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Confirming the identity of the *Hypogeococcus* species (Hemiptera: Pseudococcidae) associated with *Harrisia martinii* (Labour.) Britton (Cactaceae) in Australia: implications for biological control

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Abstract

Determining the identity of potential control agents is critical to successful biological control and can contribute to our understanding of the failures of previous introductions, especially in cases where host-associated cryptic species may be present. In 1975, a mealybug was introduced into Australia from Argentina for the classical biological control of the invasive cactus *Harrisia martinii* (Cactaceae). This cactus also originates from Argentina and is an environmental and agricultural weed in parts of Australia. Since its release, the imported mealybug species has been incorrectly referred to as *Hypogeococcus festerianus* (Hemiptera: Pseudococcidae) in the applied literature, and its performance as a biological control agent has been considered poor in some locations. In this study, the identities of mealybug specimens collected from 10 locations in Queensland and New South Wales, Australia, were assessed. The genetic, morphological and reproductive characteristics of these specimens were compared with those of two congeneric mealybug species, *Hypogeococcus pungens* sensu stricto (Hemiptera: Pseudococcidae) and *Hypogeococcus festerianus*. Specimens from the different Australian localities examined were all very similar to each other morphologically and genetically, based on comparisons of mitochondrial and nuclear DNA sequence data. The morphological features of all the specimens were typical of *Hypogeococcus pungens* sensu stricto. *H. pungens* is now considered to constitute a species complex, and the specimens from Australia are genetically similar to the Cactaceae clade of this species complex from Argentina. In common with *H. pungens s. s.*, the insects collected in Australia can also reproduce parthenogenetically. These findings help confirm that all populations of the mealybug in Australia are not *H. festerianus*, but part of the *H. pungens* cryptic species complex. There is no mismatch between this agent and the host plant in Australia, as *H. martinii* is one of the host plants of the most closely related cryptic species of *H. pungens* in the native range in Argentina. Thus, despite the original confusion around its identity, the variable performance of the introduced mealybug as a biological control agent of *H. martini* in Australia is likely due to other factors, and these require further investigation.

KEYWORDS
genetic diversity, *Hypogeococcus festerianus*, *Hypogeococcus pungens*, integrative taxonomy, species complex

INTRODUCTION

Accurate species identification is an important component of successful biological control programmes (Foster et al. 2021; Rosen 1986; Tixier et al. 2008). This can be especially challenging in situations where cryptic species may be present (Bickford et al. 2007; Paterson et al. 2019; Rafter & Walter 2020). The advent of molecular techniques has made it increasingly clear that many phytophagous insect species that were once considered to be host plant generalists may represent cryptic species complexes of more specialised species (Rafter et al. 2013; Rafter & Walter 2020). The inability to recognise cryptic species, or appropriately identify them whilst prospecting for biological control agents, can result in poor performance against the target plant species or failure of biological control (e.g., see Annecke & Moran 1978; Barratt et al. 2018; Bartlett et al. 1978; Paterson et al. 2011).

In 1975, a mealybug from the genus *Hypogeococcus* Rau (Hemiptera: Pseudococcidae) was imported from Chaco, Argentina, and introduced into Australia as a classical biological control agent for *Harrisia martinii* (Labour.) Britton (Cactaceae) and other *Harrisia* species, including *H. tortuosa* (J. Forbes ex Otto & A. Dietr) Britton & Rose, *Harrisia regelii* (Wieng), and *H. bonplandii* (Pharm. Ex Pfeiff) (McFadyen 1986). Its effectiveness as a control agent has been variable, with lower levels of control reported in southern Queensland than in central Queensland (Tomley & McFadyen 1985).

The mealybug introduced to Australia originated from a colony reared on *H. martinii* but was sourced from *Harrisia* spp. and *Cleistocactus baumannii* (Lemaire) (Cactaceae) in the Chaco region of Argentina (McFadyen 2012). On host plants, it is found on stem tips, spine bases, flower buds and between the stem ribs (McFadyen 2012). The mealybug was initially identified as *Hypogeococcus festerianus* (Lizer y Trelles) (Hemiptera: Pseudococcidae) (Williams 1973) but was redescribed as *Hypogeococcus pungens* Granara de Willink (Hemiptera: Pseudococcidae) (Williams & Willink 1992) based on a female holotype of this species after a review of specimens collected from *Alternanthera pungens* Kunth (Amaranthaceae) in Argentina in 1992. In addition to utilising host plants in the Amaranthaceae, *H. pungens* also uses members of the Cactaceae and the Portulacaceae as hosts (Williams & Willink 1992).

The new name, *H. pungens*, was widely adopted for the species in its native range in South America and the invasive ranges of Puerto Rico and Barbados (Zimmermann et al. 2010). However, in Australia, the species has continued to be referred to as *H. festerianus* by biosecurity and biological control workers (see Biosecurity Queensland 2017; Business Queensland 2021; Houston & Elder 2019; McFadyen 2012). This is based on the report of McFadyen (2012), which argues that the Australian population is restricted to hosts in the Cactaceae in its Australian range, despite the local availability of Amaranthaceae and Portulacaceae, and the untested proposition that, unlike *H. pungens* in Argentina (Aguirre et al. 2016), the mealybugs introduced to Australia are unable to reproduce parthenogenetically: Aguirre et al. (2016) reported facultative deuterotokous parthenogenesis in *H. pungens s. s.* in Argentina based on the results of laboratory experiments. The report of the absence of parthenogenesis in the mealybug introduced to Australia by McFadyen (1979) was based on speculation, as no test was conducted nor a reference cited to substantiate the claim.

Recent studies suggest that rather than being a polyphagous species that can feed on a range of hosts in the Cactaceae, Portulacaceae and Amaranthaceae (Williams & Willink 1992; Zimmermann et al. 2010), *H. pungens* likely represents a cryptic species complex, with each of the putative cryptic species exhibiting host specificity to Cactaceae, Amaranthaceae or Portulacaceae in their native ranges (Poveda-Martínez et al. 2019, 2020). This may explain why the mealybugs introduced into Australia have remained restricted to hosts in the Cactaceae, as reported previously (McFadyen 2012), and further suggests that the name *H. festerianus* has continued to be misapplied to the mealybug in Australia.

In Argentina, the native range of the mealybugs introduced to Australia, two clades of *H. pungens* have been identified based on sequences of the mitochondrial COI gene, and these correlate with host plants in the Amaranthaceae and Cactaceae upon which *H. pungens* feed (Poveda-Martínez et al. 2019). Mealybugs feeding on Amaranthaceae are regarded as *Hypogeococcus pungens s. s.* because the type specimen used by Williams & Willink (1992) to describe the species was obtained from *Amaranthus pungens* (Amaranthaceae). Further sampling and genetic analyses of more populations from Australia, Brazil, United States and Puerto Rico, including genomic SNP data, as well as mitochondrial sequences, suggest that the *H. pungens* species complex might comprise five species (Poveda-Martínez et al. 2020), each being host-specific to either Amaranthaceae from Argentina, Amaranthaceae from Brazil, Amaranthaceae and Portulacaceae from Argentina, Brazil, Puerto Rico and the United States, Cactaceae from Argentina and Australia, or Cactaceae from Brazil.

In biological control, misidentification and misapplication of species names can have serious consequences that include wasted resources, the inadvertent introduction of ineffective agents and, in some cases, significant extra economic losses and environmental damage caused by the introduction of agents that significantly impact non-target hosts. For example, failure to recognise the existence of sympatric biotypes of the weevil parasitoid *Microtonus aethiopoides* Loan (Hymenoptera: Braconidae) before its introduction to Australia and New Zealand for the biological control of the weevil *Sitona discoides* Gallenhal (Coleoptera: Curculionidae) had serious consequences, and post-release evaluations showed that

several non-target weevils were parasitized by *M. aetiopoids* in both countries (Barratt et al. 2018). Annecke & Moran (1978) also reported that the variable success of biological control of *Opuntia ficus-indica* Mill (Caryophyllales: Cactaceae) in South Africa could be a result of taxonomic problems. Similar taxonomic problems limited the control of *Planococcus kenyae* (Le Pelly) (Hemiptera: Pseudococcidae) in Kenya and also that of *Nipaecoccus vestator* (Mask) (Hemiptera: Pseudococcidae) in South Africa (Bartlett et al. 1978). These examples highlight how taxonomic errors can critically affect the success of biological control programmes.

The investigation of the mealybug from Australia by Poveda-Martínez et al. (2019, 2020) was based on the DNA sequences of 10 individuals collected from one location (27.5955°S, 151.7751°E) in southeast Queensland. The variable performance of this agent across Australian locations suggests that further investigation is warranted to determine that the mealybug populations involved in the biological control of *H. martinii* in different Australian locations are the same species. Therefore, this study compares the identity of the mealybug from 10 locations in Australia using a combination of morphological, molecular and experimental approaches. This will help in the ongoing effort to understand the variable performance of the agent across different locations in Australia and promote usage of the correct species name. This study also contributes to our knowledge of the biology of this species by testing whether parthenogenetic reproduction occurs in *H. pungens* in Australia.

MATERIALS AND METHODS

Sample collection

Mealybug colonies were collected from *H. martinii* and *H. tortuosa* at 10 locations across Queensland and New South Wales (Table 1). Adult females were isolated from the waxy colonies of each population ($n \geq 25$ from each population) and preserved in 70% ethanol at −18°C before morphological and molecular characterisation.

Morphological analysis

Sixty adult female mealybugs ($n \geq 5$ insects from each site) were slide-mounted. The procedure of Malausa et al. (2011) was followed with some modifications: (a) An incision was made in the abdominal region of the mealybug using an insect pin to remove the abdominal content, and then the specimen was placed in 10% potassium hydroxide (KOH) for 24 h; (b) specimens were washed in distilled water for 20 min and a small spatula was used to gently press the abdominal region to dispel body contents; (c) individuals were then transferred to staining dye consisting of equal volumes of acid Fuchsin (1%), distilled water, and lactic acid; (d) individuals were transferred to glacial acetic acid for 1 h and then transferred to lavender oil for 1 h; (e) single specimens were placed in a drop of Canada balsam on a microscope slide and covered carefully with a coverslip. The slides were left to dry at room temperature for 2 months.

TABLE 1 Locations of *Hypogeococcus* species sample collections in Australia.

Location	State	GPS	*Harrisia* spp.	Date of collection
Ipswich	Queensland	Lat. −27.3321 Long. 152.4762	*Harrisia martinii*	13 May 2019
Goondiwindi	Queensland	Lat. −28.4925 Long. 150.2501	*H. martinii*	21 May 2019
Emerald	Queensland	Lat. −23.6493 Long. 147.5562	*H. martinii*	15 March 2020
Toowoomba	Queensland	Lat. −27.3259 Long. 151.5659	*H. tortuosa*	15 July 2019
Inglewood	Queensland	Lat. −28.1108 Long. 151.0814	*H. tortuosa*	7 October 2020
Millmerran	Queensland	Lat. −27.8989 Long. 151.2312	*H. martinii*	23 July 2019
Dunmore	Queensland	Lat. −27.6117 Long. 150.9423	*H. tortuosa*	23 July 2019
Moree	New South Wales	Lat. −28.9267 Long. 149.6381	*H. martinii*	23 July 2019
North Star	New South Wales	Lat. −28.7938 Long. 150.4361	*H. martinii*	24 July 2019
Peats Ridge	New South Wales	Lat. −28.7956 Long. 150.5272	*H. martinii*	24 July 2019

Specimens were viewed under a compound microscope (Zeiss Axioskop 5, Germany), and images were created using a digital camera [5.1 megapixels, colour USB 2.0 (Aptina, China)]. Morphological comparisons between the Australian specimens and the closely related mealybugs *Hypogeococcus pungens* and *Hypogeococcus festerianus* were conducted using the published species description outlined in Williams & Willink (1992): No morphometric measurements were taken. The morphological identification of the eight described *Hypogeococcus* species is based on the presence, nature and position of cerarii, the presence of trilocular pores, the number of circuli, the presence of multilocular pores, and the position of the conical setae (Williams & Willink 1992). The first major character that differentiates *H. festerianus* from *H. pungens* is the presence of only one circulus in *H. festerianus*, whereas there are three in *H. pungens*. The second major character is the arrangement of the conical setae: Both species possess them, but they are present only in the posterior ventral area in *H. pungens*, whereas in *H. festerianus* they are located on the dorsomedial areas of the head and thorax.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from 100 mealybug samples comprising at least eight individual mealybugs from each location. The DNA extraction process followed a cetyltrimethylammonium bromide and silica column protocol (Ridley et al. 2016). DNA amplification was conducted for the mitochondrial cytochrome oxidase I (COI) gene and the nuclear gene translational elongation factor [EF1 alpha (α)]: These regions were selected based on the available sequences on NCBI GenBank. To amplify mitochondrial COI, 10 μM working solutions of the primers C1-J-2183F (CAACATTTATTTTGATTTTTTGG) and CI-N-2568R (GCWACWACRTAATAKGTATCATG) were used (Brady et al. 1990; Simon et al. 1994). To amplify EF1α, 10 μM working solutions of the primers M51.9F (CARGACGTATACAAAATCGG) and RcM53.2R (GCAATGTGRGCIGTGTGGCA) were used (Cho et al. 1995).

Polymerase chain reactions (PCRs) were performed in a Veriti PCR machine (Applied Biosystems/Thermofisher, Waltham, USA). The reaction took place in a total volume of 25 μL consisting of 12.5 μL of 1× PCR buffer containing Taq polymerase, 2 μL of DNA template, 9.5 μL of distilled water and 0.5 μL of each primer working solution (10 μM). The PCR conditions for COI were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 40 s, and extension at 72°C for 30 s with a final extension at 72°C for 7 min. The PCR conditions for EF1α were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 40 s at 54°C and extension at 72°C for 30 s with a final extension at 72°C for 7 min.

The PCR products were visualised on a 1% agarose gel in 1× Tris-38 acetate-EDTA (TAE) buffer, stained with ethidium bromide (8.2 mg/mL) (Sigma, St Louis, MO, USA). Successfully amplified genes were cleaned by adding 2 μL of freshly mixed Exonuclease I (1 unit/μL) and Antarctic Phosphatase (1 unit/μL) to the PCR product. The mixture was then incubated at 37°C for 20 min, followed by 10 min enzyme denaturation at 80°C. The cleaned product was sent to Macrogen, Inc, South Korea, for Sanger sequencing.

DNA sequence editing and phylogenetic analysis

The gene sequences were edited with CodonCode Aligner (CodonCode Corporation, www.codoncode.com) and primers were removed. Twenty (two sequences per location, per gene) of the edited COI and EF1α mealybug sequences were deposited in GenBank, with the accession numbers (OP593513–OP593532) and (OP593533–OP593552), respectively.

The mitochondrial cytochrome oxidase I (COI) and translational elongation factor (EF1α) gene sequences of *H. festerianus* and *H. pungens* populations from different host plants were downloaded from NCBI GenBank. These genes included the sequences from Poveda-Martínez et al. (2019, 2020), which used the same loci as this study. The gene sequences of *Planococcus ficus* Signoret (Hemiptera: Pseudococcidae) and *Planococcus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) were used as outgroups in the EF1α and COI phylogenetic trees, respectively (Table S1). The sequences of samples from different locations were aligned using the Geneious aligner (Geneious version 2021.0.3, https://www.geneious.com). The pairwise per cent DNA sequence differences were also determined in Geneious.

The sequences were used to assess the phylogenetic relationships between the Australian *Hypogeococcus* species and those in the native range. Phylogenetic relationships were estimated by both Bayesian inference and maximum likelihood methods, as each makes different assumptions. Bayesian inference was conducted in Mr. Bayes v 3.2.6 (Huelsenbeck & Ronquist 2001), with 11 000 000 iterations of the mcmc algorithm and a burn-in of 1 000 000. The most likely model was first estimated with Jmodeltest v2.1.10 (Darriba et al. 2012; Guindon & Gascuel 2003) based on Akaike information criteria; for COI, GTR + I + G was the most likely and for EF1α it was GTR + G.

Maximum likelihood trees were constructed with IQ-TREE v 1.6.12 (Minh et al. 2020). Model finder was used to infer the most likely model using Bayesian Information Criteria (Kalyaanamoorthy et al. 2017), for COI the

most likely model was TN + F + I and for EF1α the most likely was TNe. SH-like approximate likelihood ratio test values (SH-aLRT) (Guindon et al. 2010) and ultrafast bootstrap approximation values (Chernomor et al. 2016) were obtained in IQ-TREE using 10 000 replicates each.

Tests for parthenogenesis in *Hypogeococcus* specimens collected in Australia

The test for parthenogenesis in the Australian *Hypogeococcus* specimen was conducted using colonies of the mealybug on *H. martinii* obtained from Goondiwindi (28.4925°S, 150.2501°E) in southern Queensland and maintained at room temperature in the Entomology Laboratory, School of Biological Sciences, The University of Queensland, St Lucia, Queensland.

The ability of the Australian *Hypogeococcus* specimens to reproduce parthenogenetically was tested in two stages. Firstly, virgin female reproduction was tested (first female generation). Ten newly emerged first-instar nymphs (≤24 h old) were obtained from ovipositing females in the laboratory cultures. The nymphs were placed singly on individual *H. martinii* plants ($n = 10$) grown in organic potting medium in 15 cm diameter pots in a glasshouse. The infested plants were placed in a fine mesh (96 × 26 mesh) cage (32.5 cm × 32.5 cm × 32.5 cm) and maintained in an insectary (25 ± 2°C; L: D 12: 12 h), away from other mealybug cultures to avoid contamination with males.

Mealybug development was observed daily. At the third instar, male nymphs can be distinguished from females by differences in body form and behaviour. At this stage, plants with male nymphs were discarded and replaced with newly emerged first instar nymphs and the process was repeated until a total of 10 female nymphs (each on a separate plant) were successfully reared to the adult stage. During the daily observations of nymphs, the following data were recorded for each female that completed development: (i) development time (from the emergence of neonates to adult female eclosion); (ii) pre-oviposition time (from the start of the female adult stage to the appearance of first nymphs [eggs hatch rapidly, <20 min after oviposition]) and (iii) realised fecundity (the total number of nymphs produced by a female during her adult lifespan). As each female was observed daily from moulting to death, the number of nymphs that each female produced daily was also recorded and neonates were transferred immediately onto new mealybug-free plants to avoid double counting. These neonates were reared to adults to confirm the presence of male and female offspring in this maternal generation, but sex ratio was not determined as the number of adult males and females was not counted. The production of offspring that complete development by unmated females indicates successful reproduction through parthenogenesis.

The reproduction of virgin and mated second female generation was then tested. To assess parthenogenetic reproduction in the second female generation, one newly emerged nymph from each female in the first experiment ($n = 10$) was transferred to a potted *H. martinii* plant and reared singly following the methods and under the conditions previously described.

To assess sexual reproduction in the second female generation, cohorts of 15 randomly selected nymphs from each female in the first experiment were transferred to a potted *H. martinii* plant and reared together (15 nymphs established on each of 10 plants). At the third instar, the nymphs were sexed to ensure that the cohorts contained both male and female nymphs: The presence of males provided the opportunity for mating and fertilisation of females, although mating was not assured. Development time, pre-oviposition time and realised fecundity were recorded as previously described. The offspring of the virgin and mated second female generation was also reared to adults to confirm the sexes, although the sex ratio was also not determined.

Statistical analyses

All analyses of the data from experiments investigating parthenogenesis were conducted in R version 4.1.0 (R Core Team 2021). Data were tested for homogeneity of variance and normality of distribution using Levene's and Shapiro Wilk's tests, respectively. As the development times were not normally distributed, the development times of the first and the second female generations were compared using the Wilcoxon test. Similarly, as the pre-oviposition periods and the numbers of offspring produced by first virgin females, second mated females and second virgin females were not normally distributed, they were compared using a Kruskal–Wallis test. The post hoc correction for multiple comparisons following a Kruskal–Wallis test was conducted with Dunn's test.

RESULTS

Morphological analysis

The morphology of the circuli, antennae, conical setae, multilocular disc pores, capitate setae and the legs (Figure 1) of all the slide-mounted mealybugs were very similar and matched the description of *H. pungens* in Williams & Willink (1992). Specifically, all specimens possessed three circuli (Figure 1a,b), numerous multilocular disc pores throughout the entire body segments, and slender capitate setae on the head, thorax and anterior

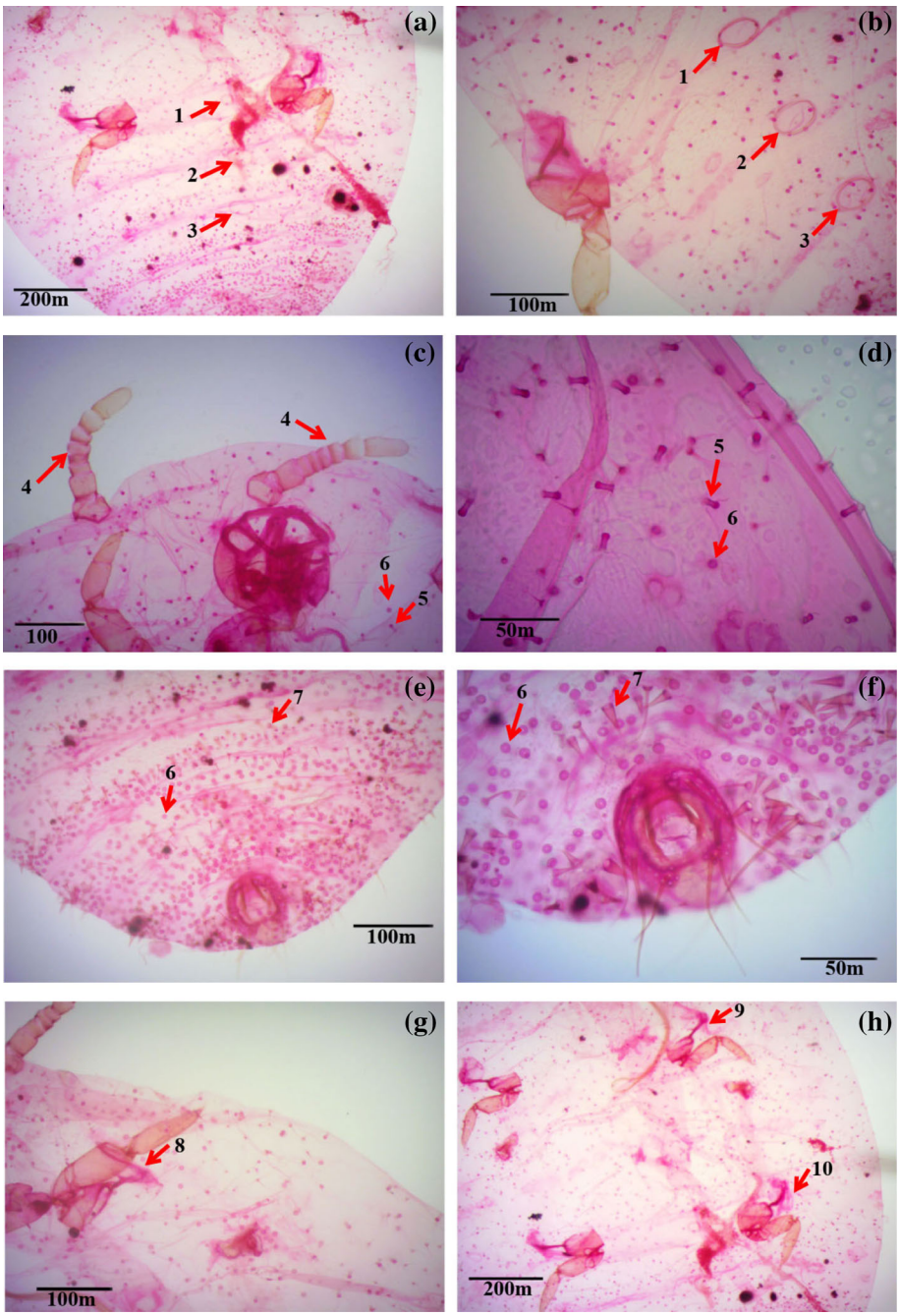

FIGURE 1 The different morphological features of the Australian *Hypogeococcus*: Panels (a) and (b) show that the abdominal region has three circuli (1–3); panel (c) shows that the head region has two antennae (4), numerous slender capitate setae (5), and numerous multilocular pores (6); panel (d) shows the presence of numerous slender capitate setae (5) and numerous multilocular pores (6) in the abdominal region; panels (e) and (f) show that the posterior ventral area possess numerous multilocular pores (6) and numerous conical setae (7); panels (g) and (h) show the foreleg (8) with the curvature attachment point, mid-leg (9) and hindleg (10), both with attachment points ending in a bifurcation. These characters are consistent with *Hypogeococcus pungens s.s*.

abdominal segments but lacked trilocular pores in all body segments (Figure 1c–f). Further, all had conical setae in the posterior abdominal segments (Figures 1e,f), but these structures were absent from the head, thoracic segments and anterior abdominal segments (Figure 1). The morphologies of the fore- (Figure 1g), mid- and hind-legs (Figure 1h) were also typical of *H. pungens*.

Molecular data and phylogenetic analysis

The mitochondrial and nuclear genes were successfully sequenced from all Australian *Hypogeococcus* populations sampled. In total, 384 base pairs of COI and 406 base pairs of the EF1α sequences remained after editing and aligning with sequences on GenBank. The sequences for all

Australian specimens were identical for each gene and all phylogenies placed them within *H. pungens* rather than *H. festerianus*. As all the gene sequences were the same, only two sequences per location and gene were used in the phylogenetic analysis ($n = 20$ per gene) (Figures 2 and 3).

In the EF1α phylogeny (Figure 2a,b), the Australian population and *H. pungens s. s.* formed a clade, with no differences in the gene sequences. Using this gene, *H. festerianus* was placed sister to *H. pungens*. There were more sequences available from GenBank for COI and, in phylogenies estimated from this gene, *H. pungens* was split into several clades representing different host plants and locations (Figure 3). In the maximum likelihood tree (Figure 3a), the relationship between *H. festerianus* and *H. pungens* was not resolved, with a polytomy representing *H. festerianus* and two clades of *H. pungens*. In the Bayesian tree (Figure 3b), *H. festerianus* was placed sister to *H. pungens* from Amaranthaceae in Brazil, but this branch was not supported (posterior probability [PP] = 0.55).

Among all the populations within the *H. pungens* species complex, the Australian population is most similar to the population from Cactaceae hosts in Argentina, with 0.8%–1.6% difference in COI and 0% difference in EF1α between Australian samples and those from Cactaceae hosts in Argentina. However, the Australian population differs markedly from other Cactaceae populations of the *H. pungens* species complex from Brazil, with up to 2.9%–3.4% divergence.

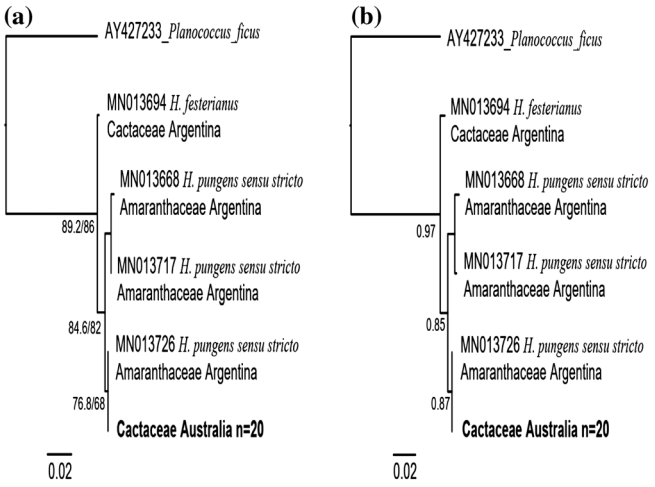

FIGURE 2 Phylogenetic trees based on EF1α sequence data (a) maximum likelihood (IQ-TREE) and (b) Bayesian (Mr. Bayes). Branch labels indicate the ultrafast bootstrap and SH-aLRT values for the maximum likelihood tree and posterior probabilities for the Bayesian tree. *Planococcus ficus* was used as the outgroup for both trees. The host plant family is followed by the country of collection. See Table S1 for further details on host plant species and specimen collection codes for *Hypogeococcus*.

Parthenogenesis

All the first virgin females, second mated females and second virgin females produced viable male and female offspring. Although the sex ratio was not determined, both male and female offspring were common within the cohorts. There was no significant difference in the time to complete development between the first maternal generation (30.3 ± 1.6 days) and the second generation (31.4 ± 1.4 days) ($W = 39$; $P = 0.0545$) (Figure 4). The mated second-generation females had a significantly shorter average pre-oviposition period (11 ± 1) days than the first-generation virgin females (21.8 ± 6.3) and the second-generation virgin females (23 ± 7.4) days ($H = 19.491$; $P = <0.0001$) (Figure 5a). The first-generation virgin females and all the mated and virgin second-generation females produced male and female offspring, with no significant difference between the average number of offspring produced by the first-generation virgin females (63 ± 21), the second-generation virgin females (72 ± 27) and the second-generation mated females (74 ± 20) ($H = 1.2429$, $P = 0.5372$) (Figure 5b). In the fertilisation experiment, mating success was not assessed; therefore, the results from this experiment may include measurements from females that had not mated.

DISCUSSION

This study indicates that there is no variation between the populations of *Hypogeococcus* involved in the biological control of *Harrisia* at different locations in Australia. The morphological data from populations of *Hypogeococcus* in Australia match those of *H. pungens s. s.* in South America, as described by Williams & Willink (1992). Furthermore, similar to the reports of Poveda-Martínez et al. (2019, 2020), the molecular data indicated that the population in Australia is most closely related to the putative cryptic species of *H. pungens* found on *Harrisia* cactus hosts in its native range.

Contrary to the suggestion of McFadyen (1979), the current study also confirms the ability of the Australian population to reproduce parthenogenetically and sexually, with both fertilised and unfertilized females producing viable male and female offspring. This indicates that the *Hypogeococcus* species in Australia also shares similar reproductive processes with *H. pungens s. s.*, as Aguirre et al. (2016) recorded facultative parthenogenesis by deuterotoky in *H. pungens s. s.* in its native range. These results reinforce that this agent is part of the *H. pungens* species complex and it is concluded that the name *H. festerianus* has been misapplied to the agent in Australia by biosecurity and biological control workers.

A key element for any successful biological control programme is the appropriate matching of the pest and its natural enemy, and this can be particularly challenging

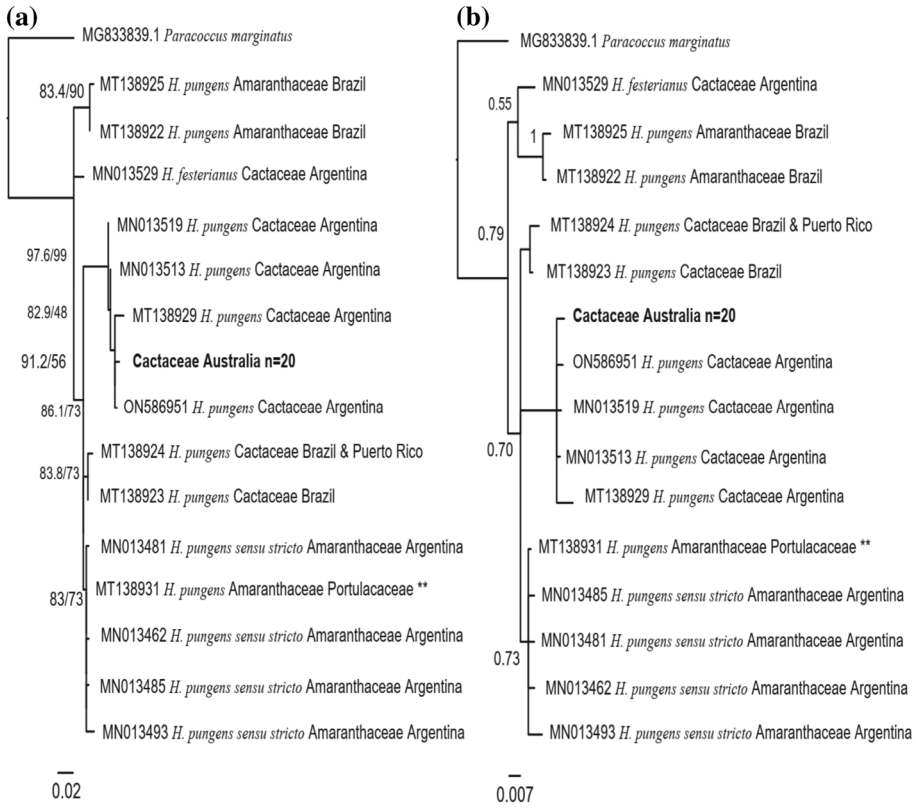

FIGURE 3 Phylogenetic trees based on COI sequence data (a) maximum likelihood (IQ-TREE) and (b) Bayesian (Mr. Bayes). Branch labels indicate the ultrafast bootstrap and SH-aLRT values for the maximum likelihood tree and posterior probabilities for the Bayesian tree. *Paracoccus marginatus* was used as the outgroup for both trees. The host plant family is followed by the country of collection. See Table S1 for further details on host plant species and specimen collection codes for *Hypogeococcus*. **Country of collection includes Argentina, Brazil, Puerto Rico, and the United States.

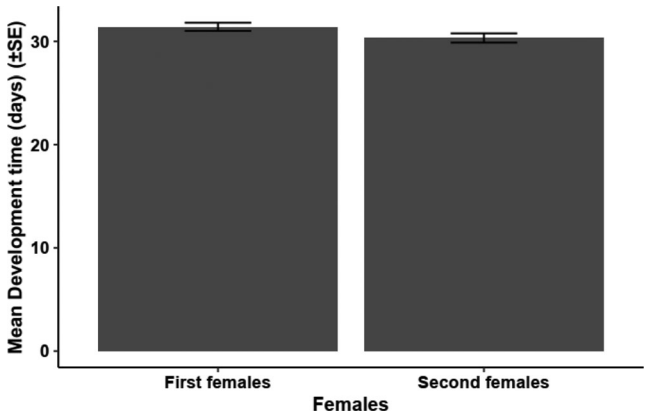

FIGURE 4 Mean (± SE) development time in days of the first- and second-generation females, from the first nymph to adult emergence of the Australian *Hypogeococcus* ($W = 39$, $p = 0.0545$).

for insect taxa where cryptic species occur (Paterson et al. 2011; Rosen 1986). This study, and in particular the molecular data presented, indicates that the agent in Australia has appropriate host plant associations. Therefore, the variable performance of this agent in Australia is not due to the presence of an inappropriate agent or variation between agent populations. These findings are important for the biological control of *Harrisia* species involving *H. pungens* in Australia and highlight the importance of studies of this nature during pre-release research to ensure the selection and release of the most appropriate lineage of a biological control agent for the target species (van Steenderen et al. 2021).

The occurrence of genetic divergence between the mealybug populations referred to as *H. pungens* (Poveda-Martínez et al. 2019, 2020) suggests that the presence of cryptic species is common and that they can adversely affect the effectiveness of a biological control agent if specimens from the most appropriate population are not introduced (Annecke & Moran 1978; Barratt et al. 2018; Paterson et al. 2011; Rosen 1986). This reinforces the importance of a thorough molecular and taxonomic investigation of the host and agent to avoid the problem of host-agent mismatches and ensure the safety of biological control practice.

The genetic results reported here, indicate that the population in Australia is genetically the same at the COI gene, suggesting that a single species was introduced and established. The Cactaceae clade of *H. pungens* from Argentina feeds on species of *Harrisia* and *Cleistocactus* in contrast to the Cactaceae clade from Brazil which feeds on different species of Cactaceae, namely, *Pilosocereus*,

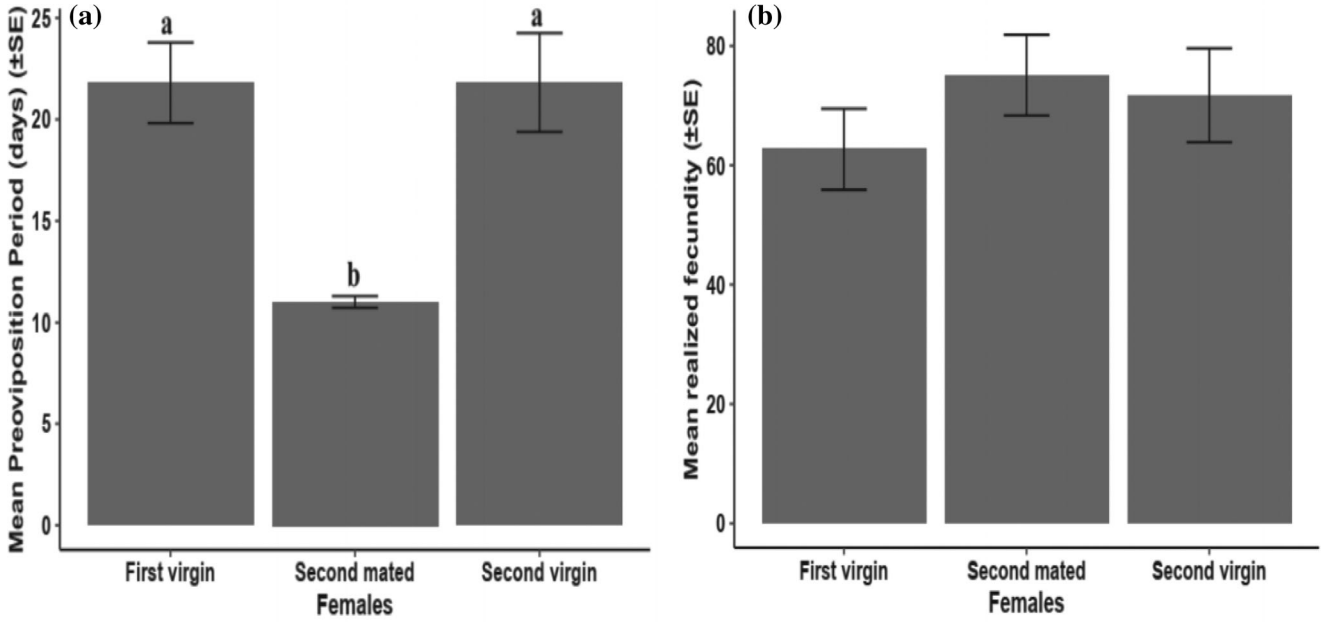

FIGURE 5 (a) Mean (± SE) pre-oviposition period (days) of the first virgin females, the second virgin, and second mated female generations ($H = 19.491$, $p < 0.0001$), means labelled with similar letters are not significantly different (Dunn's test, $p < 0.05$); (b) realised fecundity of the first virgin females, the second virgin and second mated female generations ($H = 1.2429$, $p = 0.5372$).

and *Coleocephalocereus* species (Poveda-Martínez et al. 2020). This confirms that the Cactaceae clade from Argentina is the source of the insects in Australia as reported by McFadyen (2012). The difference (0.8%) between the COI of the Cactaceae clade from Argentina and the Australian population could be due to variation in the native range that has not yet been sampled in genetic studies. In Australia, the mealybug is host-specific to cactus, thus making it a suitable biological control agent with respect to possible host range, as there are no native cactus species on the continent. However, despite being collected from within the endemic range of *H. martinii*, it is not as effective as expected for control of the target weed in some locations (Tomley & McFadyen 1985).

Climate often plays a key role in plant–insect interactions (Byrne et al. 2002; DeLucia et al. 2012; Pincebourde et al. 2017). The variable performance reported for *H. pungens* in Australia could be a result of the interactions of other factors, for example, environmental conditions, or the interactions between plant and mealybug behaviour, rather than the introduction of an inappropriate agent. In Argentina, *H. martinii* and *H. pungens* are found in the northern regions with tropical and subtropical climates (Franck 2016; McFadyen 1986). This is different from the climate in most Australian locations where the mealybug was released, especially in southern Queensland where control levels are currently reported to be relatively low. This location experiences mostly temperate climatic conditions, with very high summer temperatures but cold winter temperatures: Based on its distribution in its native range, the mealybug is unlikely to be adapted to these climatic conditions. The effects of temperature and other factors on the performance of this mealybug in Australia should be assessed. This may unravel the reasons for its variable performance between locations and help in the ongoing efforts to improve the control of *H. martinii* in Australia. There are no reports of taxonomic issues or variability between populations of the invasive weed *H. martinii*, but genetic studies of the host would be useful to confirm that one species occurs in the invasive range. Investigating how environmentally mediated changes in this weed affect the performance of the mealybug may also provide important information on the host–agent relationship in different Australian locations.

In conclusion, this work has improved our understanding of *Hypogeococcus* imported into Queensland for the biological control of *H. martinii* and will inform researchers in Australia to refer to this agent as *H. pungens* and not *H. festerianus*. It also reinforces claims for the existence of cryptic species within the species currently known as *H. pungens* (Poveda-Martínez et al. 2019, 2020). This is important information for any work involving this and other biological control agents and highlights the importance of a thorough taxonomic examination of both host and agent to avoid host-agent mismatches. Further work on the morphology of the putative cryptic species in their native range is required to further disentangle this group, and this may reveal morphological differences. Although the Australian population of *Hypogeococcus* should be regarded as *H. pungens*, it will likely require another name once the cryptic species complex is fully taxonomically revised.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

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