Final report

Determining the role of ground pearls in Pasture Dieback

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Abstract

This project aimed at determining the role of ground pearls in pasture dieback. Multiple surveys were conducted of symptomatic and asymptomatic sites, and hundreds of soil samples were processed using a methodology developed in this project. Ground pearls were found in approximately 75% of pasture dieback sites examined, but they were also present and absent in asymptomatic sites and were absent in 25% of symptomatic sites. As they do not readily move in the soil, and were absent from multiple pasture dieback sites, if pasture dieback is caused by a single agent, they cannot be the primary cause. Symptomatic sites had more ground pearls present than asymptomatic sites, which indicates that their feeding may exacerbate symptom expression. It was found that they are more likely to emerge as adults from their cysts during warmer weather, such as spring and summer. Molecular characterisation indicated the presence of four ground pearl species in Australia. A molecular diagnostic for ground pearl detection and identification was developed that can streamline soil processing and be applied to archived soil samples. There is now much greater awareness of ground pearls among graziers, and scope to better characterise their impacts and develop effective integrate management plans.

Executive summary

Background

Pasture dieback is a concerning phenomenon that emerged around 2016. It is not known whether the current pasture dieback has the same causes of a presentation with similar symptoms, buffel grass dieback, that was present in the 1990s but then disappeared. Having identified ground pearls (Hemiptera: Margarodidae) at a range of pasture dieback sites in Queensland, this project aimed at determining whether they were involved in pasture dieback.

This project has significantly raised grower awareness about the presence of ground pearls in Queensland pastures and will be useful for future identification of areas where these insect pests are impacting production.

Objectives

The primary objective was to determine if ground pearls are linked to the pasture dieback condition, and if so, how they can be effectively managed.

1. Confirm and quantify the presence of ground pearls at PD sites. Expand the number of PD sites investigated, systematically record the symptoms and history of the sites, and take paired samples from these sites and adjacent unaffected sites to determine the likelihood that ground pearls are the primary cause of PD.

ACHIEVED

- A sampling strategy and processing procedure were developed for extracting ground pearls from soil samples.
- Ground pearl presence/absence was screened for more than 50 sites, including 21 sites with paired symptomatic/asymptomatic samples.
- Ground pearls were present in 75% of sites exhibiting pasture dieback symptoms.
- Ground pearls were also found in asymptomatic areas.

- There was a significant difference (p = 0.02) in the number of ground pearls found in symptomatic sites compared to asymptomatic sites
- Given the non-mobile biology of ground pearl cysts, if they were the single cause of pasture dieback, they would be found in all pasture dieback sites. This was not the case, so they cannot be the primary cause of pasture dieback in all areas.
- Being plant root-feeders, it is likely they cause stress to infested grasses, and this stress will be exacerbated under climatic conditions conducive to plant stress.
- It is concluded that ground pearls cannot be the primary cause of pasture dieback over large areas, but their potential role in exacerbating damage or causing dieback in localised areas cannot be discounted.

2. The impact of ground pearls on a range of host plant species. Develop glasshouse screening protocols to transmit ground pearls and determine their impact on host grasses in controlled settings. This will determine the host range and susceptibility of a range of improved and native pasture species which will be essential to the long-term management of ground pearl infestations, and contribute to management of GP if proven to be a causal agent or co-factor in PD.

PARTIALLY ACHIEVED

- Rhizobox experimentation did not show that ground pearl cysts removed from plant roots could readily re-attach to new roots.
- It is likely that the swarming nymph stage is the critical stage to root attachment, and then the cyst forms.
- Adult ground pearls that had emerged from cysts were observed, particularly under warm conditions. Several adults laid eggs that hatched into the mobile nymph stage. However, we were unsuccessful in transferring these nymphs to living grass roots and were therefore unable to culture them in laboratory conditions.
- The inability to routinely culture the ground pearls prevented us from trialling their impacts on a range of host grasses.

3. Molecular characterisation of ground pearl populations. This will provide new knowledge on the population structure of white ground pearls (*M. australis*), information required for developing management strategies and facilitate the development of a future soil DNA test.

ACHIEVED

- A range of DNA extraction protocols were trialled for ground pearls, including a semi-destructive method that facilitated retention of the external covering of the cyst, thus preserving taxonomically valuable material.
- PCR testing of diagnostic loci revealed at least 4 distinct ground pearls species present in Australia.
- *In silico* analysis of microsatellite alleles present in whole genome sequences revealed negligible genetic exchange among populations of the white ground pearl.
- Long read sequences were unable to be achieved owing to the presence of unidentified co-extracted compounds in the individual ground pearl DNA extractions that clogged the

nanopores. This precluded annotation of the full genome sequence of the white ground pearl.
- Whole genome shotgun sequencing using the Illumina platform revealed the presence of bacterial endosymbionts which have co-evolved with their hosts in the same manner as mitochondria. As these endosymbionts are inherited in direct lineage through the ground pearl lifecycle, their DNA sequences are diagnostic of their host.
- A quantitative PCR protocol targeting the endosymbiont DNA has been developed and it successfully amplifies target DNA from individual ground pearls.
- A fragment has been amplified from DNA extracted from infested soil, but further optimisation using other loci are required to complete the ground pearl soil DNA test.

4. Development of Integrated Pest Management (IPM) program for ground pearl. Aims to deliver a management package that can be adopted by producers through existing MLA channels. This package is likely to include variety recommendations, soil management, and biological and chemical control options.

COMMENCED

- While nominally the development of an IPM plan was not addressed, this is because we have been unable to successfully complete Objective 2.
- However, the foundations of an IPM plan have been established. These include:
    - Development of a soil sampling and processing methodology to identify ground pearls.
    - Early development of machine learning software to enumerate ground pearls.
    - Development of molecular assay for differentiating ground pearls.
    - Initial soil DNA test developed (requires further optimisation)
    - Identification of endosymbiont targets for future management.
    - Indication that ground pearl cysts that are disassociated from grass roots do not re-attach efficiently, thus the potential for soil renovation techniques as a method for ground pearl control.
    - Indicative timing of chemical interventions around periods of adult emergence when the ground pearls are more susceptible to interventions.
    - Improved grazier knowledge of these cryptic pests.
- Further work is required to better understand the impacts of ground pearls on the Australian grazing industries, but more is now known about Australian ground pearls than ever before.

Methodology

This project employed multiple methodologies associated with the objectives. These included:

- Development of a sampling and soil processing methodology using two sieves
- Surveys of pasture dieback affected and unaffected sites and processing of samples from third parties (eg. QDAF, QUT, AHR)
- Glasshouse and laboratory methods for attempting to culture ground pearls.

- Deployment of thermogradient bed to determine optimum temperature conditions for adult emergence.
- DNA extraction protocols for individual ground pearls.
- Whole genome sequencing, bioinformatics and development of quantitative PCR protocols to detect and identify ground pearl species.

Results/key findings

The key finding is that ground pearls are vastly more widespread throughout Queensland than previously thought and they are likely having a significant impact on pasture production. While they have been identified in association with pasture dieback, we have also found them at unaffected sites, and have failed to find them at all affected sites. This is strongly indicative that ground pearls are not the primary cause of pasture dieback if pasture dieback has only one cause.

Benefits to industry

This work has largely discounted the potential that ground pearls are a primary cause of pasture dieback, which in itself is an important finding because it removes a live lead. However, there are broader industry benefits. Until this work the industry was largely unaware of the presence of ground pearls in pastures, but now graziers can investigate unthrifty pastures and potentially identify ground pearls where they are present. Ground pearl detection and identification will be streamlined by the development of DNA tests, while several targets for improved ground pearl management have been identified.

Future research and recommendations

While this project was specifically focused on the potential role of ground pearls in pasture dieback, like other scientific research it has uncovered more questions than answers. We now know that ground pearls are much more prevalent than previously thought, but we have not as an industry got information on their impacts to grazing. We have taken great strides in identifying management strategies, but these need to be further developed if they are to be deployed. Further research on these cryptic pests are warranted because we now know they are widespread, but we do not know what they are costing us.

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1. Background

1.1 Determining the role of ground pearls in pasture dieback

Pasture dieback (PD) is an extremely serious issue for northern graziers. It also appears to be growing in intensity, presumably exacerbated by changing climatic conditions that stress pastures and makes them more susceptible to attack. Previous to the current work, white ground pearls, *Margarodes australis* (Hemiptera: Margarodidae) had been associated with at least 12 PD sites throughout Queensland (Thomson 2019).

Ground pearls are cryptic soil insects that feed on the roots of plants. In Australia they chiefly feed on grasses, but some species overseas feed on grape vines (Thomson et al. 2021). In Australia, a related species, the pink ground pearl (*Eumargarodes laingi*) causes significant damage to turf grasses and sugarcane when environmental conditions lead to plant stress (Samson and Harris 1998). Given the presence of white ground pearls at multiple pasture dieback sites, and the similarity of pasture dieback symptoms to those caused by related ground pearls in other grasses, it was considered necessary to undertake research aimed at determining if ground pearls played a role in pasture dieback. Prior to this current project, virtually nothing was known about the impacts of ground pearls on Australian pastures.

Previous work on a related, if not identical, condition called buffel grass dieback was unable to determine the causes (Makiela 2008, Makiela and Harrower 2008). However, one particular observation was that soil disturbance resulted in recovery of affected buffel grass, while non-disturbance led to normal disease progression and death. This was reported to have occurred consistently over three experiments, each involving 20 treated and 20 untreated plants (Makiela 2008). All of the samples with disturbed soil recovered, and all of those with undisturbed soil died. This suggested that whatever was causing buffel grass dieback was not only associated with the roots but could also be dislodged from the roots and be unable to re-attach. This finding seemed to be consistent with what may be expected if ground pearls were causing the problem. Furthermore, throughout the course of that research there was no evidence for the involvement of other insects, nematodes or fungi, and given ground pearls are readily overlooked unless people are familiar with them, it was considered possible that they may have been responsible for the plant death.

The current project aimed to deliver knowledge that can be readily adopted by stakeholders. If it was established that ground pearls are the primary cause of PD, then factors such as host preference and resistance, pest dispersal methods, early detection systems, soil detection protocols, and integrated management strategies were to be developed. This project is distinct from other projects investigating mealybugs as the cause of pasture dieback as we are focused on a different potential agent.

2. Objectives

The primary objective is to determine if ground pearls are linked to the pasture dieback condition, and if so, how they can be effectively managed.

1. Confirm and quantify the presence of ground pearls at PD sites. Expand the number of PD sites investigated, systematically record the symptoms and history of the sites, and take paired samples

from these sites and adjacent unaffected sites to determine the likelihood that ground pearls are the primary cause of PD.

2. The impact of ground pearls on a range of host plant species. Develop glasshouse screening protocols to transmit ground pearls and determine their impact on host grasses in controlled settings. This will determine the host range and susceptibility of a range of improved and native pasture species which will be essential to the long-term management of ground pearl infestations, and contribute to management of GP if proven to be a causal agent or co-factor in PD.

3. Molecular characterisation of ground pearl populations. This will provide new knowledge on the population structure of white ground pearls (*M. australis*), information required for developing management strategies and facilitate the development of a future soil DNA test.

4. Development of Integrated Pest Management (IPM) program for ground pearl. Aims to deliver a management package that can be adopted by producers through existing MLA channels. This package is likely to include variety recommendations, soil management, and biological and chemical control options.

3. Methodology

3.1 Confirm and quantify the presence of ground pearls at PD sites

3.1.1 Site inspections

A range of pasture dieback sites throughout Queensland were investigated to determine presence/absence of ground pearls. These sites included a selection of those surveyed by the Queensland Department of Agriculture and Fisheries (QDAF), Applied Horticultural Research (AHR) and Queensland University of Technology (QUT). Soil sampling methods employed by QDAF, AHR and QUT varied, but for these sites we had access to geographic coordinates, symptomatology, pasture species, and, through this work, ground pearl species identification and abundance. Other pasture dieback sites were surveyed as part of this project. In addition to the same characteristics as the first dataset, this dataset included more detailed information on soil chemistry and climate variables for each site. These sites were surveyed between November 2020 and May 2021.

Site selection was based on recommendations by QDAF and reports from landholders detailing the decline in pasture. Confirmation of pasture dieback symptoms were made at the time of the site visit. A sample sheet and checklist was deployed to facilitate comprehensive collection of data.

For sites where pasture dieback was apparent, soil samples were collected from symptomatic and nearby asymptomatic areas. Symptomatic samples were collected along, or near, the edge of the severely affected dieback patch where either yellow or red discolouration of grass leaves was occurring (Fig 1). For samples to be characterised as asymptomatic, the plants had to have no visual decline and be a minimum of 30 m from the area considered to be symptomatic.

Fig 1. Rhodes grass (*Chloris gayana*) affected by Pasture Dieback in the Good Night locality. Affected grass exhibits reddened leaves and appears to have reduced biomass in comparison to the surrounding pasture. A few broad leaf weeds (i.e., *Bidens pilosa*) are interspersed through the dieback affected area.

Regions for sampling were determined at the timing of the site visit. Therefore, due to seasonal dry conditions, asymptomatic regions of pasture could not be accurately identified for some sites. In these instances, a sample for the location was collected only from the area successfully identified as symptomatic. Within each region, 10 random plants were selected and bulk soil samples (~ 600 g moist weight) were collected from the area encompassing the selected plant (200 mm wide x 200 mm long x 200 mm deep). Samples were individually bagged and labelled. Each sample label included details regarding sampling depth, pasture species present, a brief summation of symptom expression and any other pertinent notes. Given other developments in pasture dieback research, we specifically looked for evidence of other insects, particularly pasture mealybugs (*Heliococcus summervillei*).

The moisture content of each sample was determined. Two hundred and fifty grams (± 0.3 g) of dry weight (DW) soil was added to a rotary tumbler with 2 L of reverse osmosis (RO) water and tumbled for a minimum of 30 min. This solution was then washed through a stack of two sieves (2 mm aperture and 150 µm aperture) (see 3.1.2). Ground pearls were removed using forceps and stored in Petri dishes labelled with the location and sample number. Under a dissecting microscope, cysts were counted and characterised as either ‘live’ or ‘empty’.

3.1.2 GP enumeration

In order to determine the potential impacts of GP on pastures, it is essential to develop a consistent sampling, processing and enumeration method.

The main method previously used to separate GP from the soil is wet-sieving (Walker and Allsopp, 1993, Dominiak et al., 1998, Samson and Harris, 1998). In the published literature there are multiple methodologies, but as they are not consistent it is difficult to compare populations across studies, or to determine damage thresholds for these insects.

A series of experiments were established to compare the extraction precision and efficiency of wet-sieving compared to sucrose flotation for the removal of *Margarodes australis* cysts from heavy clay soils.

Two methods were evaluated. One employed a stack of four sieves (2 mm, 1.18 mm, 1.03 mm and 150 µm), while the other comprised two sieves (2 mm and 150 µm). To confidently determine the extraction precision, the process for each method was repeated 15 times. To determine the efficiency of each method, the time taken to wash the soil sample through the sieve was recorded.

A heavily infested area of the Gatton campus of UQ was used to source material for this experiment. A total of 50 g of dry weight soil was placed in a 500 mL Schott bottle and thoroughly mixed with 500 mL of reverse osmosis (RO) water and 2.5 g of Calgon (sodium hexametaphosphate) which was used as a clay dispersal agent. Samples were then washed through the respective nest of sieves. Each sieve was visually inspected and cysts were removed into a labelled Petri dish. Under a dissecting microscope, the total number of cysts recovered were counted and the results recorded.

The two-sieve method was also assessed in combination with sucrose flotation. Most soil-dwelling invertebrates have a specific gravity between 1.0 and 1.1, therefore using a sucrose solution with a specific gravity greater than 1.2 should enable invertebrates to separate from the soil and other matter and float to the surface (Edwards, 1991). A reference molarity point was calculated by determining the concentration of sucrose in water required to float margarodid cysts to the surface of the solution. This was done by adding margarodid cysts into water with sucrose gradients ranging from 2 M to 5 M. It was found that a 4 M sucrose solution (specific gravity of 2.39) was sufficient for maximum cyst flotation. Samples were washed through the sieves as aforementioned and the material that collected on the 150 µm sieve was washed into a 500 mL beaker using 4 M sucrose solution. The beaker was filled with 480 mL of 4 M sucrose solution and the mixture was swirled continuously as it was tipped through a 100 µm sieve. Soil and other material that accumulated at the bottom of the beaker were not tipped into the 100 µm sieve. The material collected on the 100 µm sieve was thoroughly rinsed and then transferred into a Petri dish for identification and enumeration under a dissecting microscope. Significant differences in means were calculated using Duncan's multiple range test.

3.1.3 Machine learning GP estimation

There has been a rapid expansion in machine learning technologies for identifying, counting, and classifying invertebrates (Xia et al. 2018, Ärje et al. 2020b). Counting and classifying is trained on features, such as colour, shape, and size (Wäldchen and Mäder 2018). Manually annotating the characteristics or features to train the models is labour intensive and relies on the expertise of specialists. However, with the development of artificial neural networks, the process of feature extraction can be automated and the model trained on a suitable representation of the data from a collection of examples, to then develop a robust model from that information (Wäldchen and Mäder 2018, Xia et al. 2018). For this work, we applied a deep learning technique, YOLOv5 (You Only Look Once) to enumerate and classify ground pearls.

The dataset used to train the ground pearl detection model consists of RGB images captured using a dissecting microscope (3.2 x magnification) and Olympus CellSens Standard Image Acquisition software. These images captured ground pearls in a Petri dish, with live and empty specimens, along with soil, pieces of organic matter and quartz. The images have a raw resolution of 2448 x 1920 pixels.

A range of different models were applied in collaboration with experts in the field. These essentially involved first training the software to recognise the general shape and structure of ground pearls, then re-training it to differentiate live and dead ground pearls. The scope of the mathematical modelling employed to conduct this work is to be presented in a separate report.

3.1.4 Biophysical characterisation

An analysis of soil physical and chemical properties for selected soil samples was completed by CSBP Soil and Plant Analysis Lab using standardised methods described by Rayment et al. (2011). The standard suite of tests were conducted to determine: soil colour and texture, ammonium Nitrogen, nitrate Nitrogen, Phosphorous (Colwell), Potassium (Colwell), Sulphur, organic Carbon (Walkley-Black), electrical conductivity, pH and exchangeable cations (Aluminium, Calcium, Magnesium, Potassium, Sodium).

3.2 The impact of ground pearls on a range of host plant species

3.2.1 Host range

It was initially considered beneficial to determine the host range of white ground pearls and assess their impact on the plants. In order to complete this objective it was necessary first to attempt to generate live cultures of ground pearls so that miniaturised controlled experiments could be conducted. To this end, a series of experiments were established to try and grow the ground pearls.

3.2.2 Rhizobox establishment

Rhizoboxes allow plant scientists to observe root architecture. It is well established that Australian ground pearls feed on the roots of grasses, however, very little is known about their impact on plant health. Previous studies have demonstrated that the parthenogenetic females may emerge from their cyst stage, travel a short distance, lay hundreds of eggs and then die. When the eggs hatch, the nymphs crawl through the soil and attach to a root: failure to obtain a root leads to death (see Thomson et al. 2021 for a review). However, it is not known whether or not encysted ground pearls once removed from a root can attach to a new one. Therefore a series of studies were conducted to determine whether ground pearl cysts can attach themselves to grass roots they encounter in a confined space, and, further, to determine if there is any impact on the plant.

Twenty rhizoboxes were planted with buffel grass (cv. Gayndah) in a controlled temperature room at UQ Gatton. The Gayndah cultivar was selected as it was historically subject to significant buffel dieback, which is considered potentially a precursor to what is today known as pasture dieback. Banks of LED lights were used to provide 14:10 hr day:night at 28°C:22°C. Ten of these rhizoboxes contained 500 white ground pearl cysts, evenly distributed along stratified layers of 100 separated at 10 cm intervals. The others were free from ground pearls. Rhizoboxes were covered in photo-opaque coverings, regularly inspected, weighed and topped up with water so that each plant had

exactly the same moisture, calibrated to be just below field capacity. The array of lights was periodically raised as the grass grew. After approximately 6 weeks the buffel grass was harvested, dried and weighed for analysis. A second harvest occurred before the experiment was discontinued. A schematic diagram of the rhizobox experiment is presented in Fig. 2.

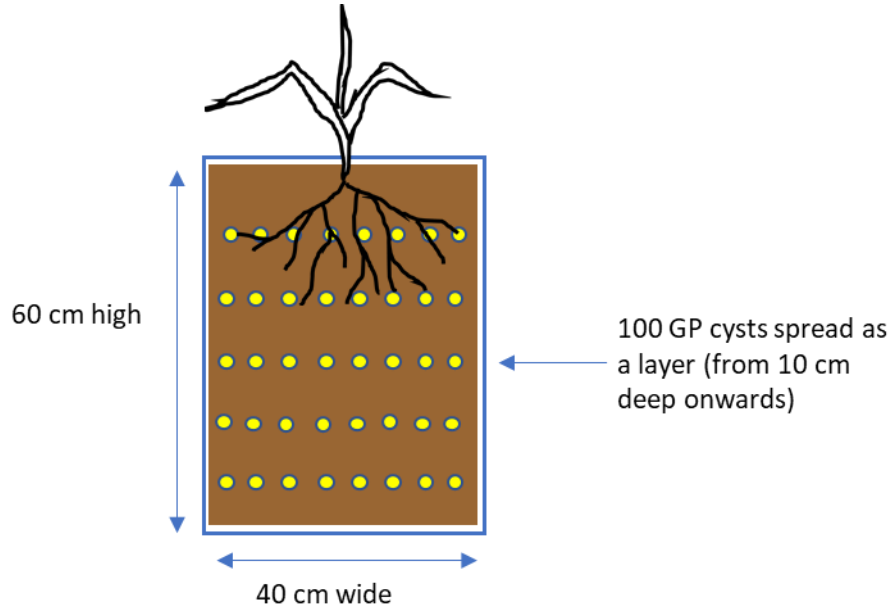

Fig 2. Pictorial illustration of a ground pearl infested treatment in a rhizobox.

3.2.3 Ground pearl emergence study

In order to conduct epidemiological studies on ground pearls it is necessary to culture them on live hosts. As there is evidence that ground pearl cysts removed from roots are not particularly efficient at re-acquiring them (4.2.2), it is likely necessary to work with nymphs. After emergence from the egg, ground pearl nymphs actively move in search of a root. Once attached, the nymph assumes the sedentary encysted form. It completes its lifecycle when it emerges as an adult female and deposits between 300-600 eggs depending on the species.

Anecdotal reports from greenskeepers and canefarmers indicate that adults of the pink ground pearl emerge at the start of summer, often following rainfall events. The factors governing adult emergence are not understood, but it is likely that environmental and potentially circannual processes are involved. There is virtually no information available on the emergence factors for the white, yellow or brown ground pearls.

Margarodes australis cysts were collected from soil sourced at The University of Queensland Gatton campus (-27.560203, 152.334330) and were extracted and counted using the methods described previously (4.1.2). Live margarodid cysts were sorted into individual Petri dishes in groups of 100 and stored at 18°C until the total number of cysts required for the experiment were amassed.

A thermogradient bar was commissioned to determine what temperature, or range of temperatures, were conducive to the emergence of *M. australis* adults. Four Petri dishes, with each dish containing 20 live cysts, were placed in different chambers of a thermogradient bar. The thermogradient bar was set for a minimum of 10°C and a maximum of 40°C (Takavarasha and Giga, 1988) which created a gradual, increasing temperature gradient across the 10 chambers of the bar, ranging from 12-35°C.

Temperature for each chamber was logged using TinyTag temperature and humidity data loggers. The cysts were maintained on moist filter paper to help prevent desiccation over the duration of the experiment.

This experiment was run outside of the main period during which *M. australis* adults would typically emerge (i.e. November and December) (Hitchcock 1965, Samson and Harris 1998). The experiment commenced on the 12th August 2021 and concluded on the 4th November 2021. Petri dishes were checked every two days. The date that adult females emerged on was recorded at which point they were transferred onto moist filter paper in individual Petri dishes and were maintained for the remainder of the experiment in the same temperature treatment from which they emerged.

3.3 Molecular characterisation of ground pearl populations

3.3.1 DNA extraction protocol for GP

A range of different DNA extraction methods were trialled to determine an effective way of extracting DNA from individual ground pearls. Initially this involved a modification of the salting out method (Sunnucks and Hales 1996).

GP cysts were surface sterilized by soaking in 5% bleach solution for 1 min then three successive 1 min washes in sterile distilled water. The GP was homogenised in a 1.5 mL microfuge tube by a glass rod after the addition of 10 µL 4 mg mL^{-1} Proteinase K to facilitate tissue breakdown. 400 µL TNES solution (50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) was added as lysis buffer by rinsing the glass rod to retain as much DNA as possible. The glass rod was sterilized by repeated wiping with clean tissue and 70% ethanol between each round of isolation. Tubes were incubated at 50 °C for over 5 hours or overnight. Following incubation, 100 µL 5 M NaCl was added to each tube to precipitate protein, followed by hard shaking for 1 minute and then tubes were centrifuged at 13,000 rpm for 10 min. Approximately 400 µL of supernatant was transferred to a new tube, then 1,000 µL cold (-20 °C) 100% ethanol (2.5x volume) was added. Tubes were centrifuged for 10 min at 13,000 rpm, the supernatant removed, and the resulting pellet was washed with 1,000 µL 70% ethanol. After centrifuging and removal of all liquid, the DNA pellet was re-suspended in 40 µL of pure water.

DNA quantity and quality were assessed using a Thermo Fisher Nanodrop Spectrophotometer.

In addition to the salting out method, two commercial kits were trialled: the QIAGEN DNeasy Blood & Tissue kit (69504) and the Meridian Bioscience ISOLATE II Plant DNA kit (BIO-52070). The ISOLATE II Plant DNA kit was employed for genome sequence quality DNA. For this work, instead of using a glass pestle to homogenise the GP, the initial tissue maceration was conducted using the bead beating approach. This involved placing the sample and buffer into a 2 mL screw-cap tube containing a 4.5 mm diameter steel ball. Tubes were processed using a FastPrep machine for 40 s at setting 6.5. When the sample had settled, the supernatant was extracted as per the manufacturers' instructions.

Following engagement with an international expert on ground pearls, Prof. Penny Gullan (ANU), it was considered advantageous to attempt a semi-destructive DNA extraction technique that would facilitate the retention of the diagnostically important cyst cuticle. As such, the salting out method was applied to a series of white ground pearl cysts with three different processing methods. These were:

1. Full destruction where the glass rod was used to grind the entire cyst as per previous extraction protocol;

2. Pin-prick where a small piercing in the cyst cuticle was made with a fine pin before placing the cyst in the extraction buffer; and

3. Gentle popping where the glass rod was used to gently 'pop' the ground pearl cyst at the bottom of the tube in the buffer.

The sample tubes for series 3 were retained after incubation and removal of the supernatant and consigned to Prof. Gullan to determine if sufficient morphological taxonomic features were present for adoption of this methodology. If this was the case, there was the potential to establish hologenophore material, whereby new species may be described based on their DNA sequences, with sufficient physical structures present to facilitate taxonomic description.

3.3.2 Molecular taxonomy of Australian GP

Initial work involved amplification and sequencing of standard diagnostic loci for insects (Table 1). It was found that while the cytochrome oxidase amplified and sequenced well, the ribosomal genes often resulted in poor sequence quality, likely owing to the presence of pseudogenes.

Table 1. General insect primers used in this study.

Gene	Primer pairs	Primer sequences (5' to 3')	T_a(°C)	Estimated product length (bp)	Reference
Cytochrome oxidase I	C1-J-1718	GGAGGATTTGGAAATTGATTAGTTCC	66.1	506	Simon et al. (1994)
	HC02198	TAAACTTCAGGGTGACCAAAAAATCA	66.9		
Cytochrome oxidase I	C1-J-1718	GGAGGATTTGGAAATTGATTAGTTCC	66.1	472	Percy et al. (2018)
	C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC	59.9		
18S rRNA	18SF	ATGATAACTCGACGGATCGC	63.9	600	Floyd et al. (2005)
	18SR	CTTGGATGTGGTAGCCGTTT	63.8		

Alignment files were generated from Australian GP using diagnostic loci identified from the genomic sequencing approach (below). These loci were nuclear (18S ribosomal RNA genes), mitochondrial (cytochrome oxidase) and also the 16S rRNA genes of a bacterial symbiont that was found to consistently be associated with the different GP samples analysed.

Phylogenetic analyses of the nuclear ribosomal, mitochondrial and endosymbiont ribosomal genes were conducted using the Maximum Likelihood algorithm of MEGAX (Kumar et al. 2018). Distance

matrices were calculated using a general time reversible model, sites were gamma distributed and trees were inferred via nearest-neighbour interchange. Trees were subjected to 1,000 bootstrap replications. As the resulting trees shared identical topologies, a concatenated tree comprising 4,673 sites was compiled for each of the samples analysed.

3.3.3 Genome sequencing of Australian GP

Given the unsatisfactory performance of the ribosomal sequencing, it was determined to develop large sequence datasets for selected GP by employing a metagenomics approach using Illumina shotgun sequencing. A total of 35 individual GP were consigned either to the Australian Genome Research Facility (AGRF) (https://www.agrf.org.au/) or to the Australian Centre for Ecogenomics (ACE) (https://scmb.uq.edu.au/centres-and-institutes/australian-centre-ecogenomics) for library preparation and paired end sequencing (2x 150bp reads on the NovaSeq6000). The raw reads were processed with Trimmomatic (ver. 0.39, ILLUMINACLIP:2:30:10, SLIDINGWINDOW:4:15 and MINLEN:50) for quality filtering. Quality-controlled reads were then assembled using MetaSPAdes (ver. 3.15) with default parameters. Contig assemblies were provided as text files.

Contig files for each of the GP samples were uploaded onto BioEdit Sequence Alignment Editor. This facilitated generation of BLAST libraries to identify homologous regions between the genomes. Ribosomal RNA gene sequences from related scale insects were retrieved from the NCBI Genbank database and used to interrogate the genome assemblies in order to secure homologous genes from the GP samples. This facilitated the production of an alignment file of over 3,000 bp covering all the ribosomal genes and internal transcribed spacer sequences of the GP in this study. From this, a new set of primers were designed to specifically amplify GP ribosomal elements. These were trialled in PCR and sequencing experiments on a range of different GP extractions from this study.

Mitochondrial sequences were also retrieved, as well as sequences associated with the newly discovered endosymbionts.

In addition to the samples analysed during this project, in collaboration with overseas partner Prof. Juang Chong (Clemson University, South Carolina, USA), the genome data for 12 pink ground pearls from the USA were generated. These data were added to the current project for comparative purposes.

3.3.4 Dispersal patterns and investigation into genetic exchange

Bioinformatic analysis on the raw contigs of the initial white ground pearl genome sequence data (Gatton1) was conducted to identify prospective microsatellite alleles for analysis of dispersal patterns. A selection of the loci identified in sample Gatton1 were then compared with the genome data for WGP collected from Banana, Takilberan, Gatton2, Good night, Eidsvold and Brian Pastures to determine if there were any variants.

3.3.5 Development of soil test for GP

Ground pearls are a cryptic root-feeding insect whose presence is generally not even suspected by graziers. This is because they are small (~2 mm), look nothing like other insects, and can readily be confused for fertiliser of quartz granules. During the course of this project we have found that virtually no graziers knew of these insects until we uncovered them on their properties. Nowhere was this more pronounced than at Brian Pastures, a property that has had an iconic involvement in

the development and support of Queensland's cattle industry, but where, until our project, it was not known that ground pearls were present.

An increasing number of graziers are now interested in ground pearls and what are their impacts on production. Under most circumstances it is expected that ground pearl damage is self-limiting. However, when climatic conditions are unfavourable for the host, the plants will be stressed and more likely to succumb to ground pearl feeding pressure.

During this study we have located ground pearls in a range of pasture settings spanning over a thousand kilometres throughout Queensland. The early part of our work demonstrated that there was no apparent chemical or physical soil signatures that could be used to predict their occurrence or abundance. Furthermore, the necessity of having to wet sieve soil and then further inspect the sieves is a significant rate-limiting step for ground pearl analysis. As such, it was considered that the development of a molecular soil test for ground pearls would facilitate more rapid assessment of ground pearl numbers and impacts.

Following the initial genetic characterisation work, it was considered that the internal transcribed spacers (ITS) of the ribosomal RNA genes of the ground pearl would be a good target for the development of a quantitative PCR (qPCR) assay for ground pearls. This was because there was sufficient variation present (Fig 3) to be able to differentiate species based on diagnostic melt curves. A set of primers were developed (GP28SF 5' GGAGGTCCGCAGCGATTCTG and GP28SR 5' AAACCCTTCTCCACGGCAGC), however, inconsistent amplification occurred likely due to the presence of pseudogenes.

Fig 3. Genetic variation in the internal transcribed spacer (ITS) of yellow, white, brown and pink ground pearls.

The next strategy was to look for other targets. Multiple GP endogenous genes were considered, but then the genomic sequencing data (4.3.3) led to a new strategy. As there was approximately 10 x more coverage of the endosymbiont DNA than the GP DNA (owing not only to copy number but the short length of the endosymbiont genome) and given that each ground pearl species had its own highly specific endosymbiont and that these were consistently maternally inherited, it was considered that an assay targeting the endosymbiont would be equally informative as one targeting the endogenous DNA of the ground pearl itself.

Endosymbiont 16S rRNA gene sequences were aligned and a range of qPCR primers spanning informative sequences were designed (Fig 4). These were tested *in silico* to determine whether diagnostic melt curves could be generated to differentiate GP species. Of these, the most promising were GPENDF1- ACCCTGGTAGTCCACGCTGT and GPENDR1- ACGACAACCATGCAGCACCT. A range of ground pearls comprising all known Australian species were tested using a SYBR Green assay. This was 6.25 µL 2 x Quantinova SYBR reagent, 0.625 µL of 20 µM forward and reverse primers, 0.104 µL of ROX passive reference dye, 4.896 µL H_2O and 1 µL of each template. Thermocycling involved 5

min at 95°C, 40 cycles of 95°C (15 s), 60°C (15 s), 72°C (15 s), then a melt stage ramping between 60°C and 95°C, with 1°C increments for 5 s each. All assays included no template controls (NTC) as well as known positive samples.

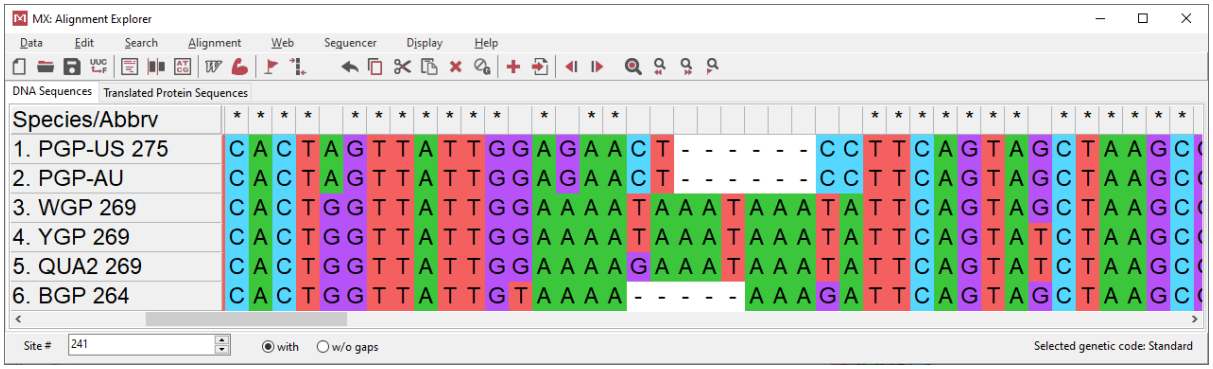

Fig 4. Partial 16S rRNA gene sequences for the endosymbionts of pink (1-2), white GP (3), yellow (4-5) and brown (6) ground pearls.

Twenty soil samples known to be infested with white GP were collected from a known site at UQ, Gatton. Most commercial soil DNA extraction kits have a maximum capacity of 250 mg of soil, but with that relatively small amount it is possible to miss GP if they were present. Therefore soil samples were weighed into 10 g subsamples, visually confirmed to contain GP, then consigned to Metagen (https://metagen.com.au/) for DNA extraction. DNA concentration and quality was checked and then DNA was received back in the UQ lab and subjected to qPCR testing using the GPEND primers. Additionally, 12 DNA extractions from soil with no history of GP were included for testing. Representative DNA from all known Australian GP species was included as controls in all of the qPCR assays, in addition to NTCs.

Following initial results from the soil extractions, another set of primers was designed targeting the endosymbiont DNA. For this, the whole genome sequences generated in this study for the endosymbionts was aligned using MAUVE (Darling et al. 2004). Included in this alignment was the full genome sequence for the closest known relative with a genome sequence available, *Sulcia muelleri.* Several regions conserved among GP but distinct from that of *S. muelleri* were selected for analysis. After identifying the regions, the respective loci from the GP endosymbiont genome datasets were retrieved and aligned using MEGAX. A new set of primers (30KF TTCAGCTCATTGGGATGG and 30KR ACTTGTTCTTTTGAAGC) were developed and tested in the same manner as the previous sets.

In addition to our in-house testing, we consigned ground pearl infested samples to Metagen for metabarcode analysis. This analysis included using primers that nominally amplify all ecdysozoans (eg. arthropods, nematodes, annelids etc), and so should amplify ground pearls. Likewise, a bacterial screening was conducted in order to determine if the diagnostic endosymbiont DNA could be detected.

3.4 Development of Integrated Pest Management (IPM) program for ground pearl

Key experimental findings can be used to commence the development of an integrated pest management program for ground pearls. These are discussed in 4.4.

4. Results

4.1 Confirm and quantify the presence of ground pearls at PD sites

4.1.1 Site inspections

In total, 48 Pasture Dieback sites were surveyed (Fig 5). Of the dieback sites surveyed, paired samples (i.e., symptomatic sample with a corresponding asymptomatic sample) were collected from 25 sites while single symptomatic samples were collected from the remaining 23 sites. A total of 76 samples were collected from symptomatic (48 samples) and asymptomatic (28 samples) regions across these dieback sites. The first dataset is comprised of 15 single sample sites and 17 paired sample sites, and the second dataset consists of 8 single sample and paired sites.

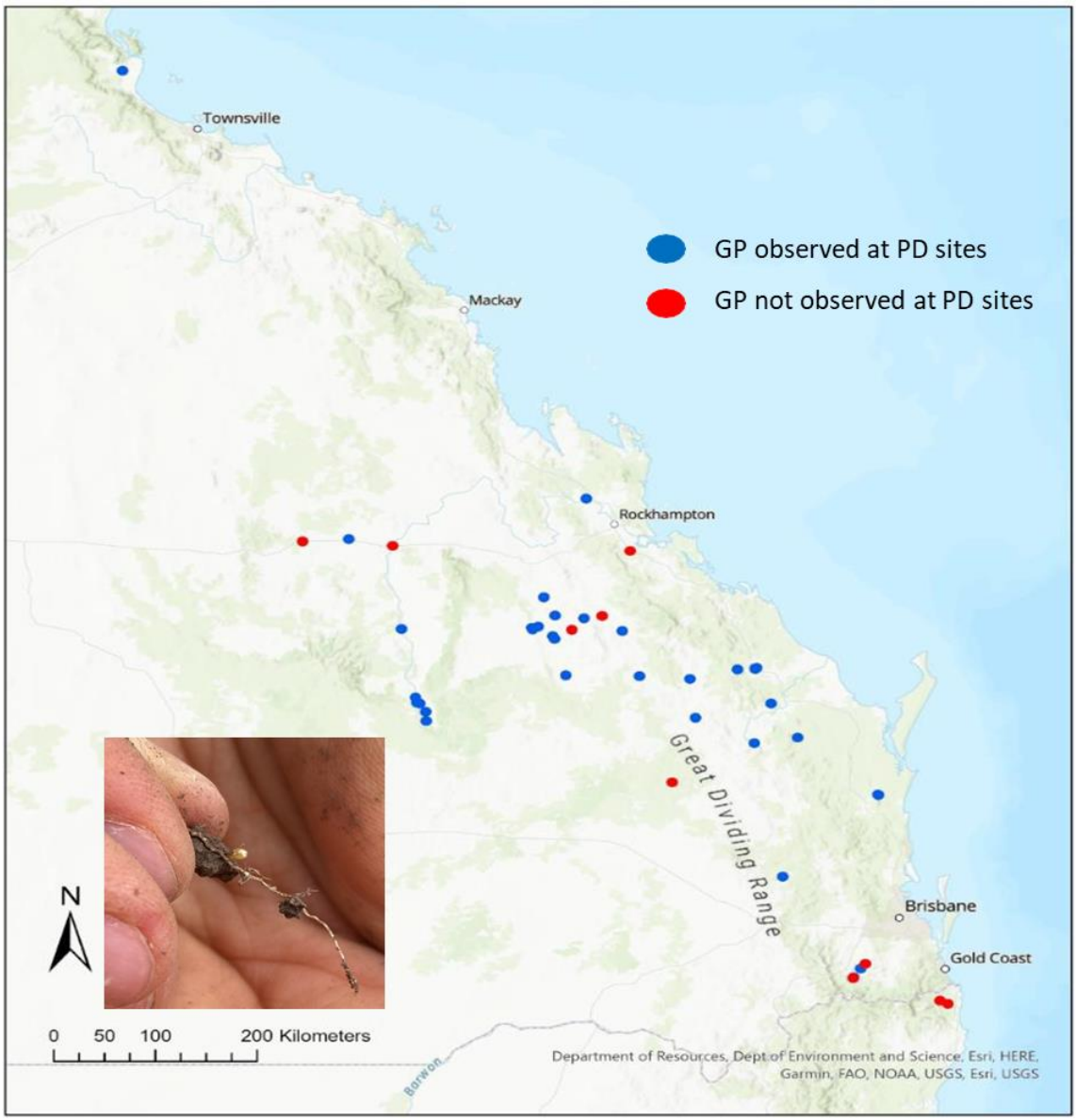

Fig. 5 Summary of pasture dieback sites sampled. Blue dots indicate where ground pearls (white, yellow and brown) were identified; red dots are sites where they were not identified associated with pasture dieback sites. Inset: cyst of *Margarodes australis* attached to grass root.

Ground pearls were identified at pasture dieback symptomatic sites, as well as asymptomatic sites. There was a statistically significant higher number of ground pearl cysts in the symptomatic sites (Fig 6). This does not suggest that ground pearls are the primary cause of pasture dieback, but they are likely having adverse impacts where they are present.

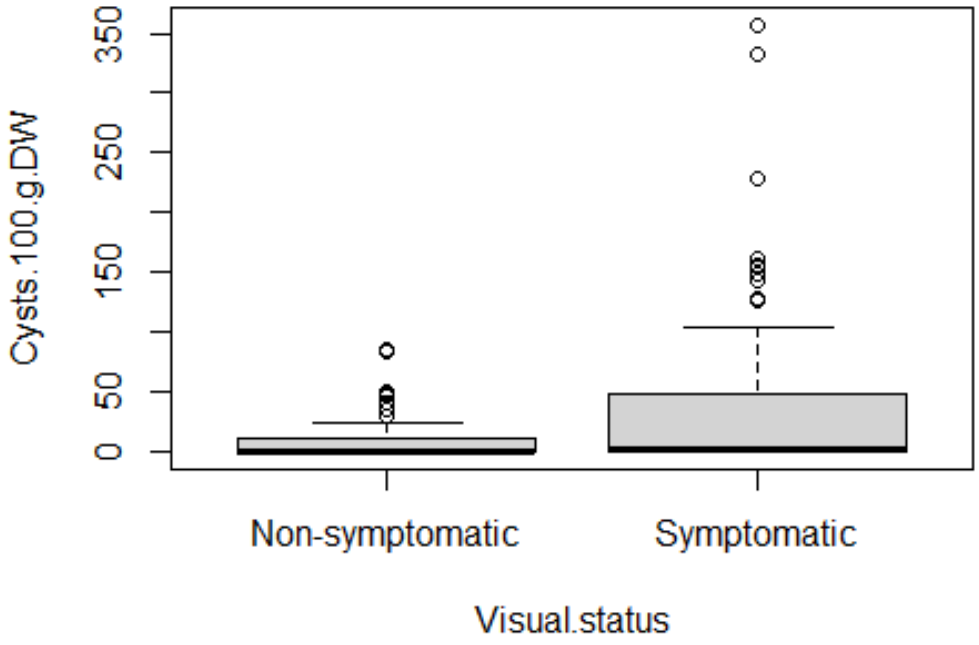

Fig 6. Variation in the number cysts found in symptomatic areas compared to asymptomatic areas. To compare across different sites and soil types, the number of white ground pearls in a sample were calculated on 100 g of dry weight (DW) soil. The rectangular box spans from the first quartile to the third quartile. The whiskers above the plots illustrate the maximum value. There are no whisker below the plots as zero is the minimum number of cysts that can be observed. The thick black lines inside the rectangle represent the median, while the dots are the outliers.

4.1.2 GP enumeration

Both of the sieving methods had high precision, but the 2 sieve method was significantly quicker (Table 2). The flotation method was significantly less efficient and was therefore abandoned. Subsequent GP extractions from soil used the 2 sieve method.

Table 2. Summary of results for GP extraction efficiency. Superscripts indicate statistically significant differences.

Extraction Method	Precision	Time (s)
4 sieve	98 [a]	368 [a]
2 sieve	98 [a]	232 [b]
2 sieve + 4M sucrose	75 [b]	282 [b]

4.1.3 Machine learning

The machine learning approach shows significant promise for future detection and enumeration of ground pearls. The initial model was trained on a limited number of images, using patches, yet achieved an average precision of 0.829 on the validation set for the first dataset. While the model was able to detect most of the ground pearls accurately, it tended to misidentify round rocks and dirt as ground pearls. This process is still under further optimisation and will be applied to unknown samples as part of its development.

4.1.4 Biophysical characterisation

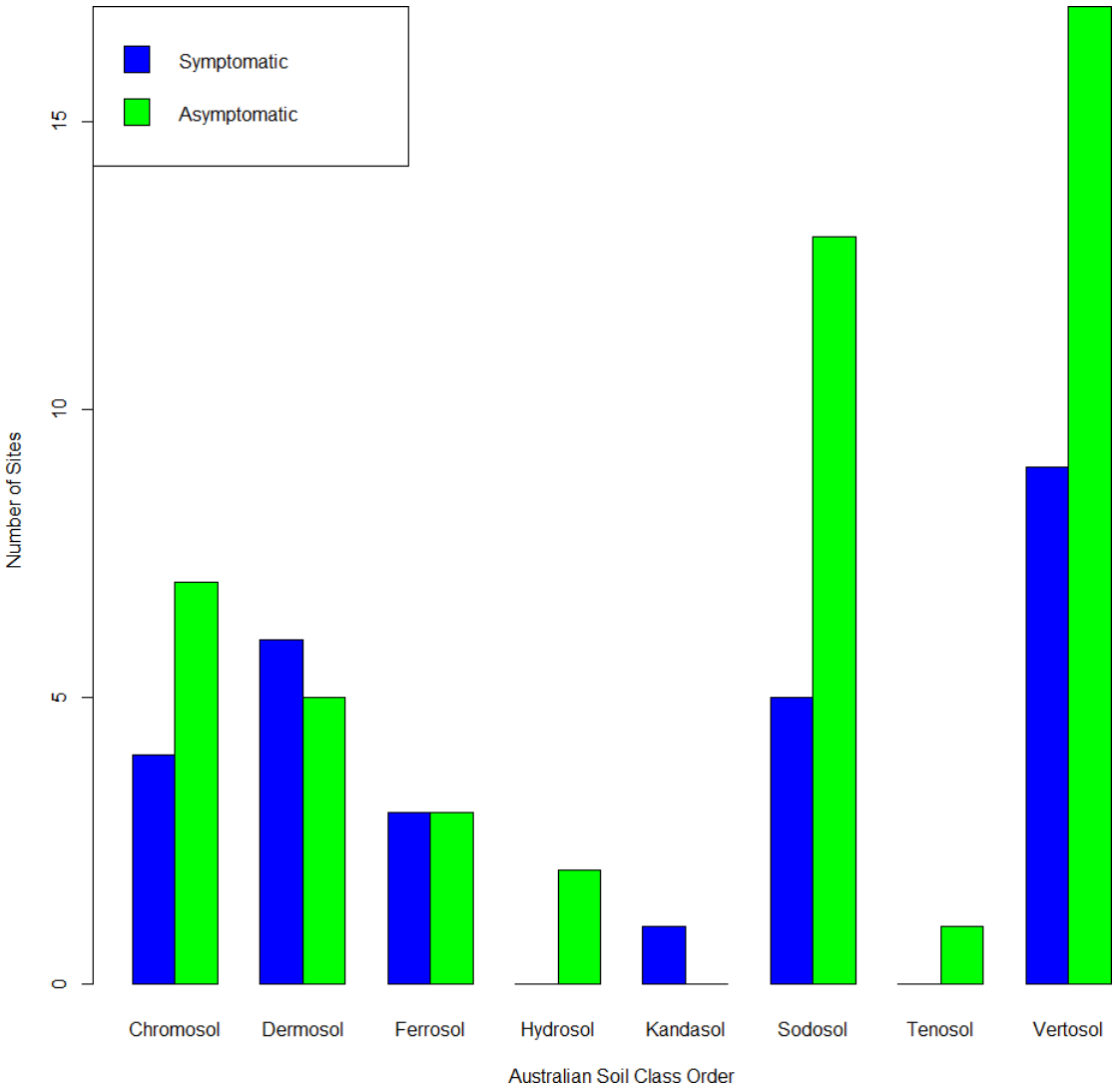

Fig. 7 Recorded soil type for the asymptomatic (green) and symptomatic (blue) sites surveyed.

Samples collected for the surveyed sites ranged across eight different soil types (Fig. 7). Vertosols and sodosols were the predominant soil types reported for samples, however, there was no significant relationship between dieback symptoms and soil type.

Similarly, no significant differences (P = 0.4) were observed for either physical or chemical soil characteristics of symptomatic or asymptomatic sites (Fig 8). A greater proportion of the variability

in the data was explained by the first two principal components for physical soil characteristics (73%) then chemical soil characteristics (56.8%).

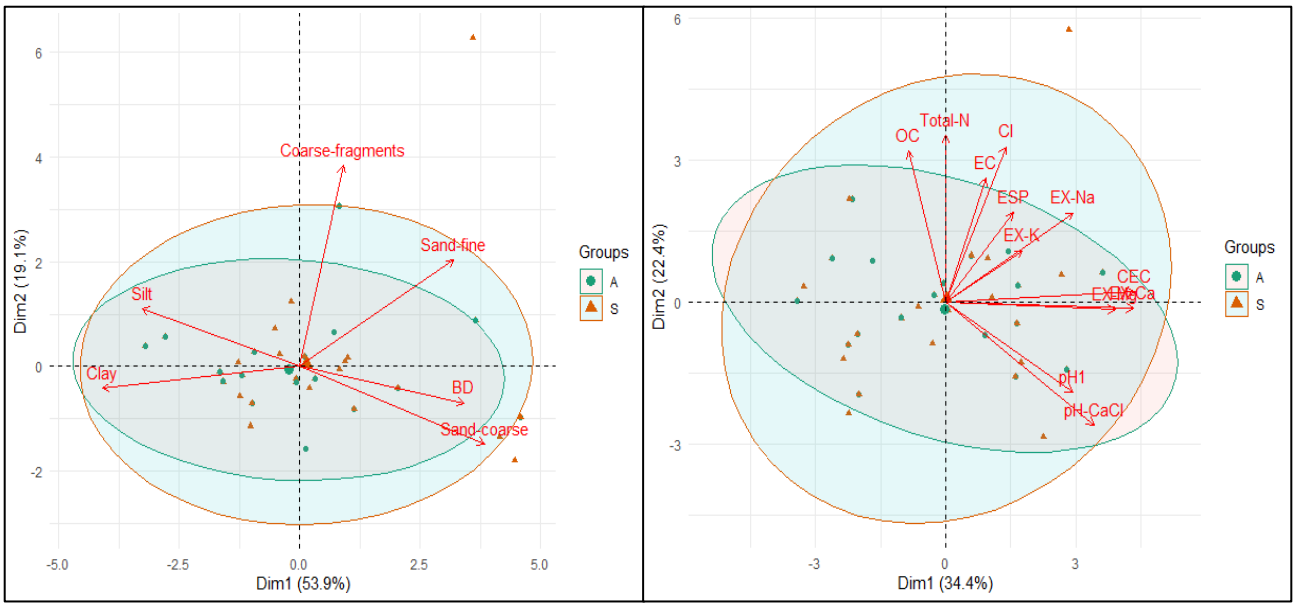

Fig. 8. PCA biplots of physical (I) and chemical (II) soil characteristics for asymptomatic (green) and symptomatic (blue) Pasture Dieback sites. The respective physical and chemical soil characteristics are loaded as eigenvectors. For the physical soil characteristics plot (I), 73% of the variation in the data is explained by principal components 1 and 2. Comparatively, only 56.8% of variation in the data is explained by the first two principal components for chemical soil characteristics (II).

4.2 The impact of ground pearls on a range of host plant species

4.2.1 Host range

While we have successfully managed to stimulate a small number of ground pearls to emerge from their cysts and oviposit, we have been unable to culture these insects sufficiently to infest new plants. As such, we have been unable to determine the host range or preferences for ground pearls.

4.2.2 Rhizobox establishment

Twenty rhizoboxes were established including 'guard' rows (Fig 9). These included treatments containing 500 dissociated cysts, with controls containing no cysts. Buffel grass (cv. Gayndah) was planted in each box. No obvious differences in plant appearance occurred throughout this study. There were no statistically significant differences in plant dry weight or any indication that the cysts had any impact on the grass.

When the rhizoboxes were inspected there was some evidence of attachment of some ground pearls, but it could not be established whether that was plant contact with the cyst, or whether the cyst's proboscis was attached to the root. Each treatment rhizobox initially contained 500 cysts, and grass roots penetrated to the bottom of the rhizobox. If cysts could actively attach to roots it might have been expected that many would have attached and there would have been a commensurate decrease in plant dry matter.

Fig 9. Rhizobox experiment established to monitor impacts of white ground pearls on buffel.

Despite each treatment box containing 500 cysts, there was little evidence of attachment. This suggests it is the nymph stage that attaches to the roots.

4.2.3 Ground pearl emergence study

Over the course of this experiment, 17 white ground pearl adults emerged at multiple different constant temperature treatments (Fig. 10). While the number of adults that emerged is not statistically significant, there are only two other published records of instances where margarodid adult emergence was achieved under controlled conditions (Gonzalez et al. 1969, De Klerk et al. 1980).

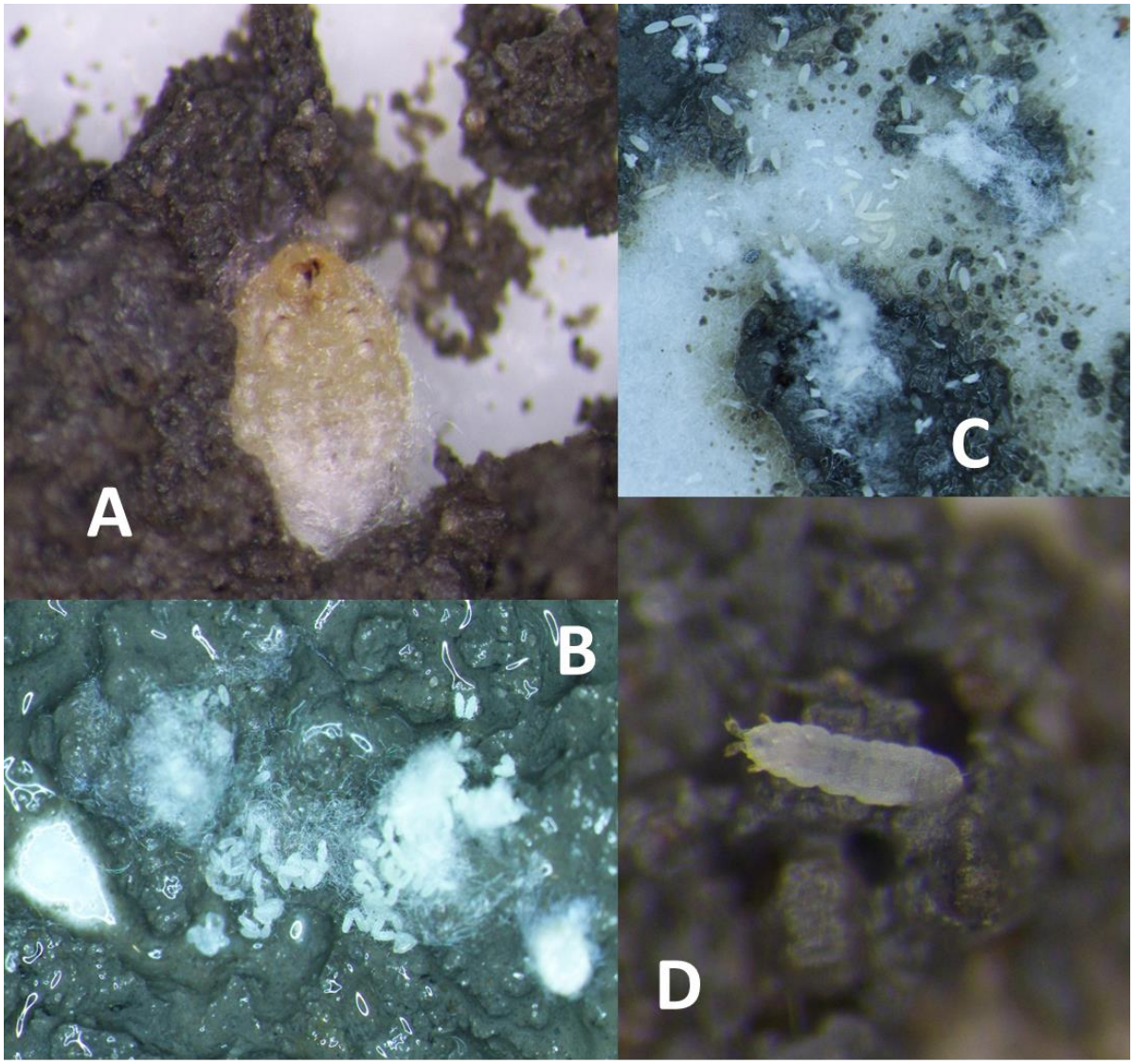

Fig. 10. Reproduction of the white ground pearl, *M. australis*. A. Emerged adult ♀ weaving her ovisac. B. Eggs associated with ovisac. C. Eggs (translucent) and hatched nymphs (off-white) associated with ovisac. D. Nymph white ground pearl. Note large club-like antennae, suggesting olfactory cues are associated with host selection.

This experiment also resulted in 11 of the female *M. australis* adults successfully ovipositing and the hatching of first-instar nymphs from the eggs of 5 of those adults (Fig. 10). This is the first time that several of these life stages for the white ground pearl have been photographed. Approximately 250 first-instar nymphs were found and successfully transferred onto seedlings of Buffel grass (cv. Gayndah) grown in McCartney bottles. However, the seedlings were unable to be successfully sustained in these bottles. Approximately 50 first-instar nymphs were collected and stored in 80% ethanol and glycerol to be used for taxonomical purposes.

4.3 Molecular characterisation of ground pearl populations

4.3.1 DNA extraction protocol for GP

PCR and Illumina quality DNA was readily extracted from GP using the salting out method and the commercial kits.

For the production of potential hologenophore material, it was found that the pin-prick method did not liberate sufficient DNA to be useful at this stage. However, the gentle popping method facilitated retention of diagnostically valuable cuticle material in addition to liberating sufficient DNA for whole shotgun sequencing. As such, this method has since been used for all subsequent extractions.

Over 300 GP samples were subjected to DNA extraction and PCR analysis.

4.3.2 Molecular taxonomy of Australian GP

This is the first study to characterise the genetic relationship among Australian ground pearls. It has previously been established that at least 4 GP species are present in Australia. There is evidence of a 5th GP species from the Northern Territory collected in January 1858, however, the samples were too degraded to allow for taxonomic certainty (Jakubski 1965).

Our genetic study confirms the previous taxonomic arrangement whereby Australia has 4 known GP species. These are the white GP (*Margarodes australis*), brown GP (*M. williamsi*), yellow GP (*M.* nr. sp. *sinensis*) and pink GP (*Eumargarodes laingi*).

Throughout this work we have liaised with Prof. Penny Gullan (ANU). She has advised us that she is in the process of reviewing the current taxonomy and sees a case for re-establishing the original genus of the white ground pearl, *Promargarodes*. Our data support this move, as well as placing the yellow ground pearl in the same genus. The brown ground pearl is clearly further removed, while the pink ground pearl serves as an outgroup to the three other species.

In addition to being recorded in Australia, the pink GP is also present in the USA. As it cannot have evolved in two places at once, it is clear that either Australia got ours from the US, the US got theirs from Australia, or that both the US and Australia received pink GP from another unidentified source. Our data suggest that if the remaining three GP species evolved in Australia, it is more likely that the pink GP was acquired from elsewhere because it is the most distantly related. Analysis of the genomic data from US specimens has not been a priority of the current work, but there is apparently virtually no differences among Australian and US specimens. The first record of the pink ground pearl was from sugarcane in Bundaberg in 1938. Out of interest, it was discovered by Reginald Mungomery, who was partially responsible for releasing the cane toad in 1935. There was significant varietal exchange during this period, and it is well established that at least one sugarcane variety (CP29-116) was received in Bundaberg in 1935 (King 1965), the year that the first sugarcane quarantine facility was opened in Brisbane (Bell 1935). Thus there is the possibility that the pink ground pearl came to Australia with sugarcane plants. It is likely that the longevity of ground pearls could see them transported long distances within grazing industries by contaminated earth and machinery.

A significant outcome of the molecular taxonomy work was the discovery of a highly specialised endosymbiont that appears to be involved in amino acid synthesis for the host. Within a ground pearl are at least three separate DNA species: that held in the nuclei of the ground pearl cells themselves, that of the mitochondria, and that of the endosymbiont. That these are transmitted as a unit is clear from the phylogenetic analysis, where the three independent lineages accurately recapitulate each other's phylogenetic arrangement (Fig 11). This has major significance for Integrated Pest Management strategies, as will be discussed in that section.

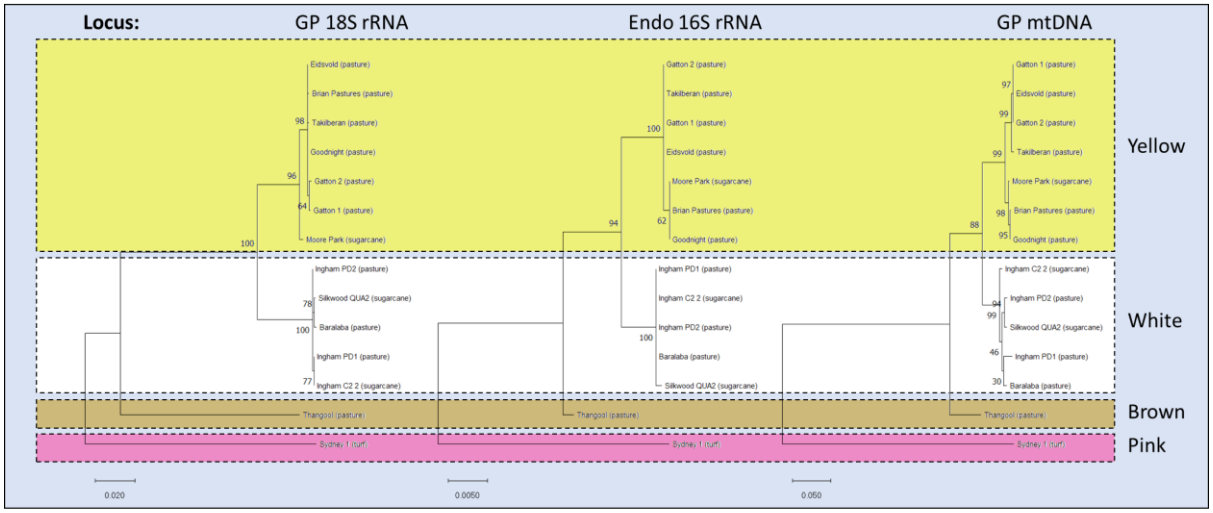

Fig 11. Phylogenetic recapitulation of three independent genetic (nuclear DNA, endosymbiont DNA and mitochondrial DNA) lineages through ground pearls. This is strong evidence for asexual reproduction and direct maternal contributions of both endosymbiont and mitochondrial lineages.

The endosymbionts found in ground pearls are related to other endosymbionts found in diverse insect assemblages. When phylogenetic analysis was completed on a broader group of GenBank accessions, it was found that the endosymbionts in ground pearls are almost as distinct from those of other Hemipterans than they are to the endosymbionts in cockroaches (Fig 12). This indicates a far more ancient divergence of ground pearls from other scale insects than what is traditionally thought.

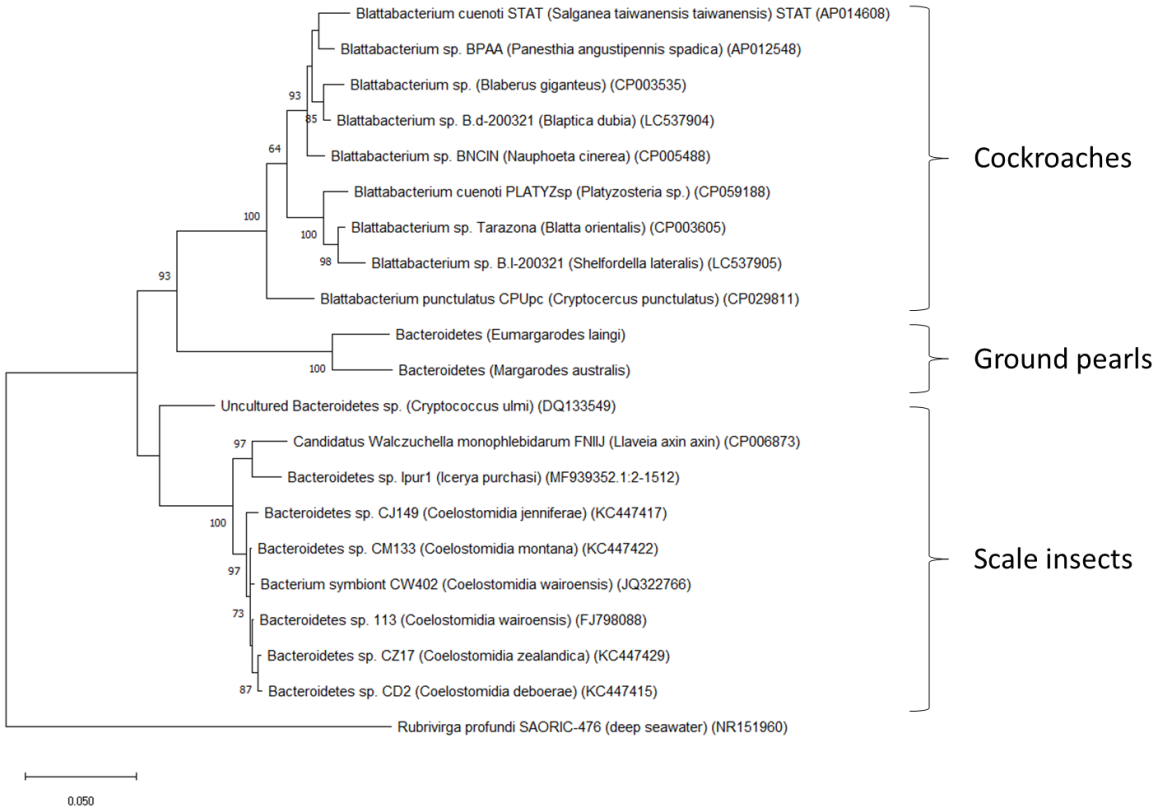

Fig 12. Relationship between endosymbionts from ground pearls, related scale insects and cockroaches. It can be inferred based on the ancient radiation of endosymbionts present, that ground pearls are more distinct from scale insects than is traditionally thought.

4.3.3 Genome sequencing of Australian GP

A total of over 1 Gb of data were generated for each of 35 samples. The number of contigs generated from each sample varied significantly between ~20k and 450k. Investigations were undertaken in an attempt to develop long read scaffolds upon which to align the contigs using the nanopore sequencing technology (*eg.* Minion, Promethion), however, insect DNA extractions appears to contain co-extracted compounds that clog the nanopores (Victoria Coyne, Genomics Project Coordinator, Central Analytical Research Facility (CARF)). While we are reasonably confident, we have captured the full sequence data associated with the GP genomes, without the chromosome assembly we are unable to produce a fully annotated GP genome as we had intended.

The coverage of the contigs varied significantly. With 1 Gb of sequence data generated for each sample, endogenous nuclear GP sequences were typically present at over 20 x coverage. In contrast, mitochondrial elements typically had x 100 times coverage. This allowed for retrieval of what is believed to be the full genome sequence (29,198 bp) for mitochondria in the white ground pearl. However, consistently the highest coverage (300-500 x) was a contig of approximately 200,000 bp from each of the samples. It was noted that this contig had a very low (25%) GC contig. This is higher than the GC content of GP mitochondria (10%), but much lower than that of GP nuclear genes (40-60%). This led to the expectation that the major contigs in each assembly were highly evolved (GC depletion akin to mitochondria) bacterial endosymbionts associated with the ground pearls.

In order to confirm the discovery of GP endosymbionts, bacterial 16S rRNA gene sequences were retrieved from GenBank and used as search items in a relaxed local BLAST of the first white ground pearl genome sequence data set. This facilitated retrieval of the complete 16S rRNA gene sequence for the GP endosymbiont, which was then used in a more stringent local BLAST against the remaining genome contigs. This allowed for retrieval of 16S rRNA genes from the assemblies of all of the species of GP analysed in this study. As expected, these were in almost all cases retrieved from the largest contig in the assembly, which also had the highest coverage. It can thus be concluded that the endosymbiotic DNA constitutes a high proportion of the DNA in the GP extractions.

Core bacterial genomes are circular. However, the implementation of shotgun sequence assembly results in linearized contigs. As such, using the pink ground pearl as a test case, outward facing primers from both ends of the contig were designed in order to provide a circularised assembly. These were: Psym1F 5’ GTCTTGATAACCCTCTCATCT and Psym1R 5’ AAGGACGAGGTTATGTACCC. PCR amplification was successful, and sequence analysis of the amplified fragments from several samples were overlaid in an alignment consisting of the known ends of the contig using MEGAX (Kumar et al. 2018). This analysis successfully demonstrated that the large contig common to most of the samples sequenced was actually a bacterial endosymbiont.

Using a similar process to that described for the pink ground pearl, the endosymbionts of the white, yellow and brown ground pearls were also circularised and aligned to the putative origin of replication (*ori*). Gene annotation for each species was completed using PROKKA software. The pink ground pearl endosymbiont had a genome size of 211,819 bp and contained a total of 197 coding sequences. The majority of these sequences were for house-keeping genes, but a significant number were involved in amino acid synthesis.

With the complete endosymbiont genomes available, it was possible to confirm very strong stabilising selection based on the extremely conserved arrangement of the genes even among the endosymbionts of distantly related species such as the white ground pearl and the pink ground pearl. In comparison with the genomes of endosymbionts from another sap sucking hemipteran insect (*eg. Sulcia muelleri*), the conserved arrangement of the endosymbiont genomes of ground pearls is even more pronounced (Fig 13). This has major significance for IPM.

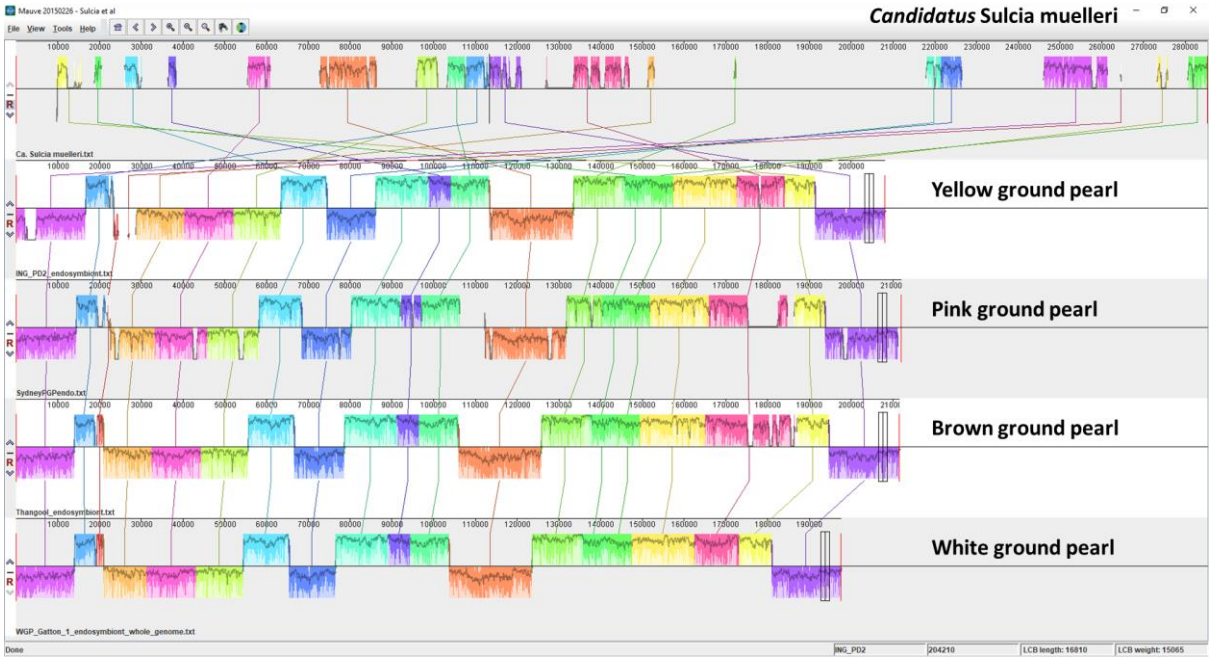

Fig 13. Comparison of genome arrangements among ground pearl endosymbionts. As is clear by the colour coding, the endosymbionts in the ground pearls have virtually all the same genes in the same order and coding in the same direction. This indicates extreme stabilising selection, which offers significant promise for future management.

4.3.4 Dispersal patterns and investigation into genetic exchange

A total of 216,186 prospective microsatellite primer pairs were identified for the white ground pearls. A subset of 106 primer sets were selected for further analysis. The microsatellite sequence (highlighted yellow, below) as well as the flanking non-microsatellite sequence, were used as inputs for local BLAST searches of the genome databases, having adjusted for being small (~140 bp) input sequences.

GGGTCAACCTACTAGCTGGAACGAAATTCAGTAGTATCGCCACACCGAGTTACCTTAGCAAACAGCAAACTGA
AAACTGAAAAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGTACACGCCACACGAGCTACAGTACC

However, this identified a very high level of relatedness among WGP collected from a wide geographic area. This finding was confirmed with the genome sequencing approach. The high level of genetic relatedness uncovered in the GP through the genome sequencing and the absence of males for the species present in Australia led the project team to focus efforts on developing consistent testing protocols for this species.

4.3.5 Development of soil test for GP

It was expected that a metabarcoding approach would be effective for detecting ground pearls in a known infested soil sample. However, although the samples had significant numbers of ground pearls, no ground pearl or endosymbiont DNA could be detected using this approach. This could be attributable to sample preparation or PCR bias in the early stages of amplification. We do not expect that the sample preparation would be adding significant biases as the DNA extraction method allows for the detection of extremely resilient bacteria belonging to the Microbacteriaceae. These typically

are difficult to extract and require significant physical forces. As such, if these are being detected, it is unlikely that the lysis steps are inadequate for ground pearls.

In parallel work examining the impacts of plant parasitic nematodes (PPN) on grass species, we have uncovered previously unsuspected PCR biases against a particular group (Tylenchidae). This resulted in metabarcodes of free-living and other nematodes, but not that of PPN which we knew were present via physical extraction using Whitehead trays. As such, we redesigned the initial PCR primers and now effectively amplify the Tylenchidae as well as the free-living nematodes. There is also the possibility that the amount of DNA present in a 10 g soil sample will lead to swamping of the target using generic metabarcoding primers. Therefore it was considered that a specific qPCR approach would be better to achieve this objective.

Initial work targeting the endogenous nuclear ribosomal genes of the ground pearls was unsatisfactory, owing to the co-amplification of pseudogenes. While we got good amplification of the expected target (Fig 14), when we attempted to sequence the target to confirm host specificity, the Sanger sequences became mixed partway through (Fig 15).

Fig 14. 1.5% agarose gel showing amplification at the expected 600 bp.

Fig. 15. The likely presence of pseudogene copies of the ribosomal genes of *M. australis*. The sequence read is good up until base 120, but then two signals appear, making the trace unreadable.

Given the high copy number of the endosymbiont genomes in GP extractions, a new strategy was developed to use qPCR to target the endosymbiont DNA. The target selected was the 16S rRNA. This was selected as there is an enormous amount of reference sequence data available on the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) database with which to select specific regions.

The primers selected were shown to differentiate the GP species based on their melting curves (Fig 16), both *in silico* and then confirmed experimentally. This was established by conducting qPCR in DNA extractions from individual GP comprising the known Australian diversity. Thus these primers can be effectively deployed on individual GP for a rapid species diagnosis.

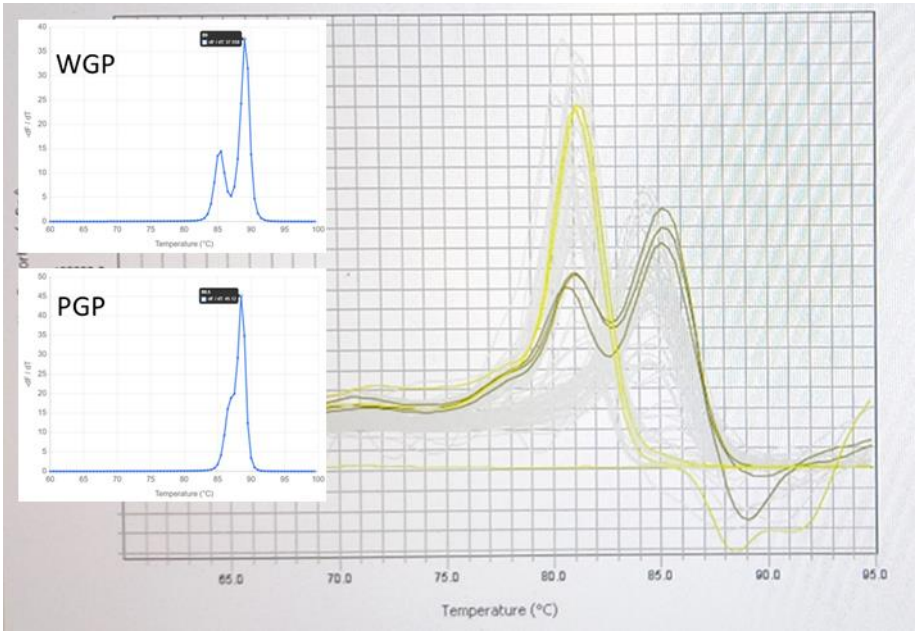

Fig 16. *In silico* testing (insets) and experimental melt curve data for white and pink ground pearls.

Primers were developed based on the 16S rRNA gene of the endosymbiont.

Having demonstrated specific and differential qPCR amplification from individual GP, the next step was to trial the assay on soil DNA extractions. While we achieved amplification using the GPEND primers from infested soil DNA extractions, we also had amplification from non-infested soil. It was therefore considered that there must be other bacteria present in soils with sufficient homology to the GPEND priming sites to result in non-specific amplification.

Therefore a new strategy was adopted. BLAST searching of the 16S rRNA genes of the GP endosymbionts revealed that a close relative, *Sulcia muelleri* (an endosymbiont of other sap sucking insects) had a complete genome available. This was downloaded and then compared with the genomes of the GP endosymbionts using MAUVE. A region (designated 30k) was identified that was present in all known GP endosymbionts, and not present in *Sulcia*. This was selected as preferable to the 16S rRNA, as all bacteria have 16S rRNA, but not all bacteria are expected to have the genes that facilitate the relationship between the endosymbionts and their GP hosts.

Another series of primers were designed from the alignments and screened *in silico* prior to synthesis and optimisation. These primers are effective at identifying GP and are currently being tested on soil samples.

4.4 Development of an Integrated Pest Management Plan for GP

Ground pearls appear to be a widely prevalent group of root-feeding arthropods that are largely held in check by the availability or otherwise of host resources. It is likely they only become a problem when adverse environmental conditions lead to plant stress. This is analogous to reports from the sugar and turf industries where the pink ground pearl causes the most problem when conditions are unfavourable to the plant.

The first step in an IPM plan is detection and enumeration of the pest. This we are well on our way to achieving, having developed an efficient sampling and extraction protocol that can soon be augmented with a machine learning approach that facilitates further efficiencies. Additionally, we have developed qPCR protocols for detecting and identifying ground pearls. We are still in the process of optimising these tests for soil DNA extractions, but we see this as achievable.

Ground pearls are notoriously difficult to control in other settings as when they are encysted, they are virtually impregnable to chemical attack. It is only during the nymph and final adult stage that they are not in encysted form. Our research on temperatures favourable for emergence supports the concept that white ground pearls are likely to emerge from cysts to breed during warmer and wetter periods such as spring and early summer. This is intuitive in that at these periods the nymphs would be less subject to desiccation, and there should be abundant root growth to promote their attachment. This knowledge can be applied for targeted chemical applications when conditions are right for adult emergence and oviposition.

Furthermore, improved knowledge of the factors governing adult emergence can be used to develop a systems approach to promote emergence and then apply interventions. This concept is analogous to the 'suicidal germination' approach that has been used for weed-seed germination, such as what has been employed in IPM for the control of a parasitic weed of sugarcane, *Striga*. This works by getting the weed to germinate in the absence of a suitable host, leading to its death. It was hoped that by using root exudates, or other stimulants, to induce adult emergence, future ground pearl IPM could take advantage of the opportunity of the relatively unprotected nature of adults to facilitate chemical or biological control programs. Further work is required on this approach.

In addition to the synthetic chemistries already used to target insect pests, our understanding of the critical nature of the endosymbionts of ground pearls now provides us with novel targets for intervention. This could be highly specific RNA interference technology targeting key amino acid synthesis services provided by the bacteria. Used in a polyphasic approach that targets more than one of the genes, this could become a sustainable method that minimises the risk of resistance. Furthermore, CRISPR approaches could be used to deliver the intermediaries directly to the ground pearl. This approach has massive potential to control not just ground pearls, but other insects that feed on grasses of other crops. There is significant scope to develop this IP as a sustainable solution for many insect pest issues.

5. Conclusion

This project showed that ground pearls cannot be causing pasture dieback in all areas where pasture dieback occurs. Ground pearls are sedentary organisms with low dispersal capabilities. While they were found in 75% of the pasture dieback sites inspected, they were absent in 25% of the sites inspected, and were also present in multiple sites where pasture dieback was not observed. Their resilient cyst structures make them stable in soil systems, so if they were the sole cause of pasture dieback, they would have been observed in all sites where pasture dieback occurs.
Pasture mealybugs were observed causing significant infestations at multiple sites. This was particularly so in northern NSW on broad leafed paspalum (*Paspalum mandiocanum*) and around Gatton on green panic (*Megathyrsus maximus*). It is acknowledged that these insects are causing significant grass death in many areas. However, at many of the sites inspected in the current project, we identified ground pearls but no evidence of mealybugs. While it is possible that the mealybugs had come through, killed the grass and moved off, we suspect that in some instances, under the

appropriate environmental stressors, the ground pearls present were responsible for the grass death.

Although explicitly not part of this research project, it was considered necessary to have some attention directed towards the climatic factors that may be involved in ground pearl development and pasture dieback in general. Plant pathologists often refer to the disease triangle (McNew 1960). This predicates that for disease to occur, there needs to be a host, pathogen/pest and appropriate environmental conditions. In pasture dieback, as there has been no significant change in the host, it is possible that a new pest/pathogen has emerged, or that environmental conditions have changed.

This phenomenon has recently been investigated in relation to the rubber leaf fall phenomenon in Southeast Asia, where it was found that the condition chiefly occurred where increased minimum temperatures and changes in rainfall patterns were experienced (Azizan et al. 2023). Incidentally, the rubber leaf fall phenomenon was first recorded at the same time as the current pasture dieback disorder. Working in collaboration with UQ colleagues, climate data were assembled for one site with extensive pasture dieback. These data were analysed in relation to ground-truthed phenological assessments of the impacted area. While as yet unpublished, this showed that the onset of pasture dieback was immediately preceded by significant changes in temperature and rainfall at the site. In the case of pasture dieback, whatever the root pest/pathogen or combination is that targets the grass, it can only operate with the level of devastation observed when environmental conditions are not favourable for the host. An improved understanding of these conditions will be useful for preparing for the next emergences of pasture dieback.

Many of the pastures examined were riddled with ground pearls. In most cases the landholders did not know that they had ground pearls in their pastures. This was most starkly apparent when ground pearls were first identified at Brian Pastures, a Queensland government pasture research facility established decades ago. Being a cryptic root-feeding pest, it is highly probable that ground pearls are causing significant production loss but are largely unnoticed. While they are not causing pasture dieback in all areas, they are likely causing problems when conditions are adverse to plant growth.

Currently there are no management strategies for ground pearls in pastures. The current project has taken steps towards developing a management plan. This includes improved grower awareness, so that we are able to better catalogue their presence, streamlined sampling and processing, development of molecular tests to identify and differentiate ground pearl species and identification of biological targets for their control. Significantly more work is required to further our knowledge of the impacts of this pest and their mitigation.

5.1 Key findings

- At least three species of ground pearls occur within Queensland pastures, while a fourth species might also be having impacts.
- They are not consistently associated with pasture dieback, and so cannot be the primary cause if pasture dieback has a single cause.
- They are present in some areas of pasture dieback where no other cause is apparent, so it is possible they may be causing localised plant death.

- Molecular assays have been developed that can identify and differentiate ground pearls present in soil.
- The role of environmental conditions needs to be understood in order to improve management of pasture dieback in the future.

5.2 Benefits to industry

This project has delivered to the industry the most up-to-date information on a significant pest of pasture production. Additionally, there is further scope to improve our understanding of the distribution and impacts of these root-feeding pests.

- Ground pearls have been revealed to be infesting broad regions throughout Queensland.
- Their impact on production is not known, but as they feed on plant sugars it is possible that they are having a significant impact, both chronically as well as acutely when conditions are adverse to pasture health.
- A new methodology has been developed to sample for and identify them.
- This project has delivered greater knowledge of this pest so that graziers can search for them and have a point of contact to send them to have them identified.
- The foundations for improved ground pearl management have been established. This could lead to improved pasture production where ground pearls are present, and enhanced profitability and sustainability of the red meat industry in Queensland.
- Extension packages should be made available to stakeholders to facilitate pasture inspection and sampling in order to further establish the range and impacts of ground pearls.

6. Future research and recommendations

There is significant scope to improve pasture productivity and resilience by furthering our management of ground pearls in pastures. Prior to this project, Queensland grazing industries had no information on their broad distribution and potential impacts. With the development of a soil sampling protocol and molecular diagnostic, there is an opportunity now to better delimit these pasture pests and their impacts on grazing. This can be deployed in a new project that specifically surveys a broader area and then maps their presence/absence back to climatic and production data for the regions.

Preliminary evidence from this project confirms findings across other agricultural commodities: changes in climate result in unexpected impacts on plant health. Pests and diseases that may not be significant under ‘normal’ climatic conditions can have a major impact when environmental conditions become stressors on the host plants. It is suspected that it has been the changed environmental conditions that have led to organisms that were already present becoming major pests.

With a changing climate comes changing pest and disease issues. There is a need for a dynamic pasture health program that responds to changing biological production constraints across grazing industries. This could involve a Cooperative Research Centre (CRC)-style collaboration that presents a ‘front of house’ for graziers with issues in their pastures, including foliar and roots diseases, nematodes and insect management. This would bring together the required expertise in these fields, which is currently fractured across states and institutes. While this will help address pests and diseases currently impacting pasture production, it can be expected that more issues like pasture

dieback will emerge, and it would be timely to implement the research framework now that will lead to their successful management in the future.

Through the current project, graziers, Extension staff and other stakeholders are now more familiar with ground pearls than they were previously. It can be assumed they are having some impact based on the fact they are root-feeders, but we still don't know whether they have host preferences, how they are transported between regions, whether some grass species are more resistant than others, and whether innovative control strategies targeting their biology can be devised. Further investment in these areas can help support the red meat industry by dealing with these broadly distributed and damaging pests.

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8. Appendix

8.1 Sampling checklist

Pasture Dieback Sampling Checklist *Date: ___ / ___ / 202_*

Site name: _____¬ GPS: _____ _____

Contact: _____ Phone: _____

email: _____ Nearby town: _____

Site Presentation (tick if present, cross if absent)

Broadly circular patches Diameter largest patch (m) # patches Interspersed affected grasses Broadleaf infestation Grass yellowing Grass reddening Grass brown Grass dead All grasses affected

Site Rating (0= no dieback observed, 9= extensive dieback): _____

Soil Type: _____

Aspect: _____

Site description: _____

_____

_____

Grasses affected and notes: _____

_____

_____

Management notes and history

PD noticed shortly after rain following dry?_____

PD following/stopping at fenceline?_____

Grazing intensity?_____

General management notes: _____

_____

_____

_____

_____

_____

Mealybugs present Y / N _____

Ground pearls present Y / N _____

Photos taken Y / N *RGB image Y / N* *Multispec flight Y / N* *Soil samples Y / N*