohort (Figure 13), most of which are common environmental organisms typically found in soil and water sources. *Ca.* Tremblaya sp. was the only species present in all samples. It is also the most dominant taxon, which is expected given that it is the primary endosymbiont in *H. summervillei*. *Ca.* Tremblaya ASVs account for at least half the amplicon reads in each sample. Some samples (C5-3-16S, G1-3-16S, S1-1-16S) have a notably low abundance of *Ca.* Tremblaya.

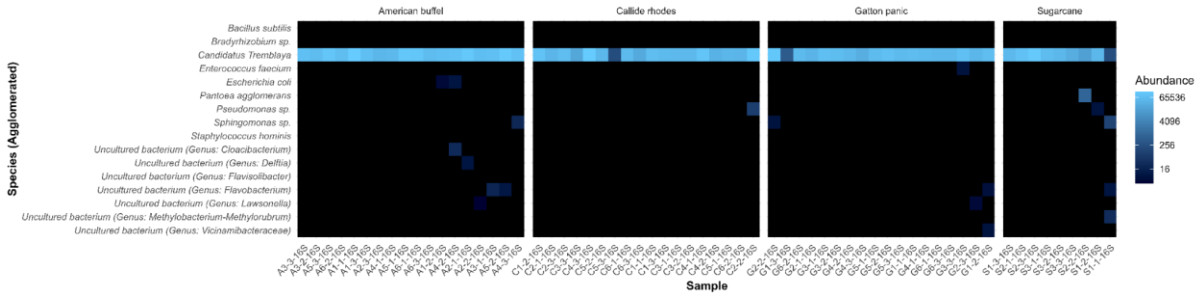

Figure 13. Heat map of ASV (16S rRNA V3-V4) abundance across the bacterial microbiome of *H. summervillei* samples collected from different plant hosts. ASVs are agglomerated at the species level for clarity; minor ASV variants are therefore not shown. ASVs that were not assigned at the species level are reported to the lowest taxonomic depth possible; unassigned ASVs here are reported to the genus level. Mealybug samples are grouped according to host plant: American buffel (*C. ciliaris* var. USA), Callide rhodes (*C. gayana* var. Callide), Gatton panic (*P. maximum* var. Gatton) and sugarcane (*S. officianarum*). *Ca.* Tremblaya is the most dominant taxon and is the only group present across all samples.

Alpha diversity analyses indicate no significant difference in bacterial diversity within individual mealybug samples (Kruskal-Wallis test, p=0.476) or within samples from the same grass variety (p=0.135). Post-hoc pairwise comparisons confirm no underlying differences (Wilcoxon rank sum test on Shannon indices, p>0.5).

Bacterial diversity is low across the entire cohort, with most samples comprising only two or three distinct taxa. Beta diversity analyses show a statistically significant difference in diversity between grass varieties (PERMANOVA, p=0.021) but this significance is lost when potential outliers (samples with relatively low abundance of *Ca.* Tremblaya ASVs, identified in Figure 13) are removed (p=0.209). Initial principal coordinates analysis (Figure 14, left) shows a highly skewed amount of variance (>99%) captured by one principal coordinate (axis 1), which generally indicates that outliers are present. Removal of outliers in subsequent analysis shows variance spread across multiple axes and no distinct clustering of sample points (Figure 14, right). These results indicate low or no difference in bacterial diversity between samples from different hosts. Post-hoc pairwise comparisons reveal no underlying differences (pairwise PERMANOVA, p>0.05).

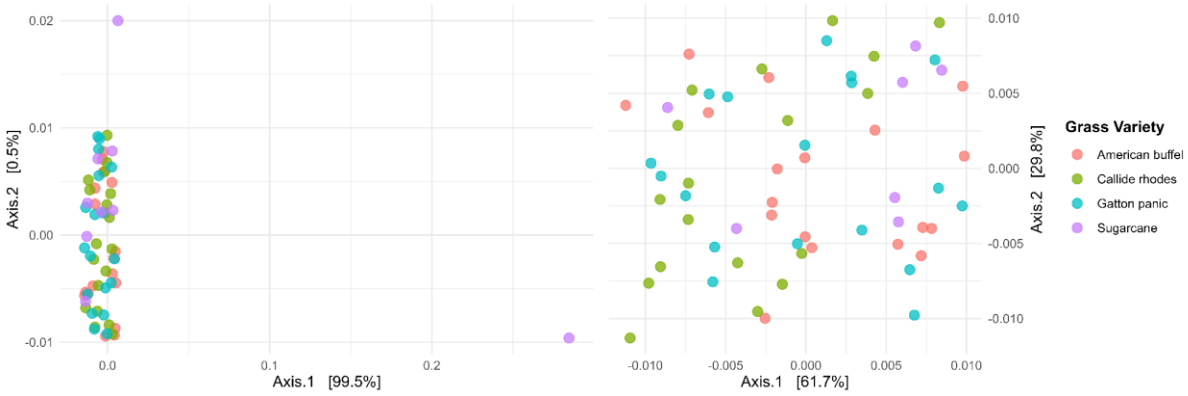

Figure 14. Ordination plots showing the results of beta diversity analysis on 16S rRNA (V3-V4) extracted from *H. summervillei* reared on different grass varieties. Ordination has been performed using an MDS (multidimensional scaling) model (also known as Principal Coordinates) and the weighted UniFrac distance method, with (left) and without (right) potential outliers. The amount of variation captured by each axis is noted as a percentage. The spatial distance between sample points reflects the differences in beta diversity - the closer two points are in ordination space, the more similar the bacterial diversity in those samples. The left-hand plot shows variance being largely captured by a single variable (99% of variation is explained by axis 1); this highly skewed variance suggests that outliers are present. Potential outliers are removed in the right-hand plot and variance becomes more appropriately spread across the axes. These results indicate little-to-no difference in bacterial diversity between samples from different host plants.

3.3.2 Non-*Tremblaya* bacterial diversity

Bacterial diversity is better visualised when *Ca.* Tremblaya is removed from analysis (Figure 15). Sugarcane demonstrates the greatest non-*Tremblaya* bacterial diversity, with one sample containing ASVs from three unique taxa. Samples from all other grass varieties comprise only one or two bacterial species. Alpha diversity is correspondingly similar within

individual mealybug samples (Kruskal-Wallis test, p=0.450) and samples grouped by grass variety (p=0.186). It should be noted that of the 63 total samples, only 15 samples contain species other than *Ca.* Tremblaya, and these are in extremely low read abundance.

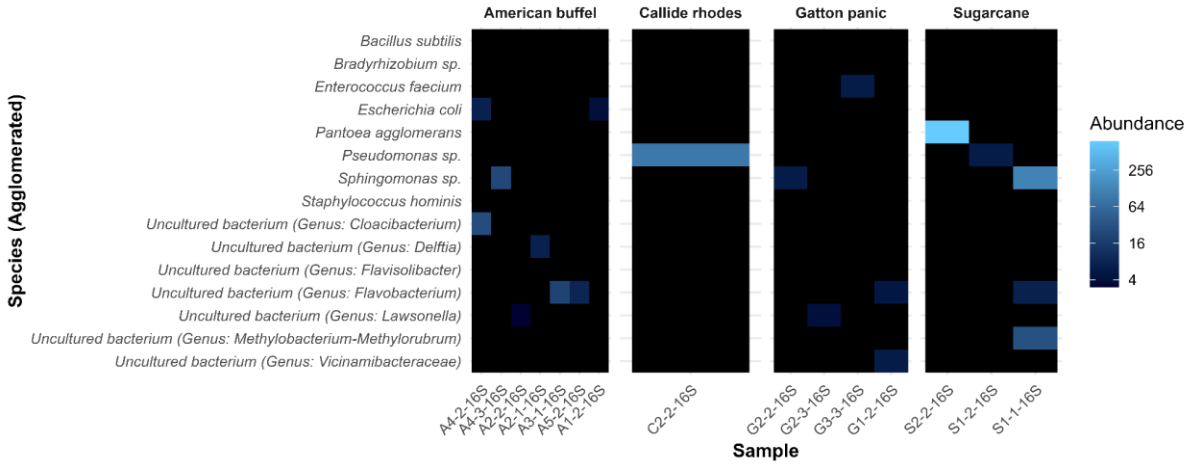

Figure 15. Heat map of ASVs (16S rRNA V3-V4) of minor bacterial species associated with *H. summervillei* mealybugs collected from different grass varieties. ASVs of the primary endosymbiont, *Ca.* Tremblaya, were removed from analysis to better visualise low abundance species and overall microbiome diversity. Note that this graph includes only 15 of the 63 total samples; in all other samples, only *Ca.* Tremblaya was detected. *Pantoea agglomerans* is the most abundant minor species and appears in only one sugarcane sample. *Sphingomonas* sp. and *Flavobacterium* sp. are the only common species between grass varieties.

Of these minor bacterial species, *Pantoea agglomerans* is the most abundant but was detected in only one sugarcane sample (S2-2-16S) (Figures 13 and 15). ASVs from *Sphingomonas* sp. and *Flavobacterium* sp. were detected in mealybug samples across three of the four grass varieties: American buffel, Gatton panic and sugarcane. American buffel and Gatton panic contain common ASVs from *Lawsonella* sp., whereas ASVs from *Pseudomonas* sp. are common across Callide rhodes and sugarcane.

There appear to be no significant differences in non-*Tremblaya* bacterial diversity (PERMANOVA, p=0.341) and there are no clear patterns in the reads indicating presence or abundance of non-*Tremblaya* taxa between grass varieties. Ordination highlights one standout American buffel sample (A4-2-16S) (Figure 16); however, this variation is likely explained by the presence of two major non-*Tremblaya* bacterial species (*Escherichia coli* and *Cloacibacterium* sp.) where most samples only have one. Given the low overall abundance of these bacterial species, this is unlikely to be a biologically significant result.

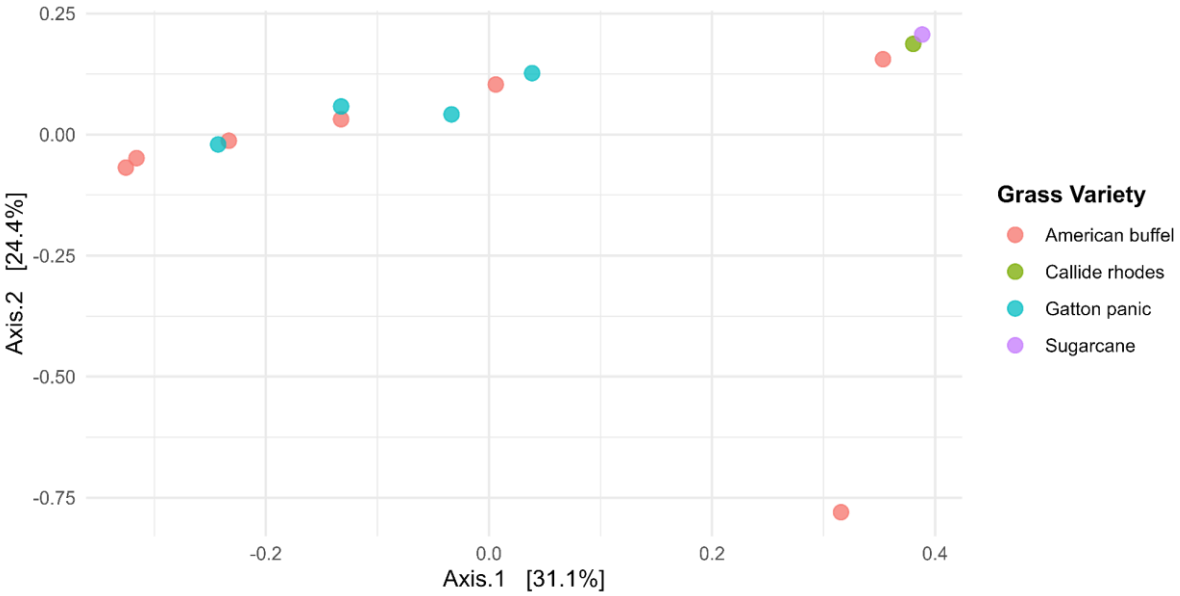

Figure 16. Ordination plot showing the results of beta diversity analysis on non-*Tremblaya* bacterial species, a subset of the 16S rRNA (V3-V4) data from *H. summervillei* reared on different grass varieties. Ordination has been performed using an MDS model and the weighted UniFrac distance method, as per Figure 14. The similar diversities between samples (i.e. low spatial proximity between points) likely reflects the extremely low abundance of non-*Tremblaya* bacteria – almost all samples are represented by only one major non-*Tremblaya* taxon, so all samples have similarly low diversities. One American buffel sample is the exception (bottom right); it contains multiple non-*Tremblaya* taxa and correspondingly falls outside the main cluster of sample points.

3.3.3 *Tremblaya*-specific diversity

59 unique *Ca.* Tremblaya ASVs were identified. There appears to be one main ASV, as indicated by its high abundance and presence in all samples, with many minor variants (Figure 17). Most samples have around 3-5 minor variants, aside from one sugarcane sample (S3-2-16S) that appears to have several more (at least 36 minor ASVs). Alpha diversity analyses reveal no significant difference in *Ca.* Tremblaya ASV diversity within individual mealybug samples (Kruskal-Wallis test, p=0.476) nor when samples are grouped by grass variety (p=0.404) and post-hoc pairwise comparisons confirm no underlying differences at either level (Wilcoxon rank sum test on Shannon indices, p>0.5).

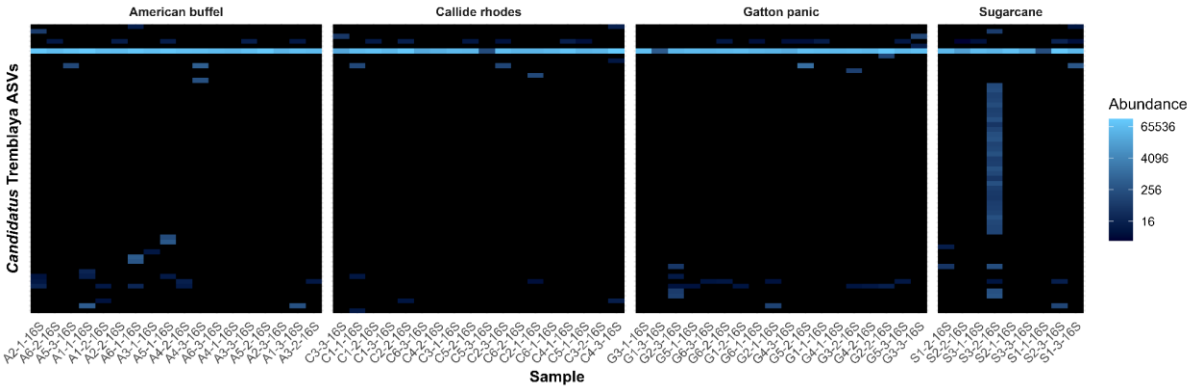

Figure 17. Heat map of *Ca.* Tremblaya ASVs (16S rRNA V3-V4) in *H. summervillei* samples collected from different grass varieties. Each row represents a unique ASV. There appears to be one 'main' ASV, present in high abundance in all samples, and many minor variants. About 3-5 minor variants are present in most samples; the only exception is sugarcane sample S3-2-16S, which appears to have several more minor ASVs.

Beta diversity analyses reveal no significant difference between grass varieties at the level of *Ca.* Tremblaya ASVs (PERMANOVA, p=0.085; pairwise PERMANOVA, p>0.100). Ordination shows two key clusters (Figure 18), the larger of which (left) represents the main *Ca.* Tremblaya ASV (present in all samples in high abundance) and the smaller (right) which is likely an ASV present in many samples but in lower abundance.

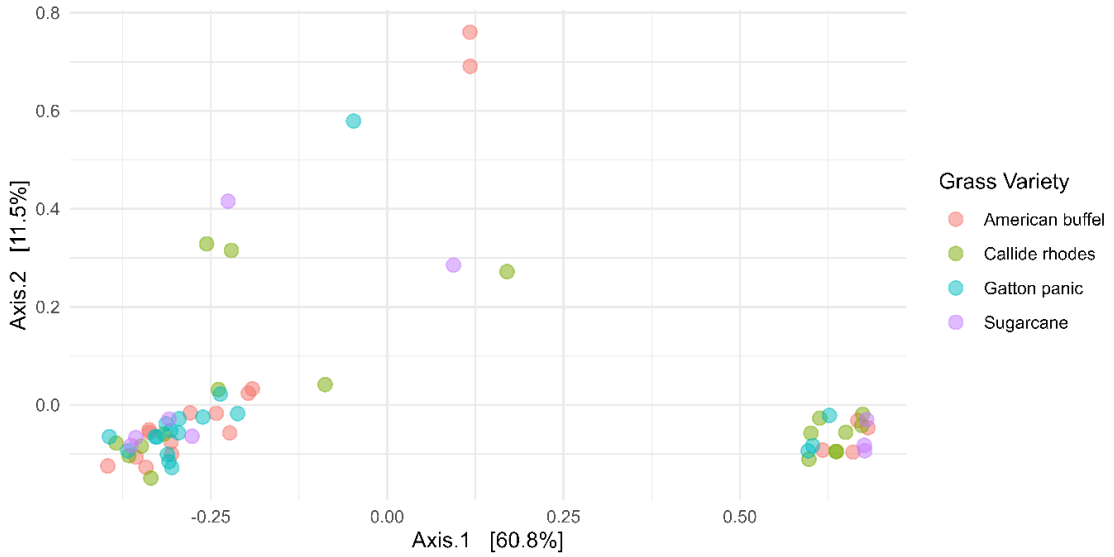

Figure 18. Ordination plot showing the results of beta diversity analysis on *Tremblaya*-specific ASVs, a subset of the 16S rRNA (V3-V4) data from *H. summervillei* reared on different grass varieties. Ordination has been performed using an MDS model and the weighted UniFrac distance method. Sample points are grouped into two key clusters that appear to represent the main *Ca*. Tremblaya ASV (left) present in all samples in high abundance and a minor variant (right) present in many samples in low abundance.

Multiple sequence alignment confirms that the *Ca.* Tremblaya ASV variants differ by number and type of single-nucleotide polymorphisms (SNPs), and only one ASV (the 'main' ASV) is present in all (n=63) samples (Figure 19).

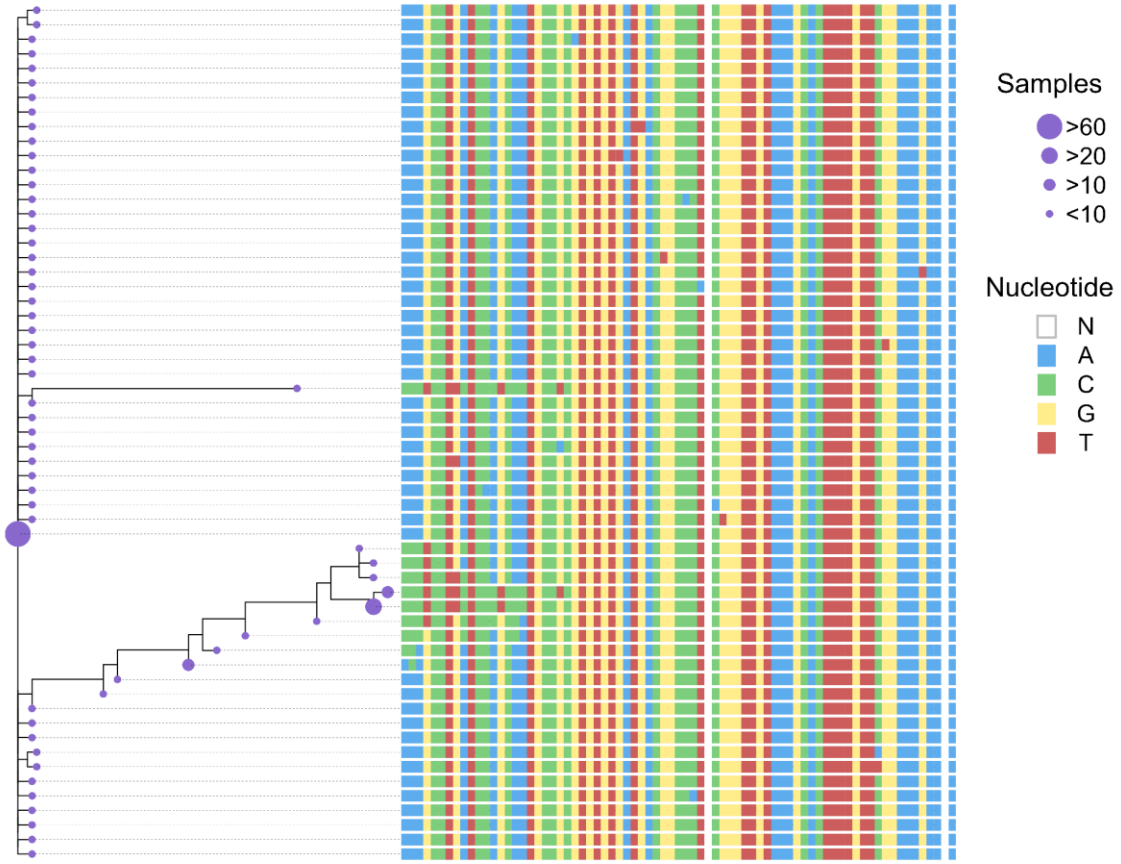

Figure 19. Phylogenetic tree of *Ca.* Tremblaya ASVs (16S rRNA V3-V4) with corresponding sequences. Each tip on the tree represents a unique ASV; the size of the tip points (purple) reflect the number of samples in which that ASV was identified. To better visualise ASV diversity, only nucleotide positions 25-100 are shown in the multiple sequence alignment, representing a region where sufficient difference was observed. Positions marked as 'N' delineate ambiguous base calls that could not be identified by the Illumina software; these have been included for completeness. One ASV appears in much greater abundance and is present in all samples (largest purple point).

3.4 Discussion

3.4.1 Low bacterial diversity across *H. summervillei* cohort

Unlike the rich microbial diversity seen in their plant hosts, some herbivorous insects possess microbiomes of limited diversity, but the reasons for this contrast are still under

investigation (Sugio et al., 2015). In this study, the diversity of bacteria detected in *H. summervillei* was particularly low, irrespective of host plant (section 3.3.1). The bacterial microbiome is dominated by *Ca*. Tremblaya (Figure 13), which is unsurprising given that *T. phenacola* is the obligate endosymbiont that performs critical amino acid biosynthesis to compensate for the nutrient-poor diet of the host insect (Gil et al., 2018; Husnik et al., 2013; Lopez-Madrigal et al., 2014).

Minor species in the *H. summervillei* microbiome are largely ubiquitous environmental bacteria commonly found in soil and natural water sources (*Bacillus subtilis*, *Bradyrhizobium*, *Cloacibacterium*, *Delftia*, *Flavisolibacter*, *Flavobacterium*, *Methylobacterium*, *Methylorubrum*, *Pseudomonas*, *Sphingomonas*, *Vicinamibacteraceae*), or in association with livestock (*Enterococcus faecium*, *Escherichia coli*, *Lawsonella*, *Staphylococcus hominis*) or plant roots (*Pantoea agglomerans*). *Bacillus* and *Staphylococcus* are common members of the gut microbiota in other *Pseudococcidae* like solanum mealybug (*Phenacoccus solani*) and passionvine mealybug (*Planococcus minor*), where they are thought to play a role in conferring insecticide resistance (Lin et al., 2019). In other phloemophagous insects like silverleaf whitefly, these bacteria are involved in reducing pathogenic microbes by maintaining the gut pH (Indiragandhi et al., 2010; Wu et al., 2014).

3.4.2 Core bacteria in the *H. summervillei* microbiome

This chapter defines ‘core’ bacteria as any taxa shared among mealybugs and consistently detected when reared on two or more grass varieties. This definition is widely thought to represent the most ecologically and functionally important microbial associates in the given host species under the experimental conditions (Neu et al., 2021). *Ca*. Tremblaya was the only taxon identified in all mealybug samples across all grass varieties and therefore constitutes the primary ‘core’ bacterium in *H. summervillei*.

Figure 15 identifies four potentially facultative/secondary bacterial symbionts, detected in multiple but not all mealybugs associated with the current outbreak of pasture dieback. These span four genera: *Sphingomonas*, *Flavobacterium*, *Pseudomonas* and *Lawsonella*.

Sphingomonas is a strictly aerobic, chemoheterotrophic genus that has been isolated from soils and plant roots, as well as many human clinical contexts (Sorouri et al., 2023). It is identified as a facultative secondary endosymbiont in papaya mealybug (*Paracoccus marginatus*) (Megaladevi et al., 2020) and a potential symbiont in birch bark scale (*Steingelia gorodetskia*) (Michalik et al., 2019b). *Sphingomonas* is also a key bacterial group in

solenopsis mealybug, though it was not one of the taxa associated with the disruption of the JA/SA pathway (Zhao et al., 2023). Recent work on the gut microbiome in cotton aphid (*Aphis gossypii*) suggests that *Sphingomonas* mediates host resistance to broad-spectrum neonicotinoid insecticides by utilising them as a carbon and nitrogen source (Lv et al., 2023). Although their role in other insect hosts has not been determined (Megaladevi et al., 2020; Michalik et al., 2019b; Zhao et al., 2023), molecular phylogenetics suggests that symbiotic *Sphingomonas* species may be a recent acquisition in scale insects (Hemiptera: Sternorrhyncha) based on their close similarity to the free-living soil bacterium *Sphingomonas echinoides* (Michalik et al., 2019b).

Sphingomonas species are also an important and typically beneficial component of the plant microbiome, and both biotic and abiotic stress may lead to an increase in their abundance as part of a stress-induced dysbiosis. Recent work on *Xylella fastidiosa* (Xanthomonadaceae) infection has shown an increase in *Sphingomonas* species in the plant microbiome that is associated with expression of symptoms in infected plants (Landa et al., 2022).

Flavobacterium species are commonly recovered from freshwater sources and rhizospheric soil, where their ability to digest inert organic matter plays an important role in geochemical cycling (Lee et al., 2023). Flavobacterial endosymbionts are thought to play a role in synthesising essential amino acids and have been found in several scale insect families, including the Diaspididae, in which they serve as primary endosymbionts (Gruwell et al., 2010; Rosas-Perez et al., 2014; Rosenblueth et al., 2012). Given that essential amino acid synthesis is fulfilled by *T. phenacola*, this may not be their primary function in *H. summervillei. Flavobacterium* may instead provide functional redundancy to compensate for compositional shifts in the microbial community associated with *H. summervillei* (Chen et al., 2022). An alternative theory is that insect-associated *Flavobacterium* species are involved in the degradation of insecticides, corresponding with their function in soil, and thus contribute to insecticide resistance of the host (Kikuchi et al., 2012).

Pseudomonas is known gut symbiont in at least three mealybug species: papaya mealybug (Krishnamoorthy et al., 2019; Megaladevi et al., 2020), citrus mealybug (Ibrahim et al., 2021) and cassava mealybug (*Phenacoccus manihoti*) (Harish & Aryalakshmi, 2022). Mealybug-associated *Pseudomonas* isolates demonstrate high proteolytic activity (Krishnamoorthy et al., 2019) and the capacity to degrade insecticides, specifically the organophosphate insecticide chlorpyriphos (Ibrahim et al., 2021) and the neonicotinoid insecticide thiamethoxam (Harish & Aryalakshmi, 2022). ASVs from *Pseudomonas* were common across Callide rhodes grass and sugarcane (Figure 15).

Mealybugs reared on American buffel and Gatton panic contain common ASVs from *Lawsonella*, a newly identified genus represented by the type species *Lawsonella clevelandensis* (Bell et al., 2016). Little is known about the distribution and function of *Lawsonella* beyond the role of *L. clevelandensis* in abscess formation in humans (Bell et al., 2016; Chudy-Onwugaje et al., 2020; Favila Menezes et al., 2018). *Lawsonella* species have been identified in the microbiomes of the cereal leaf beetle (*Oulema melanopus*) (Wielkopolan et al., 2021), turtle vein lady beetle (*Propylea japonica*) (Chang et al., 2023) and water scorpion (*Nepa rubra*) (Bektas, 2022), but their functions are unknown. Understanding the roles of rare taxa in the *H. summervillei* microbiome will require an approach that involves deeper functional characterisation.

The results of this study suggest that *Sphingomonas* and *Flavobacterium* may form part of community of potential facultative symbionts in *H. summervillei* microbiome (Figure 15). Sequence reads of *Sphingomonas* sp. and *Flavobacterium* sp. were observed in mealybug samples from American buffel, Gatton panic and sugarcane. Their absence in Callide rhodes may be a factor of small sample size in the diversity analyses of non-*Tremblaya* ASVs (section 3.3.2), where Callide rhodes was represented by only one sample (C2-2-16S). Further bacterial sequencing is required to confirm the presence of *Sphingomonas*, *Flavobacterium* and *Pseudomonas* in *H. summervillei* and determine their function in the mealybug, particularly whether they are involved mealybug-plant interactions.

This study did not detect the specific taxa identified in the salivary bacteria of solenopsis mealybug that disrupt JA/SA signalling (Zhao et al., 2023). However, there are similarities with bacteria known to affect JA/SA pathways in other insects. Modulation of JA-mediated defences in fall armyworm (*S. frugiperda*) was attributed to the presence of bacteria from five genera of the family Enterobacteriaceae, of which *Pantoea ananatis* (recently reclassified from *Enterobacteriaceae* to the family *Erwiniaceae*) was the only isolate identified to species level (Acevedo et al., 2017).

The small amount of research into the role of bacterial symbionts of insects on the JA/SA pathway of host plants suggests that several different secondary bacterial symbionts may be involved in the disruption of JA/SA pathways. In the Colorado potato beetle (*Leptinotarsa decemlineata*), induced plant defences were suppressed when at least one of three bacteria – *Stenotrophomonas*, *Pseudomonas* or *Enterobacter* – was present (Chung et al., 2013). The bacterial symbiont *Hamiltonella defensa* (*Enterobacteriaceae*) in silverleaf whitefly (*Bemisia tabaci*) saliva downregulates JA responses and upregulates SA responses, (Su et

al., 2015). Such findings imply that examination of the whole microbiome, with emphasis on Enterobacteriaceae and related taxa, is important to determine the ecology of potential bacterial symbionts that play a role in modulating plant defence responses.

Overall, the results of this study indicate that the bacterial communities associated with *H. summervillei* have extremely low diversity. Potential core bacteria other than *Ca.* Tremblaya and their role in the disruption of JA/SA pathways requires further investigation. Functional characterisation is recommended to understand the role and response of these bacteria in the mealybug and in mealybug-plant interactions, in the disruption of JA/SA pathways, and in the development of symptoms of pasture dieback.

3.4.3 Factors that may influence low bacterial diversity

Scale insects (Hemiptera: Sternorrhyncha) demonstrate great diversity in symbiotic associates (Szklarzewicz et al., 2020), even in scales from the same family (Buchner, 1965; Frago et al., 2020); however, overall gut bacterial diversity is relatively low (Malacrino, 2021; Yun et al., 2014). This appears to be true for *H. summervillei*: bacterial diversity is low (<16 species identified across the entire mealybug cohort) and the microbiome is dominated by *T. phenacola* (Figure 13), one of two known primary endosymbionts in the mealybug family.

Several factors may explain why the bacterial communities associated with *H. summervillei* are not overly diverse. Mealybugs feed exclusively on phloem, an oxygen-limited medium that is not conducive to microbial growth beyond microaerophiles, most of which are insect-vectored pathogens rather than resident microbes (Bendix & Lewis, 2018; Jing et al., 2014; Lewis et al., 2022). Since phloem is not a rich source of foodborne microbes, it may not have a significant impact on the host-adapted mealybug microbiome.

It is also important to note that the previously mentioned studies on plant-specific insect microbiomes investigate plant hosts from phylogenetically distant families - e.g. grapevine (*Vitaceae*) vs. potato (*Solanaceae*) (Iasur-Kruh et al., 2015). However, the host plants in this study are all within the grass family (*Poaceae*). Plants in the same family tend to share similar bacterial community compositions, and microbiome diversity is shown to become more distinct with increased phylogenetic distance (Lei et al., 2019). If the host plants here share a similar microbial community composition, differences in the host-adapted mealybug microbiomes may not be easily identifiable in the data. Despite their demonstrated polyphagy and successful adaptation to the different host plants in this experiment, *H. summervillei* may

not exhibit host-specific changes in bacterial diversity due to the inherent lack of microbes in phloem sap and/or potential similarities in transmissible host grass microbiota.

Another explanation may be that the structure and composition of the *H. summervillei* microbiome is influenced by the obligate symbiont, *T. phenacola*. Although insect-associated bacteria are more commonly recognised for their impacts on host nutrition and reproduction (Gupta & Nair, 2020; Singh et al., 2021), symbiotic bacteria in some insects are also known to regulate critical biological processes. Examples include larval development (Girard et al., 2023), metabolic homeostasis (Salem et al., 2014), innate immunity (Weiss et al., 2012), and microbiome assemblage (Douglas, 2014). Consider *Burkholderia* in bean bugs (*Riptortus pedestris*): *Burkholderia* is speculated to have become the primary symbiont by possessing features that facilitate establishment in the host (e.g. flagellar motility, resistance to host antimicrobial responses, ability to combat host-induced stress) (Kim et al., 2013; Lee et al., 2015) and is thought to remain the primary symbiont by upregulating antimicrobial responses to non-self bacteria (Futahashi et al., 2013; Mason, 2020; Salem et al., 2014). If *T. phenacola* exerts a similar antagonistic effect in the *H. summervillei* microbiome, this may explain the lack of abundance and consistency of any non-*Tremblaya* species (potential facultative symbionts) across the mealybug cohort and between replicates from the same host plant (Figures 13 and 15). Additionally, hosting facultative symbionts may incur an unsatisfactory cost-benefit trade-off. For example, some aphid species host symbionts that confer resistance against parasitic wasp attack, but this comes at the cost of reduced fecundity and longevity (Zytynska et al., 2021).

It is essential to recognise that this study focuses on bacterial microbiota, the most studied facet of insect microbiome research (Gurung et al., 2019). Microbiomes can comprise many more kingdoms (fungi, viruses, archaea, protozoa) that may have significant influence on the life history of the host insect. For instance, nutritional symbioses with specific bacterial lineages are found in all major sap-feeding soft scale families (Dahan et al., 2015) except the Coccidae, which appears to be dominated by an *Ophiocordyceps*-allied fungal symbiont rather than a bacterial symbiont (Gomez-Polo et al., 2017). Although research on fungal symbiosis in insects (amongst other symbioses) is still developing (Barcoto et al., 2020; Watson et al., 2022; Van Moll et al., 2021), it is highly recommended that future work on the *H. summervillei* microbiome involve screening for non-bacterial microbiota and characterisation of their roles in pasture dieback. Further recommendations include longer adaptation time for mealybugs on new plant hosts to examine whether the microbiome changes with prolonged and multigenerational exposure.

3.4.4 Host switching mechanisms in *H. summervillei*

The mechanism/s of host switching in *H. summervillei* are not apparent at the level of the microbiome in this study. Some variation in the host-adapted bacterial microbiome was expected, given the wide host range of pasture mealybugs (section 1.2.2) and the known influence of dietary changes on microbiome composition and diversity (Huang et al., 2021; Jones et al., 2019). However, only minor variations in bacterial diversity were observed across the entire cohort, with no significant differences between mealybugs from different host plants (section 3.3.1). The results of this research indicate that host plant suitability does not appear to affect bacterial composition and diversity in *H. summervillei* associated with the current outbreak of pasture dieback.

A higher degree of bacterial diversity was expected based on studies of differential colonisation of gut microbiota by food source (Huang et al., 2021; Jones et al., 2019), as well as transient or facultative symbionts acquired through horizontal transmission as the mealybugs adapted to each grass variety (Henry et al., 2013; Iasur-Kruh et al., 2015; Lin et al., 2019). Facultative (i.e. non-obligate) mealybug symbionts are relatively understudied compared to obligate symbionts like *Ca*. Tremblaya (see section 1.2.3 for further details on mealybug primary symbionts) but it is hypothesised that, when colonising new host plants, they would likely aid insect fitness through functions like the detoxification of plant chemical defences (Brady & White, 2013; Iasur-Kruh et al., 2015). This is seen in the polyphagous vine mealybug (*Planococcus ficus*) (Iasur-Kruh et al., 2015), which demonstrates markedly different microbiomes on grapevine (the preferred host) compared to potato (a common alternative host) (Cocco et al., 2021; Schulze-Sylvester et al., 2021) and suggests that host plant adaptation is associated with changes in microbial community structure. Studies on other polyphagous plant pests (e.g. cowpea aphid, cabbage looper, cotton aphid) further indicate that microbiome diversity is conditional to the host plant (Brady & White, 2013; Leite-Mondon et al., 2021; Xu et al., 2020).

Dietary adaptation often involves a cascade of changes to ensure survival in a new ecological niche (Ashra & Nair, 2022; Sudakaran et al., 2017). A host-switching insect not only needs to adapt to a new food source with a different nutrient profile – it may need to overcome a new array of host plant defences, as well as mitigate interactions with new predators or competitors. Phenotypic plasticity (the ability to produce multiple distinct phenotypes from the same genome) and transient symbioses (the acquisition of temporary microbial symbionts to enhance fitness) are common phenomena in host-switching generalists that help facilitate adaptation to multiple hosts (Santos-Garcia et al., 2020).

Neither of these strategies appear to be at play here, but *H. summervillei* possesses another trait that may explain its capacity to switch hosts.

Their effect on JA/SA signalling (Hauxwell et al., 2022d; Munro & Hauxwell, 2023) suggests that *H. summervillei* has the capacity to exploit phytohormone crosstalk to promote their own virulence. If *H. summervillei* can systematically dismantle plant defence pathways in this manner, it would negate the need for strategies like phenotypic plasticity or transient symbioses to succeed on new plant hosts. It is possible that *T. phenacola* in *H. summervillei*, with or without interactions with secondary symbionts, may disrupt the JA/SA pathways of the host grasses.

Further sequencing and network analysis of the *T. phenacola* genome with gene expression and transcriptome analyses are required to understand if the primary mealybug endosymbiont influences plant response. Network analysis may also provide insight into the interactions of *H. summervillei* and *T. phenacola* with potential secondary symbionts, in the context of JA/SA pathway disruption and induced dysbiosis in plant microbial communities. Future research could also examine molecular markers of the JA/SA pathways and measure the phytohormones directly to identify how *H. summervillei* is able to regulate plant defence mechanisms. It may also be worthwhile to investigate ethylene pathways (also involved in plant defence), which can be synergistic to the JA pathway in host plant responses to necrotrophs.

Chapter 4: Biogeography and the *H. summervillei* bacterial microbiome

4.1 Aims

Bacterial diversity in *H. summervillei* between geographic populations

Chapter 4 examines mealybug-associated bacterial diversity across the geographical range of *H. summervillei* in Australia and aims to determine whether geography affects the mealybug microbiome. Targeted next generation sequencing ('metabarcoding') of the V3-V4 region of the bacterial 16S gene was used to profile bacterial diversity in adult female *H. summervillei* specimens from dieback-affected sites across the current outbreak area. This work builds on the baseline bacterial community composition data established in chapter 3.

Like other invasive species, mealybugs from geographically separated populations are posited to develop genetic adaptations that reflect the environmental conditions and indirectly affect microbial composition and diversity (Lin et al., 2019; Wu et al., 2015). Current knowledge on mealybug diversity across geographic distributions is limited to studies based on the host genome, using molecular markers that are common in insect ecology like COI, 28S and ITS (Kaur & Singh, 2020). Intraspecific genetic variation among geographic populations is apparent in multiple Pseudococcidae systems, for example: cassava mealybug (Nopriawansyah et al., 2019), cotton mealybug (Thomas & Ramamurthy, 2014), obscure mealybug (Pseudococcus viburni) (Correa et al., 2015) and vine mealybug (Daane et al., 2018). These studies do not conclusively attribute genetic variation across mealybug distributions to any one factor; however, geography, climate and host plant selection are thought to be key influencing factors (Correa et al., 2015).

Various other hemipteran species demonstrate geographic patterns in their bacterial microbiome. The green peach aphid (*Myzus persicae*) exhibits regional variation in secondary symbionts within and between populations from Australia, Asia, Europe and Africa (Yang et al., 2023). Secondary symbionts were notably absent in global aphid populations compared to aphids in the native range of China, but the reasons for this are not clear (Yang et al., 2023). At a more localised scale, glassy-winged sharpshooters (*Homalodisca vitripennis*) from different Texas vineyards show differences in bacterial community

composition that correspond with geographic location (Welch et al., 2015). Despite this, vineyard proximity was not correlated with microbiome similarity (Welch et al., 2015), which indicates that other factors likely play a role in shaping microbiome composition. Additionally, many of the host-associated bacteria identified in this study were previously known to inhabit soil and plants exclusively, suggesting that the environment is a major source of facultative symbionts.

Although mealybugs demonstrate high capacity for passive dispersal by wind and water, they have limited capacity for active dispersal (i.e. movement through their own ability), generally only moving short distances (e.g. from plant to soil, or plant to plant) within a localised area (DAFF, 2013; Hauxwell, 2018). This implies that mealybugs are particularly susceptible to the effects of isolation by distance and may accrue local variation under geographically limited dispersal (Paddock et al., 2022). Altogether, these factors suggest that bacterial composition and diversity in *H. summervillei* may vary across a spatial scale. Corresponding with this hypothesis, chapter 4 aims to describe bacterial community composition in geographic populations of *H. summervillei* and determine whether there are differences in bacterial diversity across the geographic range of *H. summervillei* in Australia.

The QUT pasture dieback research group has collected *H. summervillei* samples across Queensland and northern New South Wales, dating back to July 2018. *H. summervillei* specimens have been identified by morphological (Biosecurity Queensland, 2022) and molecular examination (Dickson et al., 2023). A subset of this mealybug collection has been studied in this chapter. Samples have been selected to be as geographically comprehensive as possible, covering the known range of *H. summervillei* in eastern Australia.

Characterisation of *H. summervillei* biotypes

H. summervillei exhibits two known biotypes (variant phenotypes with similar or identical genotypes), as confirmed by 18S/28S DNA sequencing (Dickson et al., 2023) and light microscopy (Biosecurity Queensland, 2022). Morphologically, these biotypes differ by the presence or absence of translucent nodules on the hind tibia (Figure 20) (Schutze et al 2019, Biosecurity Queensland, 2022). The presence of translucent nodules is consistent with the description of the first recorded *H. summervillei* specimens collected in 1926 (Brookes, 1978) (referred to here as the ‘old’ biotype) but are absent in mealybugs collected from current (2015-present) outbreak (the ‘new’ biotype) (Biosecurity Queensland, 2022). New biotype mealybugs have been identified at all dieback-affected sites in the current outbreak (Hauxwell et al., 2022d). At present, Kin Kin (QLD) is the only known location of the old

biotype; a mixed population of old and new biotype mealybugs have been found here, not far (~25km) from the site of the original old biotype samples collected in 1926 (Brookes, 1978).

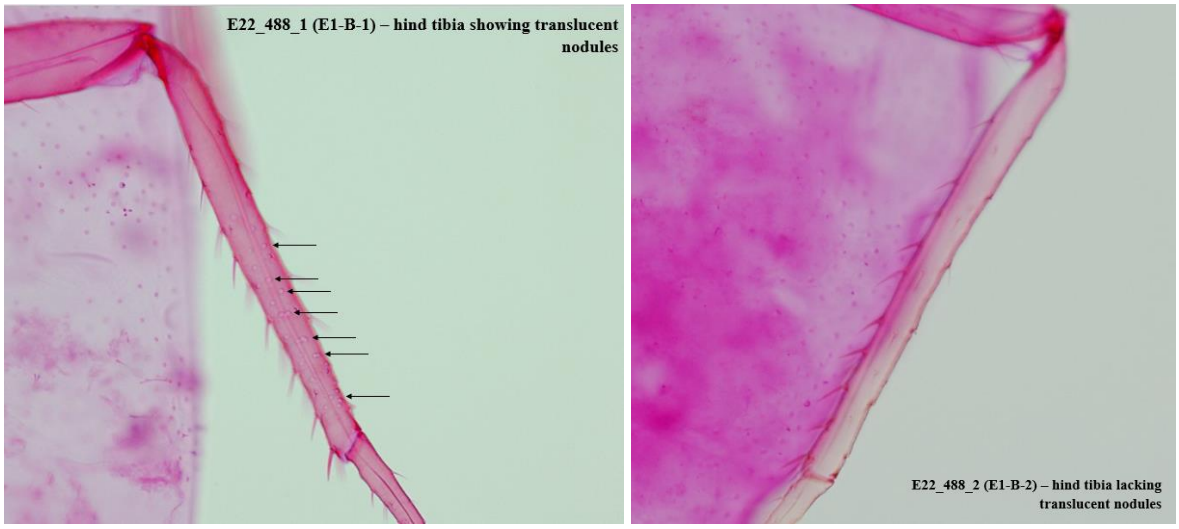

Figure 20. *H. summervillei* specimens with (left) and without (right) translucent nodules on the hind tibia, collected from *Setaria* grass in Kin Kin in 2020. The presence of translucent nodules (black arrows) is consistent with the description of specimens collected in 1926 (Brookes, 1978). These samples (E1B-1 and E1B-2) were slide mounted, stained and examined in August 2022 (Biosecurity Queensland, 2022). (Photos: M. Schutze, DAF)

Metabarcoding using a combination of the V3-V4 hypervariable regions of the bacterial 16S gene has the potential to resolve taxa to species level (Li et al., 2020) for most bacterial groups (Santos et al., 2020). Amplicon sequencing of 16S subregions has been used in the molecular characterisation of multiple hemipteran taxa. It is frequently used in studies to differentiate members of polymorphic species complexes, such as silverleaf whitefly (Gorsane et al., 2011; Lee & de Barro, 2000; Bing et al., 2013) and pea aphid (*Acyrthosiphon pisum*) (Gauthier et al., 2015; Leclair et al., 2016; Russell et al., 2013). This approach has been used in this chapter to generate novel data on bacterial community composition and diversity in the old and new *H. summervillei* biotypes and identify whether biotype differences are seen at the level of the microbiome. It focuses on differences in sequences from the primary endosymbiont, *T. phenacola*, to explore the use of symbionts in *H. summervillei* biotype characterisation. A symbiont-based approach could lend support to the host-based morphological and molecular methods that are currently being used to identify this species.

4.2 Methods

4.2.1 Sample Collection

Adult female *H. summervillei* samples were taken from material collected by the QUT pasture dieback research group from dieback-affected sites across Queensland and New South Wales from 2020 to 2022 (Figure 21) (Hauxwell et al., 2022d).

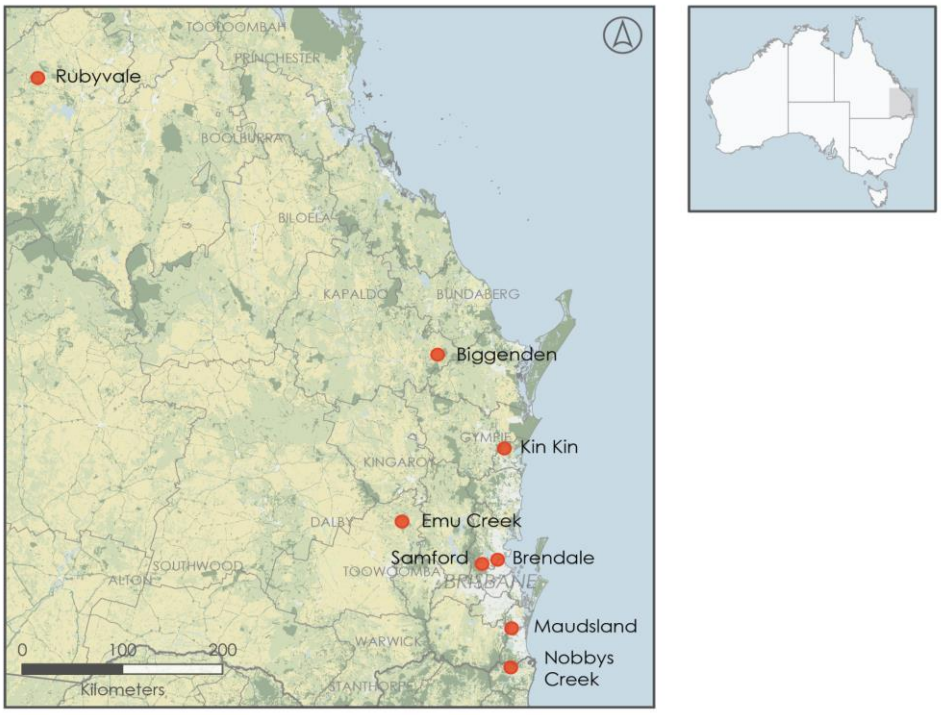

Figure 21. *H. summervillei* samples were collected from dieback-affected sites across South East Queensland and northern New South Wales, Australia. (Image: S. Richards, QUT)

H. summervillei samples were characterised into two biotypes, ‘old’ (similar to the 1926 mealybug described by Summerville) and ‘new’ (associated with the current pasture dieback outbreak). Ch was based on the presence (old biotype) or absence (new biotype) of translucent nodes on the hind tibia (Figure 20) (Biosecurity Queensland, 2022; Brookes, 1978; Schutze et al., 2019).

Samples from four (4) outgroups were included for comparison with *H. summervillei*: *Saccharicoccus sacchari* (sugarcane mealybug, A35), *Hypogeococcus festerianus* (Harrisia cactus mealybug, D7), *Coccus longulus* (long brown scale, JH3) and *Antonina graminis* (Rhodes grass mealybug, RG1-11). Sample metadata is presented in table 6.

Table 6. *H. summervillei* sample metadata, including sample ID, location, collection date, and biotype (mealybug or outgroup). Samples are ordered alphabetically by location.

Sample ID	Location	Collection Date	Biotype (Mealybug or Outgroup)
BG-3a-16S	Biggenden, QLD	26/03/2021	Pasture mealybug – new biotype
BG-TN-16S	Biggenden, QLD	26/11/2020	Pasture mealybug – new biotype
BD-1a-16S	Biggenden, QLD	12/10/2020	Pasture mealybug – new biotype
BG-UQ-16S	Biggenden, QLD	18/02/2021	Pasture mealybug – new biotype
BG-16S	Biggenden, QLD	Unknown	Pasture mealybug – new biotype
JH3-16S	Biloela, QLD	25/10/2019	Long brown scale – outgroup
RG1-11-16S	Birkdale, QLD	04/09/2018	Rhodes grass mealybug – outgroup
BR-7-16S	Brendale, QLD	22/06/2020	Pasture mealybug – new biotype
D7-16S	Dingo, QLD	30/10/2019	Cactus mealybug – outgroup
EC-1a-16S	Emu Creek, QLD	24/03/2022	Pasture mealybug – new biotype
EC-3-16S	Emu Creek, QLD	17/05/2022	Pasture mealybug – new biotype
EC-2-16S	Emu Creek, QLD	17/05/2021	Pasture mealybug – new biotype
E8b-16S	Fairfield, QLD	19/01/2020	Pasture mealybug – new biotype
K2-1-16S	Kin Kin, QLD	06/01/2021	Pasture mealybug – new biotype
K-63-16S	Kin Kin, QLD	20/06/2020	Pasture mealybug – new biotype
JL-1-16S	Kin Kin, QLD	03/03/2021	Pasture mealybug – new biotype
KK-20-16S	Kin Kin, QLD	03/04/2021	Pasture mealybug – new biotype
K-61-16S	Kin Kin, QLD	20/06/2020	Pasture mealybug – new biotype
KK1-1-16S	Kin Kin, QLD	19/06/2020	Pasture mealybug – old biotype
E1B-1-16S	Kin Kin, QLD	24/02/2020	Pasture mealybug – new biotype
E1B-2-16S	Kin Kin, QLD	24/02/2020	Pasture mealybug – old biotype
MC-6-16S	Maudsland, QLD	19/01/2021	Pasture mealybug – new biotype
MD-9-16S	Maudsland, QLD	02/03/2021	Pasture mealybug – new biotype
MC-9-16S	Maudsland, QLD	02/03/2021	Pasture mealybug – new biotype
MD-7-16S	Maudsland, QLD	05/01/2021	Pasture mealybug – new biotype
MC-5-16S	Maudsland, QLD	05/01/2021	Pasture mealybug – new biotype
MD-8-16S	Maudsland, QLD	14/01/2021	Pasture mealybug – new biotype
UNE-16S	Rubyvale, QLD	22/05/2022	Pasture mealybug – new biotype
SE-PD-4-16S	Samford, QLD	04/12/2020	Pasture mealybug – new biotype
SE-PD-1-16S	Samford, QLD	09/08/2021	Pasture mealybug – new biotype
S1-1-16S	Samford, QLD	10/02/2022	Pasture mealybug – new biotype
S2-2-16S	Samford, QLD	10/02/2022	Pasture mealybug – new biotype
SP-1-16S	Nobbys Creek, NSW	02/07/2020	Pasture mealybug – new biotype
SP-9-16S	Nobbys Creek, NSW	02/03/2021	Pasture mealybug – new biotype
A35-16S	Yerra, QLD	19/07/2019	Sugarcane mealybug – outgroup

Mealybug samples were originally collected in propylene glycol and maintained at room temperature for long-term storage as part of the QUT mealybug collection. Samples were transferred to 70% ethanol and stored at -20°C until required for further processing.

4.2.2 Sample Processing

Pretreatment, DNA extraction and sequencing were performed as per sections 3.2.2-3.2.4.

4.2.3 Microbiome Analysis

Microbiome analyses were performed as per section 3.2.5. Additional phylogenetic reconstruction was performed in the IQ-TREE web server (based on v1.6.12; Nguyen et al., 2014; Trifinopoulos et al., 2016). Phylogenies were inferred by maximum likelihood under a K2P+G4 nucleotide substitution model, as determined by ModelFinder (Kalyaanamoorthy et al., 2017), using 100 bootstrap replicates.

4.3 Results

4.3.1 Total Microbiome Diversity

Bacterial diversity within individual samples was again low, with the most diverse samples only containing only 5 unique taxa. *Ca.* Tremblaya was the only taxon identified across all *H. summervillei* samples, and was the most abundant taxon in all but one non-outgroup sample (KK1-1) (Figure 22). Most other identified taxa appear to be ubiquitous environmental organisms that are commonly isolated from soil, plant roots, natural water sources and agricultural leachate. These findings are consistent with those in section 3.3.1.

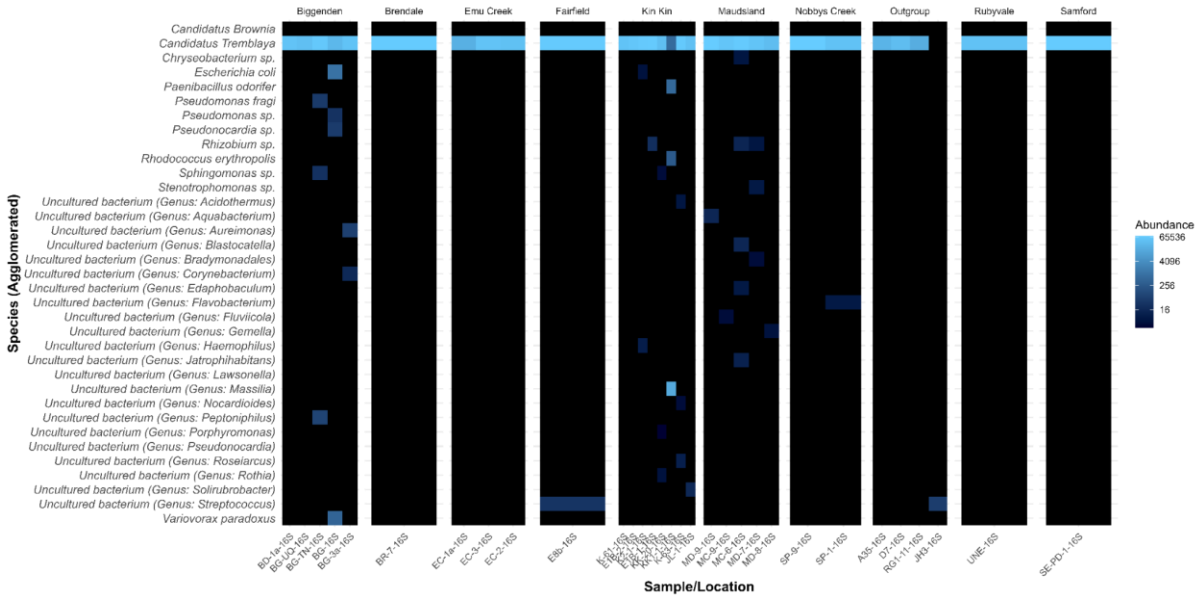

Figure 22. Heat map of ASV abundance across *H. summervillei* samples from dieback-affected sites across South East Queensland and northern New South Wales. ASVs are agglomerated at the species level for clarity; ASVs not assigned at the species level are reported to the lowest taxonomic depth possible. *H. summervillei* samples are grouped by geographical location; outgroup samples are grouped together for ease of comparison with the mealybug of interest. *H. summervillei* samples are dominated by *Ca.* Tremblaya – it represents over 60,000 reads in each sample, whereas most other bacterial taxa are represented by less than 4,000 reads.

Alpha diversity analyses indicate no significant difference in microbiome diversity within individual samples (Kruskal-Wallis test, p=0.466) or within samples grouped by location (p=0.371) or biotype (p=0.360). Post-hoc pairwise comparisons confirm no underlying differences (Wilcoxon rank sum test on Shannon indices, p>0.1).

Microbiome diversity is low across much of the cohort, aside from one sample collected at Kin Kin, identified through morphology and host sequence data as the old *H. summervillei* biotype (Biosecurity Queensland, 2022; Dickson et al., 2023) (Figure 23). Correspondingly, beta diversity analyses indicate a statistically significant difference in microbiome diversity between samples from Kin Kin vs. other locations (PERMANOVA, p=0.017), and between the old and new *H. summervillei* biotype (PERMANOVA, p=0.015). Post-hoc pairwise comparisons reveal variation in microbiome diversity between the new and old *H. summervillei* biotypes found at Kin Kin (pairwise PERMANOVA, p=0.040), but no difference in the new biotypes at Kin Kin vs. other locations (pairwise PERMANOVA, p=0.681).

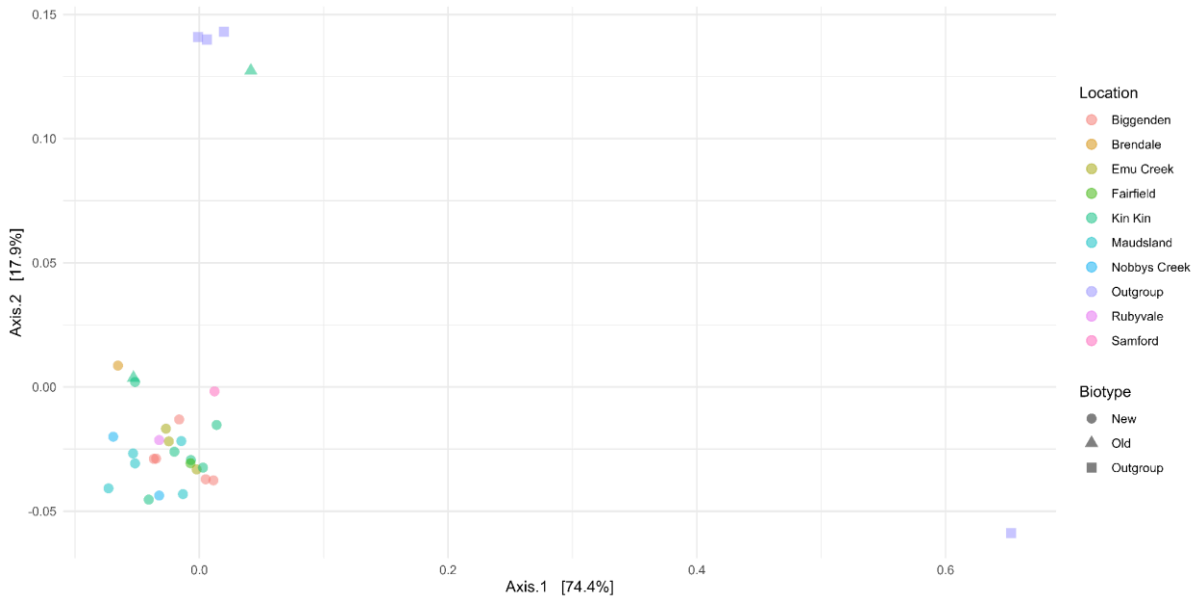

Figure 23. Results of beta diversity analysis on 16S rRNA (V3-V4) extracted from adult female *H. summervillei* samples collected from dieback-affected sites across South East Queensland and northern New South Wales, Australia. Ordination has been performed using an MDS model and the weighted UniFrac distance method. The amount of variation captured by each axis (%) is noted. The spatial distance between sample points reflects the differences in beta diversity - the closer two points are in ordination space, the more similar the microbial diversity in those samples. Clustering patterns indicate clear differences in bacterial diversity between the new biotype (bottom left) and the old biotype (top left).

4.3.2 Non-*Tremblaya* Microbiome Diversity

Ca. Tremblaya was again the dominant taxon across all samples (Figure 22) and microbial diversity is better visualised when it is removed from analysis, as demonstrated in Figure 24. Alpha diversity analyses indicate that within-group microbiome diversity is not significantly different within individual samples (Kruskal-Wallis test, p=0.453) or when samples are grouped by location (p=0.517) or biotype (p=0.479).

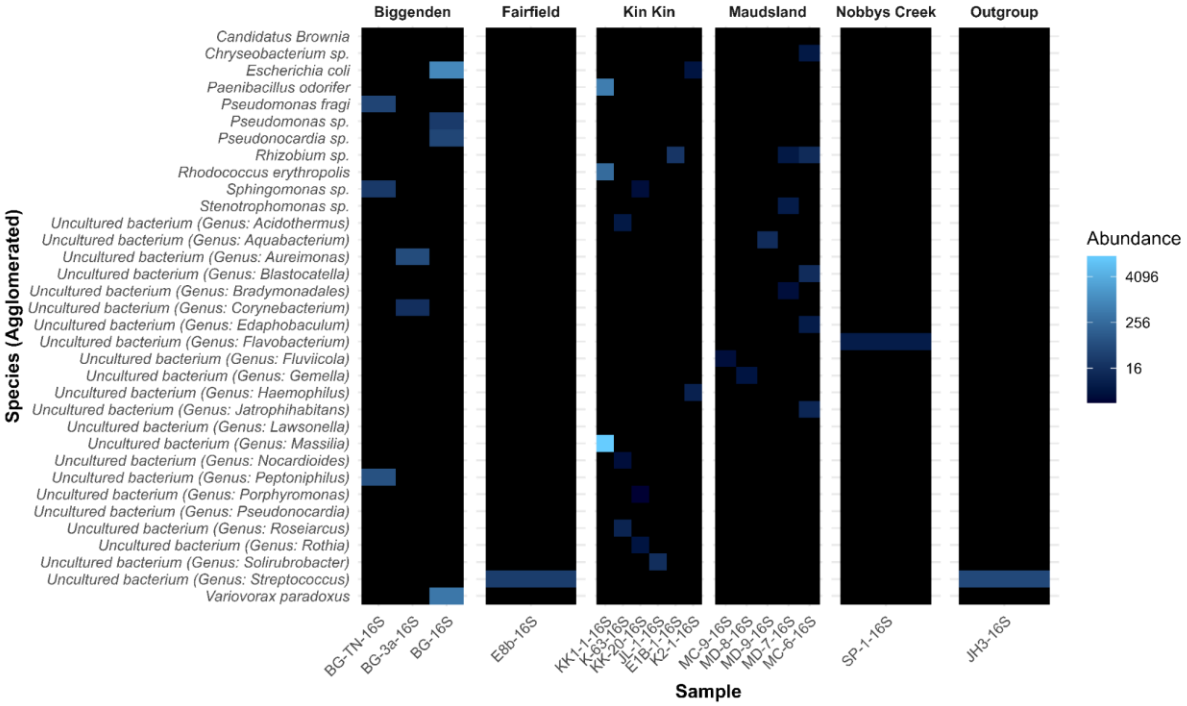

Figure 24. Heat map of ASVs (16S V3-V4) of minor bacterial species observed in the microbiome of *H. summervillei* collected from dieback-affected sites across South East Queensland and northern New South Wales, Australia. ASVs of the primary endosymbiont, *Ca.* Tremblaya, were removed from analysis to better visualise low abundance species and overall microbiome diversity. Note that this graph shows a subset of the total samples; in the excluded samples, only *Ca.* Tremblaya was detected. There are no appreciable patterns in the presence or abundance of non-*Tremblaya* taxa across the cohort.

Ordination reveals no identifiable patterns or grouping amongst samples when *Ca.* Tremblaya is removed from analysis (Figure 25). There is no significant difference in beta diversity when samples are grouped by location (PERMANOVA, p=0.274) or biotype (p=0.502). Post-hoc pairwise comparisons reveal no underlying differences within either grouping (pairwise PERMANOVA, p>0.05).

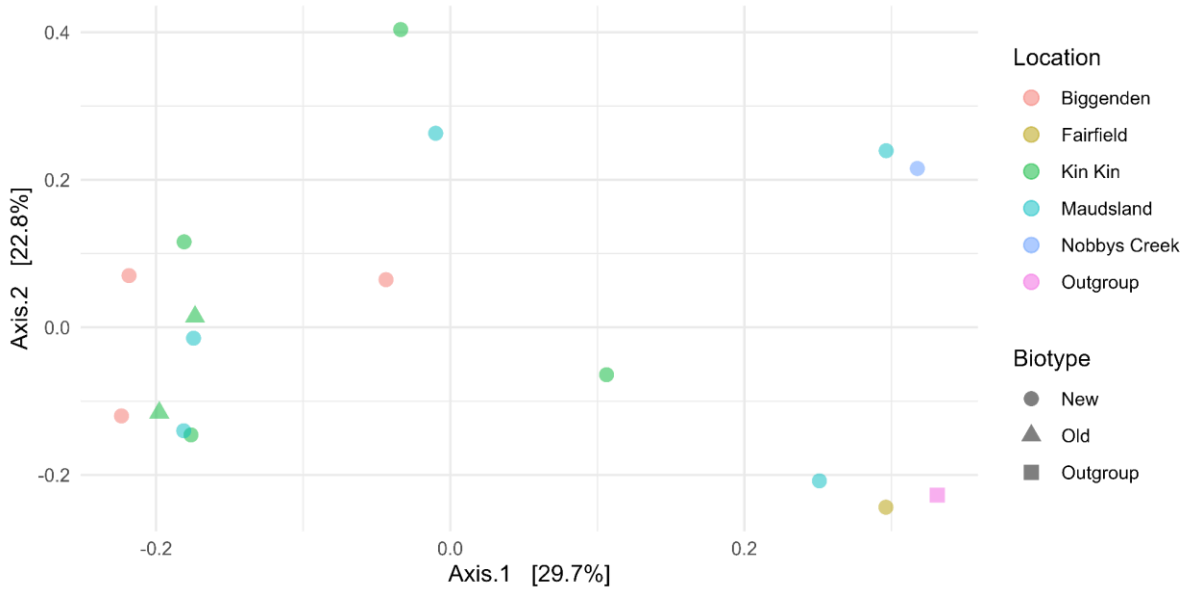

Figure 25. Ordination plot showing the results of beta diversity analysis on non-*Tremblaya* bacterial species, a subset of the 16S rRNA (V3-V4) data from *H. summervillei* samples collected from dieback-affected sites across South East Queensland and northern New South Wales, Australia. Ordination has been performed using an MDS model and the weighted UniFrac distance method. There are no appreciable differences in non-*Tremblaya* bacterial diversity between samples.

4.3.3 *Tremblaya*-Specific Diversity

27 unique *Ca.* Tremblaya ASVs were identified. Two (2) ASVs were found in high abundance across most samples, with the remaining 25 ASVs representing multiple minor variants (Figure 26). Alpha diversity analyses indicate no statistically significant variation in *Ca.* Tremblaya ASV diversity within individual samples (Kruskal-Wallis test, p=0.465), nor when samples are grouped by location (p=0.634) or biotype (p=0.686).

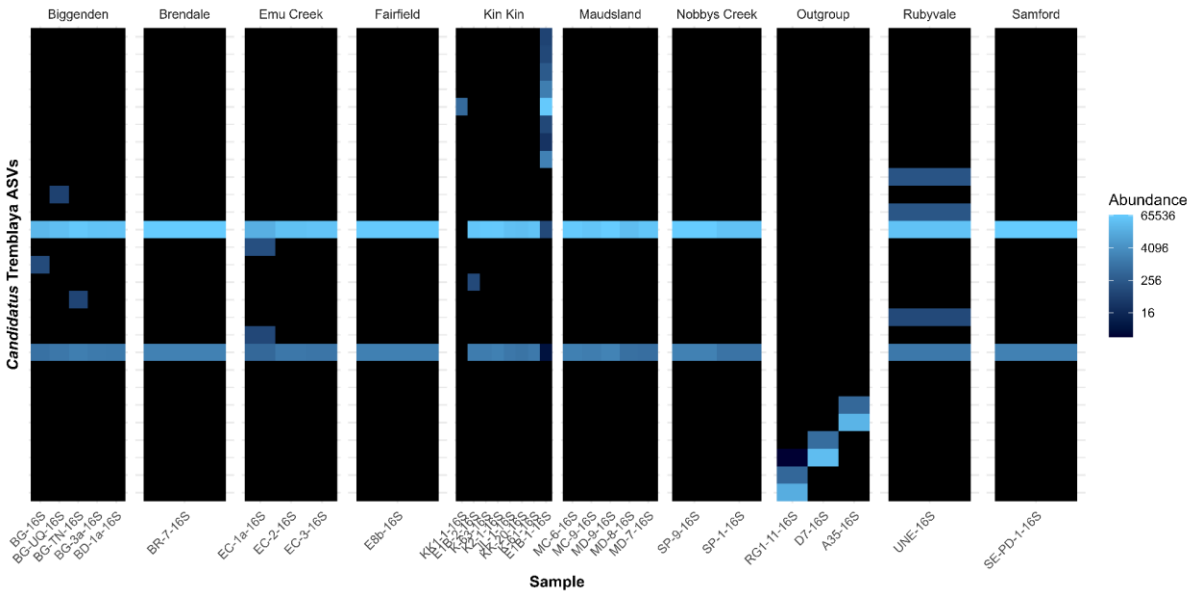

Figure 26. Heat map of *Ca.* Tremblaya ASVs (16S V3-V4) in *H. summervillei* samples collected from dieback-affected sites across South East Queensland and northern New South Wales, Australia. Each row represents a unique ASV. Samples are partitioned by geographic location; outgroup samples have been placed together for ease of comparison with *H. summervillei* samples. Two (2) ASVs are in high abundance across most samples; the remaining ASVs appear to represent minor variants.

Beta diversity analyses indicate significant variation in *Ca.* Tremblaya ASV diversity between samples from different locations (PERMANOVA, p=0.003), specifically between the ‘old’ and ‘new’ *H. summervillei* biotypes collected at Kin Kin (p=0.001). The *Ca.* Tremblaya ASVs from two old biotype samples collected at Kin Kin are significantly different from those in the new biotype (PERMANOVA, p=0.003). However, the *Ca.* Tremblaya ASVs from new biotype samples also collected at Kin Kin are not significantly different from those in new biotype samples at all other locations (pairwise PERMANOVA, p=0.956). These findings are reflected in the results of ordination (Figure 27), which show *Ca.* Tremblaya ASVs from the old biotype clustered together within the larger spread of ASVs from the new biotype.

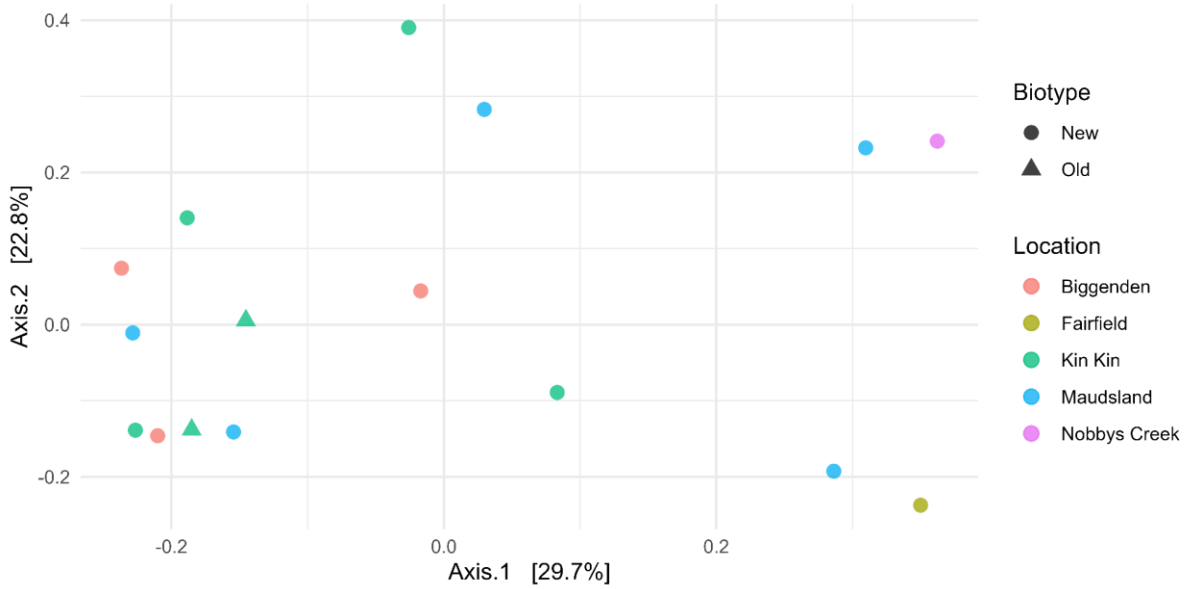

Figure 27. Ordination plot showing the results of beta diversity analysis on *Tremblaya*-specific ASVs, a subset of the 16S rRNA (V3-V4) data from *H. summervillei* samples collected from dieback-affected sites across South East Queensland and northern New South Wales, Australia. Ordination has been performed using an MDS model and the weighted UniFrac distance method. *Ca.* Tremblaya ASVs from the old *H. summervillei* biotype are clustered together within the larger spread of ASVs from the new biotype.

Phylogenetic reconstruction shows *Ca.* Tremblaya ASVs grouped according to host biotype (Figure 28). Two (2) ASVs were present in all locations where *H. summervillei* was found, though not in all samples from each location (Figure 28, new biotype clade).

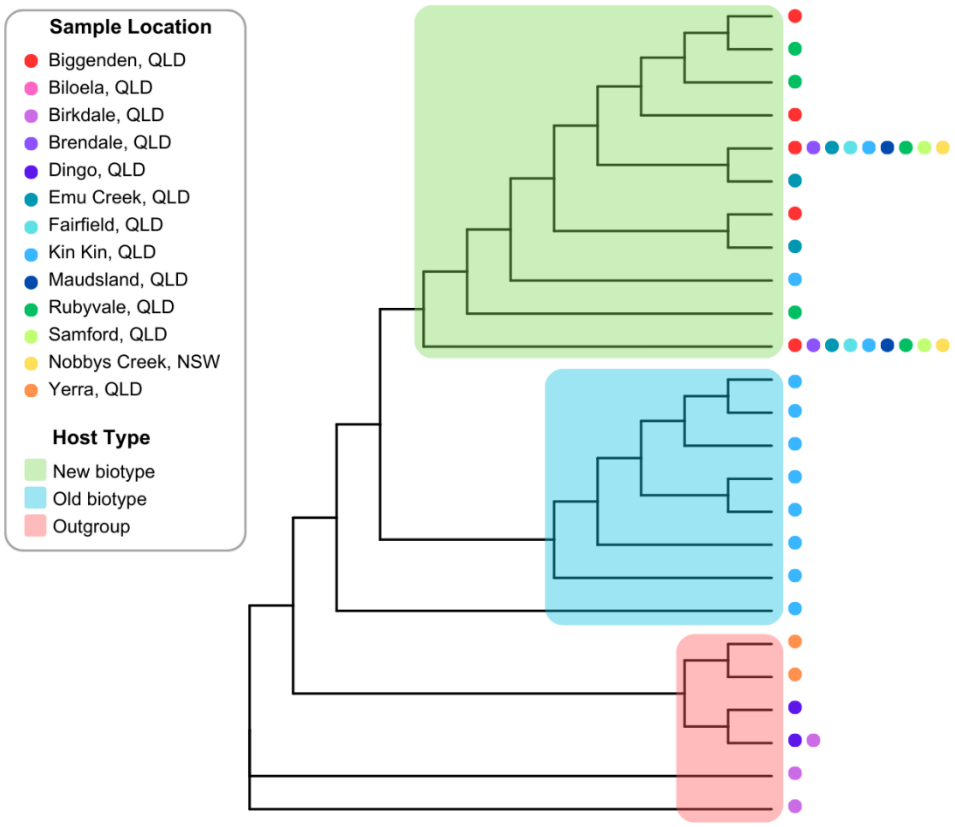

Figure 28. Cladogram of *Ca.* Tremblaya ASVs. Each tip on the tree represents a unique ASV. Clades are highlighted to distinguish *Ca.* Tremblaya ASVs from the new *H. summervillei* biotype (green), old *H. summervillei* biotype (blue) and outgroup species (red). Tip points are coloured by sample location. *Ca.* Tremblaya ASVs appear to group according to host biotype, with the old and new biotypes forming sister clades.

Multiple sequence alignment shows that differences in the ASVs between *H. summervillei* biotypes are small (as little as one SNP) (Figure 29) but statistically significant (PERMANOVA, p=0.003).

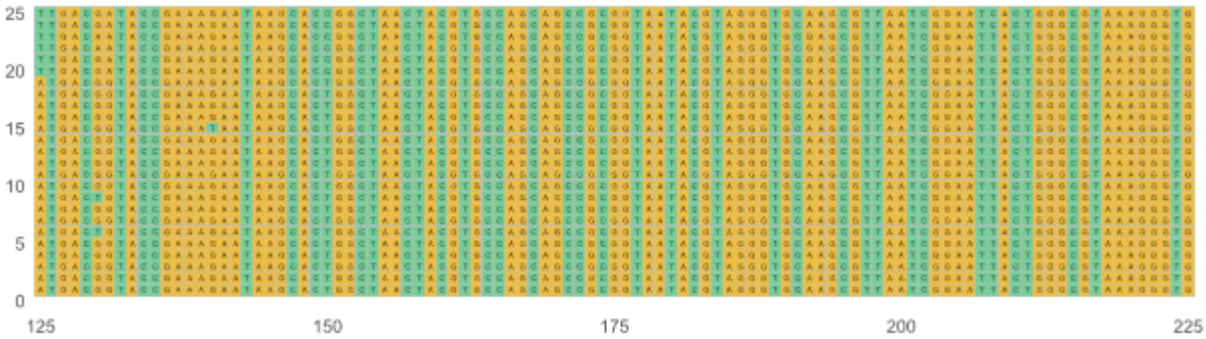

Figure 29. Multiple sequence alignment of Ca. Tremblaya ASVs. Each line represents a unique ASV. Sequence length has been scaled down to make nucleotide differences easier to identify; only nucleotide positions 125-225 are shown here. ASVs appear to vary by only a few nucleotides at most.

4.4 Discussion

4.4.1 Geographic distribution and pasture mealybug population diversity

As in the analysis of mealybugs reared on different host plants in chapter 3, the microbiome of the geographic *H. summervillei* cohort is dominated by *Ca*. Tremblaya. The remaining taxa consist of bacteria commonly isolated from soil (e.g. *Corynebacterium*, *Pseudonocardia*, *Rhizobium*, *Sphingomonas*), plant roots (e.g. *Massilia*, *Variovorax*), natural water sources (e.g. *Bradymondales*, *Chryseobacterium*, *Flavobacterium*) and agricultural leachate (e.g. *Paenibacillus*, *Pseudomonas*), or microbiota associated with livestock (e.g. *Gemella*, *Peptoniphilus*, *Porphyromonas*) (Figure 22). As discussed in section 3.4.2, several of these bacteria are components of the plant microbiome: they may form part of a community of facultative symbionts in the mealybug, and their detection in this study may be attributed to increased abundance in the plant microbiome as a result of stress-induced dysbiosis.

Despite differences in minor bacterial species, the biogeographic mealybug cohort has low overall diversity, similar to the baseline data from mealybugs on different host plants described in section 3.3.1. The results indicate that *Rhizobium* sp. and *Sphingomonas* sp. are potential facultative bacteria in the geographic *H. summervillei* samples, defined in this chapter as any taxon shared among mealybugs from two or more locations.

Rhizobium is a genus of nitrogen-fixing soil bacteria that form endosymbioses with the roots of certain plants, primarily legumes (Yang et al., 2022). Its presence is reported to have a negative impact on feeding performance in leaf-chewing insects, but no clear effect is seen in phloemophagous insects (Gadhave & Gange, 2018). It is unclear whether *Rhizobium* sp. are truly associated with the mealybug microbiome in this study or constitute contamination from the field. Future work could involve microbial profiling of rhizospheric soil alongside the host insect to better understand the dynamics of insect-environment interactions and the acquisition of facultative bacterial associates.

Escherichia coli, although present in samples from at least two locations (Figure 24), has been identified as a likely contaminant in this study. *E. coli* does not appear to be a typical member of the microbiome in mealybugs (Iasur-Kruh et al., 2015; Lin et al., 2019) or other hemipteran species (Lima et al., 2018; Welch et al., 2015; Yang et al., 2023), and its

presence in insects is associated with decreased growth and high mortality (Koga et al., 2022). The potential role of *Sphingomonas* in mealybugs is outlined in section 3.4.2.

The results of this research show no appreciable patterns in mealybug microbiome composition across spatial scale. This differs from the significant variation seen in other insects across distinct geographical locations (Correa et al., 2015; Daane et al., 2018; Landry et al., 2022; Nopriawansyah et al., 2019; Paddock et al., 2022; Thomas & Ramamurthy, 2014; Welch et al., 2015; Yang et al., 2023). As discussed in section 3.4.3, this may be attributed to the low microbial load of phloem sap and/or potential similarities in transmissible microbiota between host grasses. Alternatively, mealybugs may maintain only small communities of mainly facultative bacteria alongside their primary endosymbionts, acquired from the plants on which they feed.

Microbiome composition and diversity may also be influenced by the dominant presence of *Ca.* Tremblaya. The fruit fly parasitic wasp *Asobara japonica* (Belokobylskij, 1998) demonstrates a markedly different microbiome in the presence of the endosymbiont *Wolbachia*, a common invertebrate bacterium that alters reproductive biology (Landmann, 2019). The bacterial composition of the fruit fly without *Wolbachia* appears to be primarily shaped by geography (i.e. genetic divergence between populations as a result of isolation by distance or environment), whereas *Wolbachia* carriers share highly similar bacterial compositions across all locations with no apparent geographic effect (Brinker et al., 2022). A similar pattern is seen in cinnabar caterpillars (*Tyria jacobaeae*), which demonstrate consistent microbiomes across geographically distinct habitats (>100km apart) that are dominated by *Ralstonia*, a possible symbiont, with other bacterial taxa reflecting those from local soil communities (Gomes et al., 2020).

The bacterial communities associated with *H. summervillei* may be of similarly low diversity due to the dominating effect of the primary endosymbiont, rather than the effects of geographic population structure and/or host-plant switching. Further research on *T. phenacola*, particularly gene function and transcriptome analyses, is recommended to understand its influence on microbiome composition and diversity in *H. summervillei*.

4.4.2 Population structure associated with pasture mealybug biotype

The ongoing outbreak of pasture dieback in Australia appears to be associated exclusively with the new biotype of *H. summervillei* (Hauxwell et al., 2022d). The new biotype was collected at all locations in this study, while the old biotype was found only in a mixed

population with the new biotype at Kin Kin (see Figure 21 for a map of sample locations). This is an interesting finding, given its proximity to the site of the oldest known outbreak of pasture dieback in Australia. As summarised in section 1.2.1, *H. summervillei* was first observed in the Cooroy district in 1926 and described in a 1928 paper published by its namesake, Sir William Alan Thompson Summerville. Kin Kin and Cooroy form part of the Noosa Hinterlands and are located approximately 25km from one another. These findings suggest that the 1926 *H. summervillei* variant is still present around the original outbreak location. The results of this work support morphological and molecular taxonomy that indicate that the current outbreak of pasture dieback in Australia is associated with a new mealybug biotype (Dickson et al., 2023). Biosecurity Queensland have also identified a mixed population of old and new biotypes in *H. summervillei* samples collected in Atherton in 2016 (Hauxwell et al., 2022a; Schutze et al., 2019), which corresponds with a historical outbreak of pasture dieback in the area in 1938 (Brookes, 1978).

Phylogenetic reconstruction shows *Ca.* Tremblaya ASVs grouped according to *H. summervillei* biotype (Figure 28). Differences in microbiome diversity are not statistically significant when samples are partitioned by location (p=0.226), but they become significant when analysis is restructured by biotype (p=0.015) (Figure 23). Interestingly, there is a significant difference in diversity between the old and new biotypes at Kin Kin (p=0.040), but not between the new biotypes across all locations (p=0.681). The compositional homogeneity of the bacterial communities associated with new biotype mealybugs suggests that there is no geographic population structure across the current outbreak of pasture dieback. However, there does appear to be an infraspecific population structure across the total *H. summervillei* population based on biotype, in which the old biotype is localised to historical outbreak areas and the new biotype is spread across the current outbreak area.

Recent phylogenetic work by Dickson et al. (2023) indicates clear geographic population structure at the level of the *H. summervillei* genome (as well as infraspecific population structure based on biotype), but the research here suggests that this geographic effect is not seen at the level of the *H. summervillei* microbiome. Further research is required to understand the absence of diversity in the bacterial associates of the new *H. summervillei* biotype. Concurrent sampling of soil and host plant (i.e. factors that likely influence the mealybug microbiome) alongside the mealybug may be a more holistic approach for future microbiome diversity analyses.

4.4.3 Differential dispersal and persistence between mealybug biotypes

The absence of the old *H. summervillei* biotype across the current outbreak area (aside from their co-location in historical outbreak areas, Kin Kin and Atherton) suggests potential differences in virulence or persistence with the new biotype. Factors that could explain the discrepancy in dispersal include divergent host ranges and variable life histories. The new biotype was found on a range of host grasses – bristle grass (*Setaria*), creeping bluegrass, digit grass, green panic, paspalum, rhodes grass, signal grass and sugarcane – while the old biotype was limited to bristle grass (Table 1). Hypotheses about biotype-specific host ranges cannot be confirmed in this study due to a lack of available sampling data on the old biotype (i.e. our specimens on bristle grass at Kin Kin and the original specimens on paspalum at Cooroy), but it may be that the old biotype has a relatively narrow host range compared to the new biotype (see section 1.2.2 for more details on host range in the current outbreak).

Alternative life histories may also explain the differential dispersal patterns. Time to maturity and first reproduction appears to be shortened in the new biotype, from 70 days (Summerville, 1928) down to about 40 days (Hauxwell et al., 2022d). Such change may have disrupted stable predator-prey oscillation between *H. summervillei* and key natural enemies like *C. montrouzieri* (Figure 3a), which were reported at Kin Kin alongside the old biotype. The existence of a long-term predator-prey cycle would also be consistent with the limited spread of pasture dieback in outbreaks prior to the current outbreak (see chapter 1 for further context on pasture dieback distribution in Australia). If *C. montrouzieri* was controlling *H. summervillei* and vice versa in historical outbreak areas, the evolution of a faster-maturing biotype could have triggered an increase in the *H. summervillei* population sharp enough to overwhelm the existing *C. montrouzieri* population – thus providing an opportunity for uncontrolled mealybug population growth and widespread dispersal of the new biotype.

Broadly speaking, the evolution of microbial diversity is attributed to the long-term interactions between an organism and its environment, and continual adaptation to one another (Henry et al., 2021). Differential diversity between the *H. summervillei* biotypes – greater diversity in the old and absence of diversity in the new (section 4.3.1) – therefore suggests that the ‘new’ biotype is evolutionarily younger than the ‘old’ biotype. The emergence of the new biotype may have been the result of bottleneck or founder effects in the old biotype population, in which a marked decrease in the total mealybug population (e.g. due to environmental effects or human activities) led to the formation of remnant populations that were more susceptible to genetic drift (i.e. the acquisition of random mutations due to low genetic diversity within the population) (Szucs et al., 2017). By chance, these processes

may have produced the seemingly more virulent and persistent *H. summervillei* biotype that is found across the current pasture dieback outbreak area. Further omics-based sequencing and phylogenetics on *H. summervillei* is required to reconstruct and understand its evolutionary history.

Chapter 5: *H. summervillei* endosymbiont cophylogeny

5.1 Aims

This chapter reexamines endosymbiont sequence data from the previous chapter alongside host sequence data, with the aim of exploring host vs. symbiont diversity and evaluating patterns of evolution between *H. summervillei* and *T. phenacola*. These host and endosymbiont genomes are expected to demonstrate similar evolutionary trajectories given their long-term (ancient) association. Corresponding changes in the *H. summervillei* and *T. phenacola* genomes would further support the existence of two mealybug biotypes and highlight the need for revised biosecurity measures. If cophylogenetic patterns are present, this work would also substantiate the use of endosymbiont genes as additional molecular markers in population monitoring strategies for parthenogenetic species with low genetic diversity. The findings from this chapter are the first of their kind (at the time of submission of this thesis) to describe cophylogeny in the *H. summervillei-T. phenacola* symbiotic system and have the potential to enhance population monitoring of the pasture mealybug.

Analyses are based on *T. phenacola* 16S short read sequences from the biogeography data in chapter 4 and *H. summervillei* 28S short read sequences generated by the QUT pasture dieback research group (Dickson et al., 2023). Unlike the destructive DNA extraction method used to obtain the *T. phenacola* sequence data (section 3.2.3), *H. summervillei* data were obtained using a non-destructive DNA extraction method described by Martoni et al. (2021) (‘method 8’). *H. summervillei* specimens were incubated overnight (~17 hours) in QuickExtract DNA Extraction Solution (Lucigen, USA), then the intact specimens were removed and stored for further investigation. This method was used to enable morphological characterisation of the mealybug biotypes by Biosecurity Queensland, whose processes involve destructive staining of intact specimens to visualise the necessary features (see Figure 20) and thus preclude DNA extraction after morphotyping.

5.2 Methods

Microbiome analyses were performed on *T. phenacola* 16S sequences extracted from the biogeography data (chapter 4) and existing *H. summervillei* 28S sequences (Dickson et al., 2023).

Multiple sequence alignments were performed in the Clustal Omega program (Madeira et al., 2022). Phylogenetic reconstruction was performed in the IQ-TREE web server (based on v1.6.12; Nguyen et al., 2014; Trifinopoulos et al., 2016). Phylogenies were inferred by maximum likelihood under K2P and TNe+G4 nucleotide substitution models (for host and symbiont phylogenies, respectively), as determined by ModelFinder (Kalyaanamoorthy et al., 2017). 100 bootstrap replicates were generated for each analysis. Cophylogenetic reconstruction and tree annotation were performed with the R statistical software (v4.2.2; R Core Team, 2022) in RStudio (v2022.07.2; RStudio Team, 2020), using the 'ggtree' (Yu et al., 2017) and 'phytools' (Revell, 2012) packages.

Nucleotide BLAST searches (Morgulis et al., 2008; Zhang et al., 2000) were performed against the NCBI GenBank nucleic acid sequence database (Sayers et al., 2022) using *T. phenacola* 16S sequences from both *H. summervillei* biotypes.

5.3 Results

Cophylogenetic reconstruction suggests divergence between the 'old' (1926) and 'new' (2015) mealybug biotypes in both 16S (*T. phenacola*) and 28S (*H. summervillei*) datasets, with the old biotype clustering outside of the new biotype (Figure 30, green). Grouping by location is somewhat apparent in the mealybug genome (Figure 30, left); this pattern does not appear to hold at the level of the endosymbiont genome (Figure 30, right). BLAST searches reveal no high identity (>95% percent identity) results for the *T. phenacola* query sequences from either mealybug biotype (Figure 31).

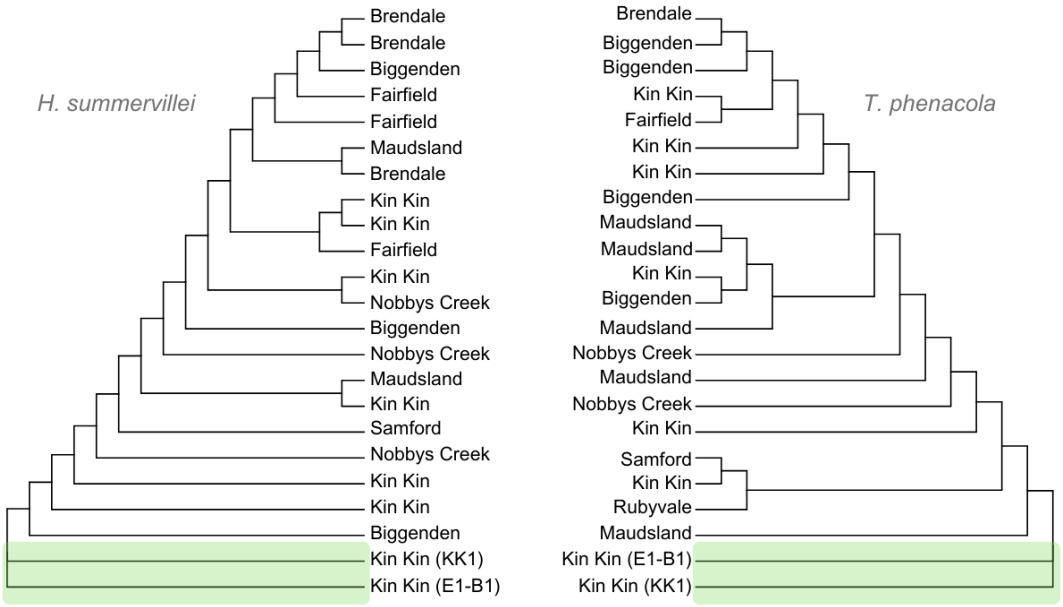

Figure 30. Cophylogenetic cladograms for *H. summervillei* (left) and *T. phenacola* (right). Tree tips denote sampling location. Old biotype samples (i.e. mealybugs whose morphology is consistent with the description provided by Summerville (1928)) are highlighted in green; sample ID is also provided. Corresponding *H. summervillei* and *T. phenacola* sequences from the same mealybug samples were used wherever possible; where this was not possible, replicates from the same sample groups were used as a proxy. A cophylogenetic pattern is apparent in the divergence between mealybug biotypes, seen in both the host and symbiont data.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	592	592	100%	3e-164	91.61%	1351	MZ890435.1
Candidatus Tremblaya phenacola clone PG74 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	553	553	100%	2e-152	89.98%	872	HM449981.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	505	505	100%	4e-138	87.94%	1356	MZ890433.1
Candidatus Tremblaya phenacola isolate Cc5 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	499	499	100%	2e-136	87.70%	1578	MK193743.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	499	499	100%	2e-136	87.70%	1356	MZ890434.1
Candidatus Tremblaya phenacola clone PMAD 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	477	477	100%	1e-129	86.84%	1467	KF444180.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	477	477	100%	1e-129	86.84%	1347	MZ890438.1
Candidatus Tremblaya phenacola clone NH112 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	477	477	100%	1e-129	86.87%	1231	HM449977.1
Candidatus Tremblaya phenacola clone NH17 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	477	477	100%	1e-129	86.81%	1287	HM449974.1
Candidatus Tremblaya phenacola isolate Pha42 16S ribosomal RNA gene, partial sequence	Candidatus Tr…	475	475	99%	4e-129	86.74%	1512	MK193747.1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	575	575	100%	3e-159	90.91%	1351	MZ890435.1
Candidatus Tremblaya phenacola clone PG74 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	547	547	100%	7e-151	89.74%	872	HM449981.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	505	505	100%	4e-138	87.94%	1356	MZ890433.1
Candidatus Tremblaya phenacola isolate Cc5 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	499	499	100%	2e-136	87.70%	1578	MK193743.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	499	499	100%	2e-136	87.70%	1356	MZ890434.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	486	486	99%	2e-132	87.21%	1356	MZ890442.1
Candidatus Tremblaya phenacola clone NH112 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	483	483	100%	2e-131	87.10%	1231	HM449977.1
Candidatus Tremblaya phenacola 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	481	481	99%	8e-131	86.98%	1356	MZ890437.1
Candidatus Tremblaya phenacola gene for 16S ribosomal RNA, partial sequence	Candidatus Tre…	481	481	99%	8e-131	86.98%	1477	AB627026.1
Candidatus Tremblaya phenacola clone PMAD 16S ribosomal RNA gene, partial sequence	Candidatus Tre…	477	477	100%	1e-129	86.84%	1467	KF444180.1

Figure 31. Results of BLAST searches for *T. phenacola* 16S sequences against the NCBI GenBank nucleic acid sequence database. Query sequences from both *H. summervillei* biotypes, old (top) and new (bottom), were used; the top ten (10) hits are presented here. The results show no high identity matches in the database.

5.4 Discussion

5.4.1 *T. phenacola* appears to be a novel member of *Ca.* Tremblaya

The *T. phenacola* associated with *H. summervillei* does not closely match *T. phenacola* from any Phenacoccidae mealybugs published to date (Figure 31). This suggests the emergence of a new strain of the endosymbiont. We propose the designation "*Tremblaya phenacola* HSUM" in line with the naming conventions of published strains - e.g. *T. phenacola* PPER from *Phenacoccus peruvianus* (Gil et al., 2018) and *T. phenacola* PAVE from *Phenacoccus avenae* (Husnik et al., 2013).

5.4.2 Cophylogenetic pattern associated with *H. summervillei* biotype

The concordant phylogenies of *H. summervillei* and *T. phenacola* (Figure 30) are consistent with the assumption that similar patterns of evolution arise in the participants of long-term symbioses (Baumann & Baumann, 2005; Gruwell et al., 2010; Lopez-Madrigal et al., 2015; Szabo et al., 2017). Cophylogeny between host and endosymbiont implies the significant biological role of the latter to the former (Lin et al., 2019). This is true for *T. phenacola* (and all other mealybug-associated members of *Ca.* Tremblaya), which performs critical amino acid and vitamin biosynthesis to satisfy the nutritional deficiencies of the phloem-based diet on which mealybugs subsist (McCutcheon & von Dohlen, 2011; Sudakaran et al., 2017). The persistence and overwhelmingly high abundance of *T. phenacola* compared to any other taxa in the *H. summervillei* microbiome, regardless of plant host (section 3.3.1) or geography (section 4.3.1), further substantiate its biological significance as a key nutritional symbiont. This agrees with the current understanding that the evolutionary history of Pseudococcidae is shaped more by their relationship with *Ca.* Tremblaya than by host usage or historical biogeography (Hardy et al., 2008).

There is no distinct grouping by location aside from at the original 1926 outbreak area (Kin Kin/Cooroy), where the old biotype was exclusively identified in this study. The major cophylogenetic pattern appears to be associated with *H. summervillei* biotype. There is clear divergence between biotypes in both host and endosymbiont genomes (Figure 30, green). Further sequencing work is recommended to increase phylogenetic resolution and provide additional support for the genetic divergence between *H. summervillei* biotypes.

As discussed previously (section 1.2.3), standard population genetic analyses with polymorphic microsatellite markers generally have limited resolving power for parthenogenetic species due to their low levels of genetic diversity. This can hinder effective monitoring of pest species like *H. summervillei*, which appears to be capable of both sexual and asexual reproduction, as is common in mealybugs (Hauxwell et al., 2022a, 2022d; Summerville, 1928). Given the concordance between phylogenies, which suggests a high degree of coevolution and genetic interdependence, genetic markers based on the *T. phenacola* genome could potentially be used to enhance monitoring of *H. summervillei*. Targeting the naturally more rapidly evolving bacterial endosymbiont genome, rather than the slower evolving insect host genome, is advantageous as it would offer polymorphic genetic markers with greater resolution. This may be a promising strategy for monitoring *H. summervillei* populations in Australia, where the mealybug continues to expand its range along the eastern coast. More complete sequencing of the *T. phenacola* genome is required to identify suitable genetic markers.

5.4.3 Implications of a new *H. summervillei/T. phenacola* variant

The new *H. summervillei* biotype and its associated *T. phenacola* strain (hereafter collectively referred to as the 'new' variant) appears to be highly virulent. Pasture dieback associated with the new variant has the capacity to spread at least a hectare a week under ideal conditions (i.e. warm and wet) (MLA, 2021b). This is almost four times more rapid than dieback associated with the 1926 variant, which reportedly spread about 5 hectares (12 acres) over the course of 10 months (Summerville, 1928). *T. phenacola* is the only taxon detected consistently across the microbiomes of the new and old variants, and there appears to be no other bacteria present in the new variant that could explain the change in virulence. If virulence is attributed to the microbiome, and the change in *H. summervillei* virulence is associated with the change in *T. phenacola*, this could explain the sudden hypervirulence of the new variant and the rapid spread of pasture dieback in the current outbreak. Further research is required to understand the relationship between *T. phenacola* and *H. summervillei* as well as the mechanisms underlying pathogenicity in this system.

Given the marked increase in transmission rate and host range compared to historical outbreaks, it is recommended that the current outbreak of pasture dieback be considered a new incursion, attributed to the new *H. summervillei/T. phenacola* variant. This new incursion should also be considered a new biosecurity risk – it demonstrates an increased capacity for widespread, rapid pasture death and therefore poses a serious threat to global pasture productivity and food security. This risk escalates further with the effects of climate change:

the projected increase in hot weather and extreme rainfall events in Australia (CSIRO, 2020) typify the ideal conditions for mealybug activity and dispersal. More effective and proactive management strategies will be required to mitigate the growing impact of pasture dieback on the agricultural industry. Continued research on the biology of *H. summervillei* and the role of *T. phenacola* in plant immunity is recommended to better inform future pasture management.

Chapter 6: Conclusions

6.1 Pasture variety screening for dieback susceptibility

Field testing of pasture grasses is essential to determine the multiple effects of variety, environment and management on susceptibility to *H. summervillei* and pasture dieback. However, comprehensive field testing of the many varieties of pasture grass is not feasible, as the costs of establishing, maintaining and monitoring large-scale and long-term field trials would quickly become prohibitive. Glasshouse and screenhouse infestation assays may provide a more rapid assessment but still require several months, are labour intensive, and generate categorical data. A more rapid, statistically robust screen to prioritise of mealybug-tolerant pasture varieties for long-term field trials is therefore necessary.

The rapid bioassays in this study show statistically significant differences in mealybug survival time and predicted dieback susceptibility on different grass varieties (Figure 12). Survival time was highest on American buffel (Table 2), which suggests greater suitability as a host for *H. summervillei* and thus increased susceptibility to pasture dieback. Gatton panic and Mekong brizantha were less suitable hosts, with the latter demonstrating the lowest relative susceptibility.

Further validation of this test is required, particularly since the mealybugs were all obtained from adults reared on American buffel grass. This could lead to a bias in results, since the second instar nymphs may be less well-adapted to the other varieties. Future research should focus on validating the rapid bioassay presented here, for example through reciprocal testing of mealybugs reared on a range of host varieties, or by testing pasture varieties with known equivalent or greater susceptibility as well as those with less susceptibility as a host.

Nevertheless, overall confidence in this rapid bioassay method is high. The results support those of field and screenhouse trials (Hauxwell et al., 2022c-e), which report greater host suitability and severity of dieback symptoms in buffel varieties compared to panic and brachiaria varieties. Plants with thick stems, erect leaves and/or pubescence (Table 3) appeared to be less suited as hosts for *H. summervillei* and were associated with lower dieback susceptibility. Similar observations about plant phenotypic traits and host suitability were also made in screenhouse trials (Hauxwell et al., 2022e).

This research has demonstrated proof of concept of a rapid (2-3 week) laboratory-based bioassay that uses regression analysis of mealybug survival time on different pasture grass varieties (relative to a standard variety) to identify grasses that are less suitable hosts for *H. summervillei* (the primary causative factor in pasture dieback) and are therefore predicted to be less susceptible to dieback. These results may inform selection of varieties for long-term field trials to reduce the scale and associated costs of such research.

6.2 *H. summervillei* baseline microbial profile

This research has generated novel data on bacterial community composition and diversity in *H. summervillei*. It presents a baseline microbial profile for this insect pest, for which there is no equivalent in the current literature, and highlights the need for further work on *T. phenacola* and its interactions with the host plant and secondary bacterial symbionts.

Very low diversity was detected in the *H. summervillei* bacterial microbiome based on 16S microbial profiling of mealybugs from a range of pasture varieties and geographic locations (Figures 13 and 14). The mealybug microbiome was dominated by ASVs from the primary endosymbiont *Ca.* Tremblaya (identified only to genus in this study) and this was consistent across all *H. summervillei* samples. Bacterial community composition and diversity may be influenced by the dominant presence of *Ca.* Tremblaya rather than the effects of geographic population structure or host adaptation. Further research on *T. phenacola* is recommended to understand its influence on microbiome composition and diversity in *H. summervillei*.

Potential (non-*Tremblaya*) ‘core’ bacteria or secondary bacterial symbionts were detected across four genera: *Sphingomonas*, *Flavobacterium*, *Pseudomonas* and *Lawsonella*. Functional characterisation is required to understand the exact roles of these bacteria in *H. summervillei* and in plant-insect interactions. This study did not detect the specific taxa identified in the salivary bacteria of solenopsis mealybug that are involved in disrupting JA/SA signalling (Zhao et al., 2023); however, there were similarities with bacteria known to affect JA/SA pathways in other insects, and bacteria associated with dysbiosis in plants suffering biotic and abiotic stress.

Future work that explores the whole microbiome, with emphasis on Enterobacteriaceae and related taxa, is important to determine the interactions of these bacteria in mealybug-endosymbiont-plant interactions, in the disruption of the JA/SA pathways, and in the development of symptoms of pasture dieback. This could include full sequence analysis of

the *T. phenacola* genome with gene expression and transcriptome analysis, and network analysis to explore the full microbiome interactions with the host plant. The results of this work, specifically the low diversity across the entire mealybug cohort, could be verified more rigorously through whole-genome shotgun sequencing and haplotype network analysis, but were beyond the scope of this thesis.

The sequences from *T. phenacola* associated with *H. summervillei* do not appear to match sequences from *T. phenacola* from any Phenacoccidae published to date. We propose the designation "*Tremblaya phenacola* HSUM" in line with the naming conventions of published strains.

6.3 New variant of *H. summervillei* and *T. phenacola*

An important finding of this work is the significant difference in the short-read sequences of *T. phenacola* and cophylogeny with two distinct biotypes of *H. summervillei*: the 'old' biotype first identified in 1926 (Summerville, 1928) and the 'new' biotype associated with the recent, widespread and economically damaging outbreak of pasture dieback. This result supports those of morphological and molecular differentiation of the two biotypes of *H. summervillei.*

Although there is no discernible mealybug population structure based on whole-microbiome sequencing across the known *H. summervillei* geographic range, there is some infraspecific population structure based on biotype (Figure 23). This is attributed to sequence variation in the primary endosymbiont rather than the bacterial microbiome as a whole (Figure 27). Phylogenetic reconstruction shows that *Ca.* Tremblaya ASVs from new biotype mealybugs are significantly different from those in the old biotype (Figures 28 and 30).

The ongoing occurrence of pasture dieback in Australia appears to be associated exclusively with the new biotype of *H. summervillei* (Hauxwell et al., 2022d; Dickson et al., 2023; Schutze et al., 2019). In this study, the old biotype (Brookes, 1978; Summerville, 1928) was found only once, in a mixed population of old and new biotypes at one location in Kin Kin, close to the site of the 1926 outbreak in Cooroy (Figure 21). The old biotype has also been identified by morphology in a mixed population with the new biotype, collected in 2016 from the Atherton Tablelands, close to a minor outbreak of *H. summervillei* in 1938 (Schutze et al., 2019; Brooks, 1978), but no DNA sequence analysis has been conducted in these samples.

The new *H. summervillei* biotype and its *T. phenacola* strain may be collectively considered as a new variant of the *H. summervillei-T. phenacola* system. The cause of the rapid and extensive spread of the new *H. summervillei* variant in the current outbreak is difficult to prove conclusively, and could be due to changes in environmental conditions or management practices, rather than genetic change in the mealybug or endosymbiont. However, the limited spread of the old *H. summervillei* variant beyond the areas of the 1926 and 1938 occurrences and the uniform association of the new variant with the current outbreak suggests a difference in virulence in the new variant. Further omics-based sequencing, phylogenetics and haplotype network analysis of *H. summervillei* and *T. phenacola* may help to construct and understand their evolutionary history and virulence.

Mealybugs are recognised as a significant international biosecurity threat (DAWR, 2019) and the identification of a new hypervirulent pasture mealybug variant in Australia is a major concern for international tropical and subtropical pasture industries. This is especially pertinent as *H. summervillei* has now been identified in association with pasture dieback in Puerto Rico in 2019 (Hauxwell et al., 2022b) and Barbados in 2020 (Gibbs, 2020). The association of a new mealybug variant with a virulent outbreak of pasture dieback calls for revision of the current biosecurity status of the host insect, *H. summervillei*, and its proposed endosymbiont strain, *T. phenacola* HSUM.

Further work on the molecular sequence of *H. summervillei* and *T. phenacola* across its global geographic rage will better inform future biosecurity diagnostics and enhance the speed of responses to these mealybug incursions. The method used here to differentiate between mealybug biotypes based on morphology, mealybug barcode sequences, and short sequence reads of the primary endosymbiont may improve the detection of emerging biotypes of mealybugs globally and better inform international biosecurity responses.

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