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Research

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An invasive variant of the pasture mealybug *Heliococcus summervillei* (Hemiptera: Pseudococcidae) and its obligate endosymbiont *Tremblaya phenacola* (Enterobacterales: Enterobacteriaceae)

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The pasture mealybug *Heliococcus summervillei* Brookes (Hemiptera: Pseudococcidae) is the cause of extensive pasture dieback in Australia and is an emerging biosecurity threat to pasture and crop grasses in the Americas. In this study, we present a morphological and molecular comparison of contemporary Australian specimens of *H. summervillei* and the primary bacterial endosymbiont *Candidatus* Tremblaya phenacola Gruwell (Enterobacterales: Enterobacteriaceae) with reference to historical and published specimens. Molecular analyses were conducted using three host nuclear markers (18S, 28S-D2, dynamin) and the universal bacterial marker (16S V3–V4 region). Our results identify a new, highly invasive variant of *H. summervillei* associated with the ongoing dieback outbreak in Australia. This work uses 16S barcoding to support host-based morphological and molecular approaches to differentiate this new variant from the type specimens.

Keywords: mealybug, pasture dieback, diagnostics, phylogenetics, taxonomy

Introduction

Mealybugs (Hemiptera: Pseudococcidae) are recognized pests of tropical and temperate grasses, including important pasture and crop species (Department of Agriculture and Water Resources 2019, Subramanian et al. 2021, Ahmed et al. 2025). The pasture mealybug *Heliococcus summervillei* Brookes (Hemiptera: Pseudococcidae) is the cause of ongoing, extensive, and economically damaging dieback in pastures across eastern Australia, a condition characterized by red, purple and/or yellow leaf discoloration and subsequent plant death (Summerville 1928, Hauxwell 2022a, 2022b, 2022c). The first records of *H. summervillei* causing pasture dieback describe localized outbreaks in South East Queensland in 1926 (Summerville 1928) and Far North Queensland in 1938 (Brookes 1978). Subsequent reports describe collections from sugarcane in Pakistan in 1975 (Brookes 1978), from rice in India in 1978 (Ghosh & Ghose 1987) and from pastures in New Caledonia in 1998 (Brinon et al. 2004). More recently, *H. summervillei* has been reported in Puerto Rico (G. Evans, United States Department of Agriculture, *personal communication*, 8 September 2020), Barbados (Gibbs 2020; I. Gibbs, Ministry of Agriculture and Food Security, *personal communication*, 2020), Mexico (Aca-Martinez et al. 2025) and the US mainland (Biles et al. 2025, Powell & Hauxwell 2026).

The host range of *H. summervillei* is substantial. It includes both tropical and temperate pasture grasses, and crop grasses like sugarcane, sorghum and rice (Brookes 1978, Hauxwell et al. 2022c). This broad polyphagy, combined with increasing national and international distribution, presents a biosecurity threat of global concern.

The ongoing outbreak of pasture dieback in Australia dates to at least 2012 (Queensland Department of Agriculture and

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Fisheries 2017), though some reports suggest it may date back to 1993 (Makiela & Harrower 2008). It has affected approximately 4.48 million hectares of productive land in Queensland and New South Wales (Meat & Livestock Australia 2021, Gibson et al. 2024, McKenna et al. 2024), causing losses estimated at AUD$2 billion (AgForce Queensland 2021, Halter 2023). *Heliococcus summervillei* was first identified in association with this outbreak of dieback in 2017 (Hauxwell 2018, Schutze et al. 2019) and subsequently confirmed as the causal agent (Hauxwell et al. 2022a, 2022b). It continues to expand across eastern Australia, with recent detections as far south as the Hunter Valley in New South Wales (R. Mann, MLA, *personal communication*, 28 May 2025) and as far inland as the Queensland/Northern Territory border (North Australia Beef Research Council, *personal communication*, 26 March 2025).

Morphological examination of specimens of *H. summervillei* from this outbreak identified differences from the type specimens, with thoughts that this might represent a new species (Schutze et al. 2019). Further examination of specimens across the current outbreak is required to determine the extent of variation within the population.

In this study, we compare the morphology of specimens of *H. summervillei* from the ongoing Australian outbreak with type material and descriptions of international specimens, and use Sanger sequencing of genomic markers from *H. summervillei* and metabarcode sequences from the primary (P-) endosymbiont *Candidatus* Tremblaya phenacola Gruwell (Enterobacterales: Enterobacteriaceae) (*T. phenacola* hereafter) to differentiate variants within the mealybug population.

Materials and Methods

Sample Collection

Mealybugs were collected at sites from Central and Southern Queensland and the Northern Rivers region of New South Wales over a 7-yr period (July 2018 to May 2025) (Fig. 1). Live insects were collected with host plant leaves into 50 mL Sarstedt tubes and kept in portable coolers during fieldwork and

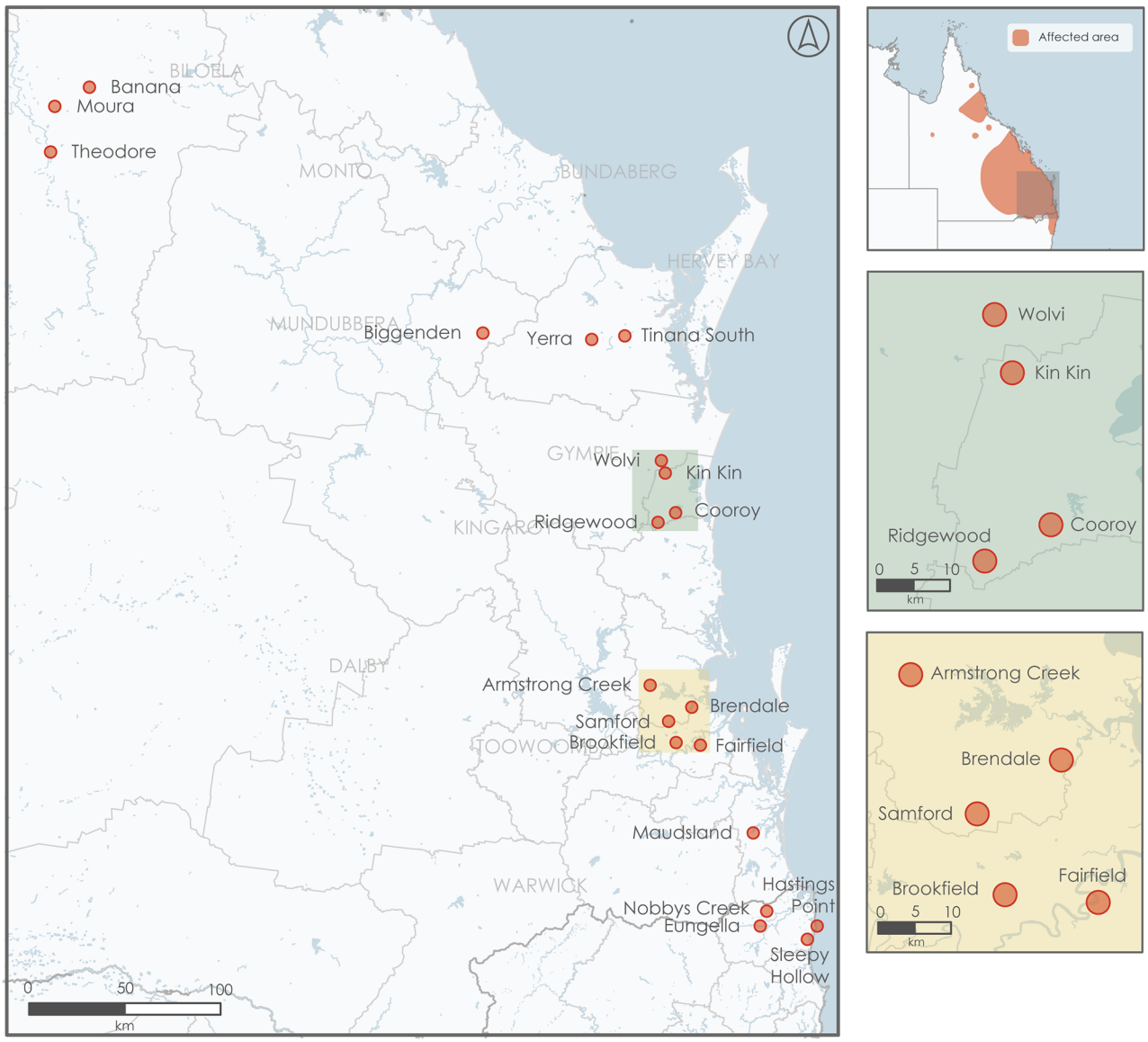

Fig. 1. Geographical distribution of *Heliococcus summervillei* collected by QUT in Queensland and northern New South Wales. Map: Sophie Richards.

transport to the laboratory. Host plants were identified in the field to the lowest taxonomic level (species/variety) where possible. Additional insect samples were provided by graziers and agronomists.

Specimens collected between 2018 and 2020 were stored in 70% to 80% ethanol to maintain cuticular integrity for morphological examination and preserve DNA quality for molecular analyses. From 2020 onward, specimens were stored in 75% propylene glycol to allow safer transport and storage (Nakamura et al. 2020). All samples were stored at −20 °C.

DNA Extraction

Specimens stored in propylene glycol were rinsed in 80% ethanol and briefly air-dried before extraction. DNA was extracted from 352 insect specimens using a nondestructive method to retain the cuticle as a morphological voucher. DNA was extracted from intact specimens using a modified QuickExtract protocol (Lucigen) with an overnight incubation (17 hr), as described by Martoni et al. (2021). Cuticles were transferred to 80% ethanol and stored at −20 °C.

Destructive extraction was performed on an additional 29 adult female specimens to increase DNA output for 16S metabarcoding of the P-endosymbiont. As per Wang et al. (2019), whole insects were pretreated with trichloromethane (30 min) to remove wax, then soaked in ultrapure water (6 hr) to soften tissues. Individual insects were homogenized by dry grinding with a micropestle. DNA extraction was then performed using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer protocols, with an extended lysis incubation step (8 hr) to increase yield. Total DNA was quantified by spectrophotometry (Thermo Fisher Scientific NanoDrop One) and stored at −20 °C.

Marker Selection and Sequencing

Molecular markers for Sanger sequencing were selected based on their use in phylogenetic studies of mealybugs (Downie & Gullan 2004, Hardy et al. 2008, Kaydan et al. 2015, Lin et al. 2019, Choi & Lee 2022). The following ribosomal, mitochondrial and chromosomal markers were used: 18S, 28S (D2 region), dynamin and COI. PCR amplicons were generated from 179 insect specimens collected by QUT using the primers in Table 1 and cycling parameters in Table 2. Amplicons were sequenced by Macrogen Inc. (Seoul, South Korea) using BigDye Terminator v3.1 chemistry (Applied Biosystems) on an ABI PRISM 3730XL DNA Analyser (96-capillary array).

Thirteen (13) unique Sanger-generated 18S sequences from *H. summervillei* specimens were provided by the New South Wales Department of Primary Industries and Regional Development (NSW DPIRD) in 2020. Reference specimens are lodged in the NSW Biosecurity Collections (ASCU) (Supplementary Table S1).

Table 1. Primer sequences used in this study

Target	Primer name	Sequence (5′ to 3′)	Source
COI, HCO-LCO region (c. 710 bp)	LCO1490 (F)	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
	HCO2198 (R)	TAAACTTCAGGGTGACCAAAAAATCA	
COI, region 2 (c. 490 bp)	C1-J-2183 (F)	CAACATTTATTTTGATTTTTTGG	Gullan et al. (2003)
	C1-N-2568 (R)	GCWACWACRTAATAKGTATCATG	
COI, barcode region (c. 650 bp)	PCO-F1 (F)	CCTTCAACTAATCATAAAAATATYAG	Hebert et al. (2003)
	LEP-R1 (R)	TAAACTTCTGGATGTCCAAAAAATCA	
COI, HCO-LCO region (c. 380 bp)	LCO-M-2d (F)	ATAACTATACCTATYATTATTGGAAG	Malausa et al. (2011)
	LCO-M-2d (R)	AATAAATGTTGATATAAAATTGG	
Dynamin (c. 300 bp)	3006F1.1 (F)	CCGGAYATGGCGTTCGAAGCTA	Hardy (2007)
	3006R2.1 (R)	TCTTCGTGGTTGGTGTTCATGTACGC	
18S (c. 600 bp)	2880 (F)	CTGGTTGATCCTGCCAGTAG	von Dohlen and Moran (1995)
	B (R)	CCGCGGCTGCTGGCACCAGA	
28S, D2 expansion region (c. 650 bp)	S3660 (F)	GAGAGTTMAASAGTACGTGAAAC	Downie and Gullan (2004)
	A335 (R)	TCGGARGGAACCAGCTACTA	
16S, V3–V4 region (c. 460 bp)	341F (F)	CCTAYGGGRBGCASCAG	Klindworth et al. (2013)
	806R (R)	GGACTACNNGGGTATCTAAT	

Table 2. PCR cycling parameters used to generate 18S, 28S-D2 and dynamin amplicons for Sanger sequencing by Macrogen Inc. Protocols were modified from Lin et al. (2017)

Primer/s	Cycling parameters
LCO1490 (F)/HCO2198 (R) **LCO-M-2d (F)/LCO-M-2d (R)**	95 °C for 3 min; (95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s) for 35 cycles; 72 °C for 5 min
C1-J-2183 (F)/C1-N-2568 (R)	95 °C for 3 min; (95 °C for 30 s, 48 °C for 30 s, 72 °C for 60 s) for 35 cycles; 72 °C for 5 min
PCO-F1 (F)/LEP-R1 (R)	95 °C for 3 min; (95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s) for 35 cycles; 72 °C for 5 min
2880 (F)/B (R) **S3660 (F)/A335 (R)**	95 °C for 3 min; (95 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s) for 10 cycles, ramping down −1 °C per cycle; (95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s) for 25 cycles; 72 °C for 5 min
3006F1.1 (F)/3006R2.1 (R)	95 °C for 3 min; (95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s) for 10 cycles, ramping down −1 °C per cycle; (95 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s) for 25 cycles; 72 °C for 5 min

Table 3. Plants that were observed as hosts of *Heliococcus summervillei* in the QUT collection and showed symptoms of pasture dieback. Plants were identified in the field to the lowest taxonomic level (species/variety) where possible

Genus/species	Common name (and variety, if applicable)
Bothriochloa insculpta	Creeping bluegrass (var. Bisset)
***Brachiaria/Urochloa* sp.**	Signal grass
***Carex* sp.**	Sedge
Cenchrus ciliaris	Buffel grass (var. USA, Biloela, Gayndah)
Cenchrus clandestinus	Kikuyu grass
Chloris gayana	Rhodes grass
Dichanthium sericeum	Queensland bluegrass
Digitaria didactyla	Queensland blue couch
Digitaria eriantha	Pangola grass
***Digitaria* sp.**	Digit grass
Lolium rigidum	Annual ryegrass
Megathyrus maximus	Guinea grass
Panicum maximum	Panic grass (var. Green, Gatton)
Paspalum dilatatum	Dallis grass
Paspalum mandiocanum	Broad-leaved paspalum
Paspalum urvillei	Vasey grass
Paspalum notatum	Bahia grass
***Paspalum* sp.**	Paspalum
Setaria sphacelate	South African pigeon grass
***Setaria* sp.**	Setaria
Sorghum bicolor* × *Sorghum sudanese	Sorghum (var. Jumbo)

For bacterial endosymbiont sequencing, 16S (V3–V4 region) amplicons were generated from the total DNA of 28 insect specimens (26 specimens processed by destructive extraction, two specimens by nondestructive extraction), including one outgroup specimen (*Coccus longulus* Douglas), on a Veriti Dx 384-well thermal cycler (Applied Biosystems) using the Platinum SuperFi II PCR Master Mix (Invitrogen, Australia) and primers and conditions outlined in Tables 1 and 2. Amplicons were quantified using the Quantifluor dsDNA System (Promega) and normalized to 5 nM prior to sequencing using a Qubit 4 Fluorometer (Thermo Fisher Scientific). Paired-end sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600-cycle) by the Australian Genome Research Facility (Melbourne, Australia).

Morphological Comparison

Adult female mealybugs, including cuticles kept as morphological vouchers after DNA extraction, were slide-mounted in Canada balsam according to the method described by Williams and Granara de Willink (1992) and lodged in the Queensland Primary Industries Insect Collection (QDPC) (Supplementary Table S1). Specimens were compared with type material (holotype and paratypes, accessions T7832 to T7836 in the Queensland Museum collection) and QDPC reference material (QDPC accession 174662), with reference to the species description of *H. summervillei* (Brookes 1978) and available keys (Williams 2004). Non-target mealybugs and other scale insects collected incidentally in the field were also identified by morphology (Supplementary Table S2).

Key morphological characters from *H. summervillei* specimens outside Australia were reviewed to confirm diagnostic consistency and assess morphological stability across regions. This included microscopy and descriptions of two specimens from Puerto Rico identified by the United States Department of Agriculture (USDA) (G. Evans USDA, *personal communication*, 2020) and 10 specimens from Barbados collected by the Ministry of Agriculture and Food Security (MAFS) and identified by the Florida Department of Agriculture and Consumer Services (FDACS) (I. Gibbs MAFS, *personal communication*, 2019), together with published descriptions of 12 specimens from Pakistan (Brookes 1978) and 11 specimens from India (Ghosh & Ghose 1987).

Molecular Data Processing and Phylogenetic Analysis

NGS data (16S) were pre-processed in QIIME2 (Bolyen et al. 2019) following the recommended workflow, with SILVA SSU Ref NR99 v138.1 (Quast et al. 2013) as the reference database for taxonomic assignment. Specimens were inspected for the presence of amplicon sequence variants (ASVs) from the P-endosymbiont *T. phenacola* and other bacterial symbionts, such as *Wolbachia* (Rickettsiales: Ehrlichiaceae) and *Candidatus* Moranella (Enterobacterales: Enterobacteriaceae). Taxonomic assignments were verified by BLASTn against the National Center for Biotechnology Information (NCBI) nucleotide collection (nr/nt). ASVs identified as *T. phenacola* were exported for phylogenetic analysis. Low-abundance ASVs (<100 reads) were filtered out as potential sequencing artifacts or contamination.

All sequence datasets (18S, 28S-D2, dynamin, 16S) were processed in RStudio 2024.12.0 (R version 4.5.0) (Posit team 2020). Raw Sanger traces were imported using *sangeranalyseR* 1.13.0 (Chao et al. 2021), with base calling and quality trimming performed under default parameters. Sequence length distributions were visualized in *ggplot2* 3.5.1 (Wickham 2016) to establish minimum length thresholds of 600 bp (18S, 28S-D2), 150 bp (dynamin) and 400 bp (16S). Alignments were generated using *DECIPHER* 2.30.0 (Wright 2016) and trimmed by visual inspection. Aligned region lengths were approximately 550 bp (18S), 600 bp (28S-D2), 200 bp (dynamin) and 430 bp (16S).

Unique sequence variants in each marker-specific dataset were identified from the trimmed alignments for use in phylogenetic reconstruction. Additional sequences from other mealybug species were retrieved from GenBank (Sayers et al. 2025) to test monophyly of *H. summervillei* within genus *Heliococcus* and *T. phenacola* within genus *Ca.* Tremblaya. These included sequences for all available *Heliococcus* species and any other Pseudococcidae species recorded in Australia (Garcia Morales et al. 2016), as well as their corresponding P-endosymbionts where available. All additional sequences were trimmed to the same length as our Sanger- and NGS-generated sequences. Accession numbers for the additional sequence data are listed in Supplementary Table S1.

Phylogenetic analyses were conducted using *phangorn* 2.12.1 (Schliep 2011), with trees inferred by maximum likelihood under the GTR substitution model and 1,000 bootstrap replicates. Where sequences were available (18S, 28S-D2, 16S), trees were rooted by species in tribe Rhizoecini (Hemiptera: Rhizoecidae) or their corresponding P-endosymbiont *Candidatus* Brownia rhizoecola Gruwell (Flavobacteriales: Blattabacteriaceae) using *ape* 5.8 (Paradis & Schliep 2019). No outgroup sequences were available for dynamin, and this tree

was left unrooted. Trees were visualized using *ggtree* 3.10.1 (Yu et al. 2017), with metadata (sample site, collection date, pasture type sampled, insect life stage) overlaid to explore potential associations with phylogenetic structure. Final trees were annotated in iTOL 6.9.1 (Letunic & Bork 2024), with clades collapsed where bootstrap support (BS) fell below 80%.

Neutrality statistics (Tajima's *D*, Ramos-Onsins & Rozas R_2) were calculated using *pegas* 1.3 (Paradis 2010).

Results

Host Range and Geographical Distribution

Mealybug specimens were collected from 48 sites across Queensland and northern New South Wales (Fig. 1). Insects showed a high degree of polyphagy and were recorded feeding on the leaves of 16 confirmed grass species, including forage sorghum and four grasses identified only to genus, as well as one sedge (Table 3). All host plants showed symptoms of dieback.

Heliococcus summervillei Morphology

One hundred nine (109) specimens were identified from morphology as *H. summervillei* and lodged in the QDPC (Supplementary Table S1). Morphological data indicate the presence of two consistent forms within *H. summervillei*, which we have designated here as types A and B. Only two specimens in the QUT collection (QDPC accessions 177398 to 177399) exhibited morphology fully consistent with the type material (QM accessions T7832 to T7836) and species description (Brookes 1978). These were designated as type A, and the individuals as specimens A1 and A2. Both were collected from mixed populations at Kin Kin (QLD) in 2020, which is approximately 25 km from the 1926 outbreak site (Cooroy, QLD). The remaining 107 specimens displayed consistent morphological differences from the type material and species description, and are designated as type B. This is the dominant variant in the ongoing Australian outbreak.

Examination of material in the QDPC identified eight specimens of *H. summervillei* collected from Tolga (QLD) in 2016, 5 km from the 1936 outbreak site (Atherton, QLD) (Brookes 1978). This included one further type A specimen (QDPC accession 174662) within an otherwise type B population ($n = 7$) (Schutze et al. 2019). To our knowledge, this represents the first record of the new type B variant.

We identified one consistent morphological difference between the new type B and all other *H. summervillei* specimens. Translucent pores in conspicuous nodules present on the hind

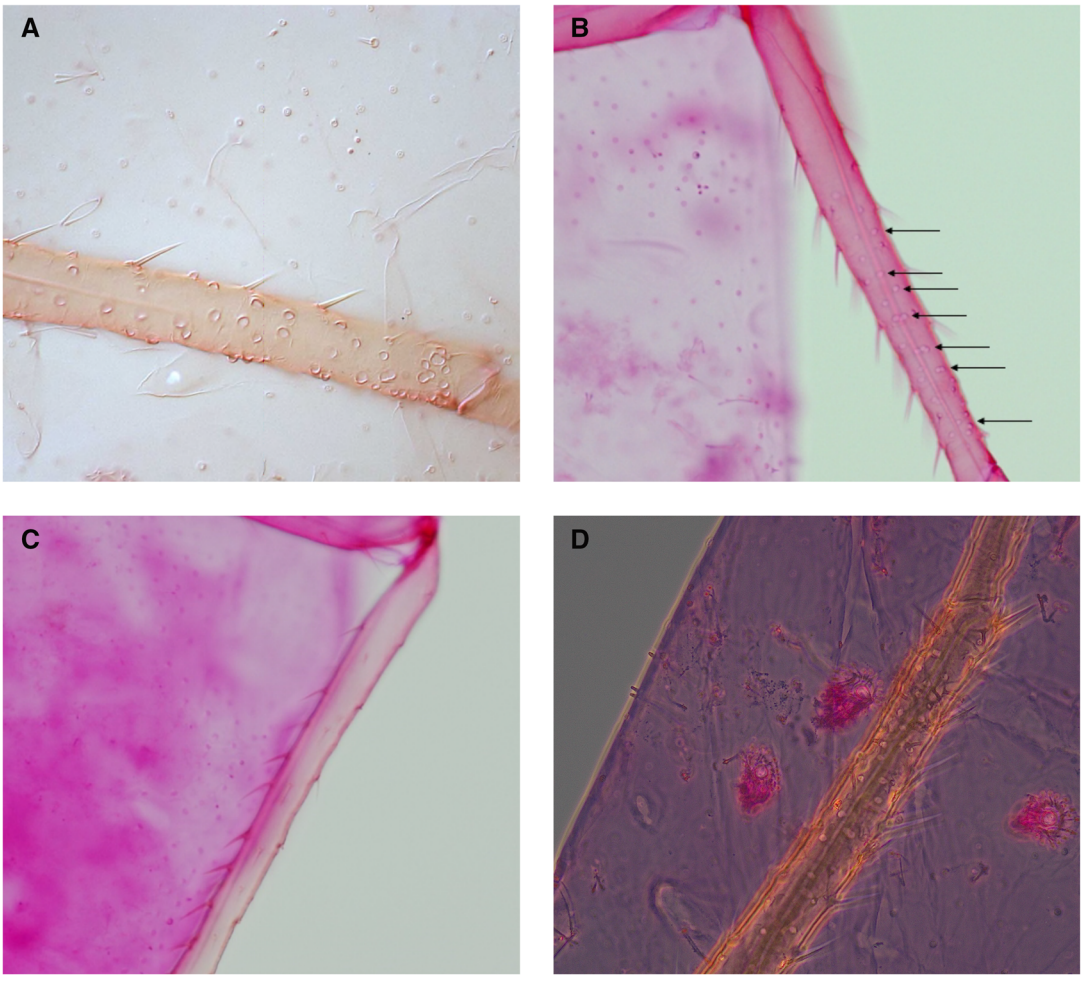

Fig. 2. Light microscopy of *Heliococcus summervillei* specimens examined in this study. Specimens are designated type "A" or type "B" based on concordance with the species description (Brookes 1978) and type material (QM T7832 to T7836). The presence/absence of translucent pores in conspicuous nodules on the hind tibiae distinguishes type A (pores present) from type B (pores absent). Panels show: A) Paratype specimen from Australia; B) Type A specimen from Australia; C) Type B specimen from Australia; and D) Type A specimen from Barbados. Specimen accession numbers are provided in Supplementary Table S1. Images: Mark Schutze (A–C) and Zee Ahmed (D).

tibiae of the type material and all contemporary type A specimens are absent in all type B specimens (Fig. 2). A comparison of key recognition characters across the examined specimens and published descriptions (Brookes 1978, Ghosh & Ghose 1987) is presented in Table 4. A full comparison of all recognition characters is provided in Supplementary Table S3.

The morphology of the *H. summervillei* type A lineage appears to be stable across regions. Specimens from Puerto Rico and Barbados corresponded closely with the Australian type A morphology, with only minor variation in pore/nodule distribution. Descriptions of specimens from Pakistan and India also corresponded with the Australian type A morphology, with minor differences in cerarii structure and slender crateriform duct distribution.

Molecular Phylogenetics of *Heliococcus summervillei* within Pseudococcidae

Sequence recovery varied strongly among host markers. Specimens collected by QUT and NSW DPIRD yielded 142 sequences of sufficient quality and length (n_{18S} = 75, $n_{28S\text{-}D2}$ = 50, $n_{dynamin}$ = 17). Only one of the two type A specimens (A1) yielded a usable dynamin sequence, and many type B specimens (n = 83) yielded dynamin sequences too short to be phylogenetically informative (<150 bp). There were similarly few dynamin sequences amongst those retrieved from GenBank for relevant Pseudococcidae taxa (n_{18S} = 36, $n_{28S\text{-}D2}$ = 39, $n_{dynamin}$ = 14).

Attempts to amplify COI in *H. summervillei* were unsuccessful. Both universal and Pseudococcidae-specific primers (Table 1) for various regions of COI (HCO-LCO, region 2, barcode region) failed to amplify during primer testing and PCR optimization with a randomized subset of specimens from the QUT collection (n = 17). COI sequences were therefore not available for analysis.

Sequence variation was structured by host morphotype: sequence variants were unique to each type, and none were shared between types A and B across any marker, ie all type A sequences were distinct from all type B sequences across all markers (Supplementary Table S4). For the type B population, one "major" unique sequence variant (identified in >50% of specimens) was evident in each of the 18S and 28S-D2 datasets, whereas dynamin had two variants of about equal frequency. Unique sequence variants did not correlate with any other factors of interest (sample site, collection date, pasture type sampled, insect life stage).

Indel structure and neutrality statistics were consistent with a recent population expansion or selective sweep within the *H. summervillei* population. Alignments of unique sequences showed a high proportion of indels in the 28S-D2 dataset, with most appearing at the same positions in the majority of sequences (1 to 29 bp in length, 10+ indels per sequence). A similar but less pronounced pattern was observed in the 18S dataset (1 to 23 bp in length, 6 to 10 indels per sequence). Dynamin sequences retrieved from GenBank contained a large gap (100 bp) not present in the sequences generated by QUT but otherwise demonstrated no consistent distribution of indels. Neutrality tests returned negative Tajima values (D_{18S} = −3.37, $D_{28S\text{-}D2}$ = −3.32, $D_{Dynamin}$ = −3.82, $P \leq 0.05$) and low R_2 values ($R_{2\ 18S}$ = 0.00, $R_{2\ 28S\text{-}D2}$ = 0.03, $R_{2\ Dynamin}$ = 0.01, $P \leq 0.05$) for the total population as well as the type B subpopulation alone (D_{18S} = −3.37, $D_{28S\text{-}D2}$ = −3.33,

Table 4. Comparison of key recognition characters between *Heliococcus summervillei* types in Australian and international populations, with respect to the type specimens described by Brookes (1978)

Recognition character	Australia (n=6) (Brookes 1978)	Pakistan (n=12) (Brookes 1978)	India (n=11) (Ghosh and Ghose 1987)	Australia (n=3) (Schutze et al. 2019)	Australia (n=132) (Schutze et al. 2019)	Barbados (n=10) (I. Gibbs, *personal communication*, 2020)	Puerto Rico (n=2) (G. Evans, *personal communication*, 2020)
Translucent pores in conspicuous nodules on hind tibiae, more numerous distally	Present	Present	Present	Present	Absent	Present	Present but fewer distally
Trilocular pores around the spiracles	Present	Present	Present	Present	Absent	Present	Absent
Posterior cerarii with 4 to 6 small lanceolate setae	Present	Poorly developed	Present	Present	Present but fewer in number	Present	Poorly developed
Large crateriform oral rim ducts	Present	Present	Present	Present	Present but larger in size	Present	Unknown
Slender crateriform ducts surrounding each of the large crateriform oral rim ducts	Present	Present	Present but fewer around posterior large crateriform ducts	Present	Present but restricted to the marginal oral rim ducts; absent or fewer in number around posterior ducts	Present	Unknown
Diagnosis	Paratype	Paratype	Type "A"	Type "A"	Type "B"	Type "A"	Type "A"

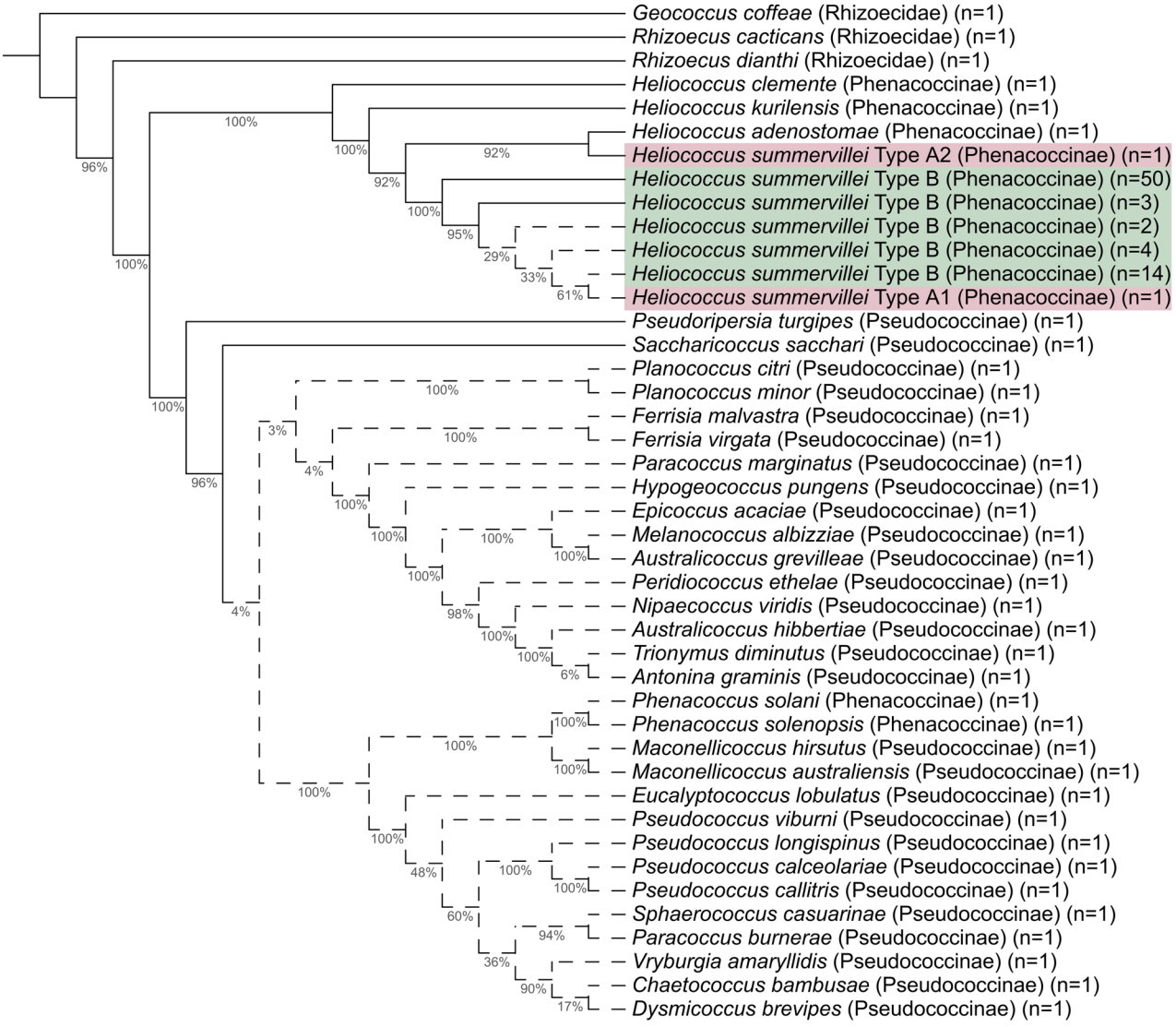

Fig. 3. Maximum-likelihood cladogram of *Heliococcus summervillei* specimens from the QUT, QDPC and ASCU collections, together with GenBank accessions for all available *Heliococcus* species and Pseudococcidae species recorded in Australia. Analysis is based on unique 18S sequences (~550 bp); the number of taxa represented by each leaf is indicated (*n*). Label colors denote *H. summervillei* morphotypes: type A (red) and type B (green). Subfamily names are included for clarity.

$D_{\text{Dynamin}} = -3.84$, $P \leq 0.05$; $R_{2\ 18S} = 0.00$, $R_{2\ 28S\text{-}D2} = 0.01$, $R_{2\ \text{Dynamin}} = 0.01$, $P \leq 0.05$). No base compositional bias was detected in any sequences used for phylogenetic analyse (χ^2 test, df = 3, $P > 0.05$).

Gene trees were constructed using host sequence data (Figs 3–5). A phylogenetic supermatrix approach was precluded, with concatenation constrained by low sequence quality: few specimens yielded usable sequences across all host markers. Congruence between gene trees was assessed by visual comparison, as taxa were not equally represented across the datasets when structured by unique sequence variants and the one-to-one mapping required of many formal tests could not be achieved. The resultant gene trees appear largely congruent. The two major Pseudococcidae subfamilies, Phenacoccinae and Pseudococcinae, are fully resolved (100% BS) as distinct groups in the 28S-D2 and dynamin trees (Figs 4 and 5). In the 18S tree (Fig. 3), two known Phenacoccinae species (*Phenacoccus solani* Ferris and *Phenacoccus solenopsis* Tinsley) fall within an unresolved clade (4% BS) of Pseudococcinae taxa ($n = 12$). Despite this unsupported placement, the broader topology still supports clear subfamily separation. Across all markers, *H. summervillei* consistently formed a well-supported lineage within Phenacoccinae.

Despite clear differences in the sequence variants between *H. summervillei* types A and B, phylogenetic analysis of host-based markers did not consistently separate the morphotypes, with only dynamin partially resolving type A from type B. Taxa of the genus *Heliococcus* consistently formed a monophyletic group within Phenacoccinae, emerging with high support (≥93% BS) from a relatively basal position in all trees. *Heliococcus summervillei* representatives form a further monophyly within the genus in the 28S-D2 and dynamin trees (100% BS). The 18S and 28S-D2 trees (Figs 3 and 4) show the type A1 specimen aligning closely with the type B specimens (≥99% BS) and the type A2 specimen separating out from this group (≥92% BS). The bifurcation between types A1/B and A2 in the 28S-D2 tree arises from a moderately less supported internal

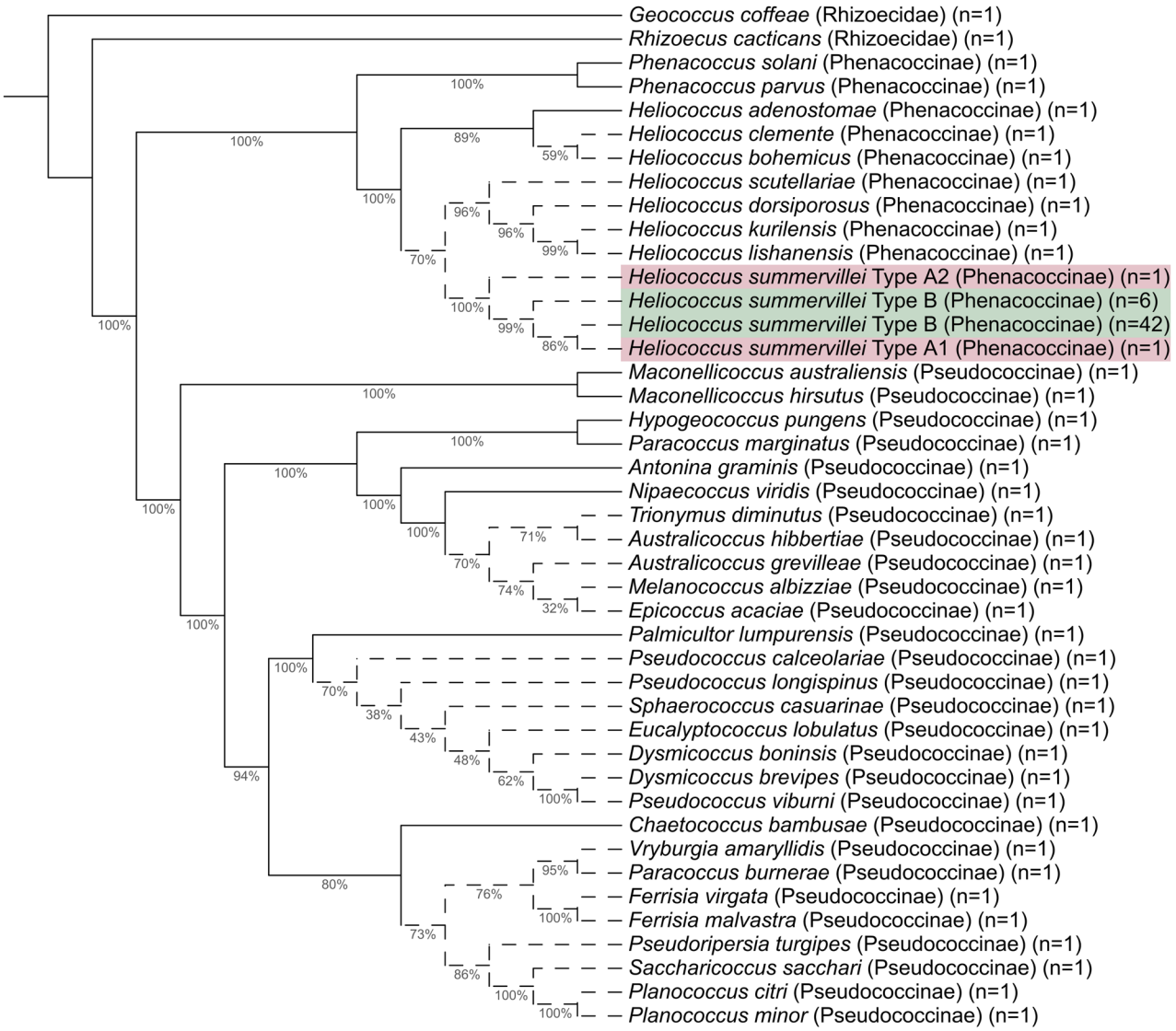

Fig. 4. Maximum-likelihood cladogram of *Heliococcus summervillei* specimens from the QUT and QDPC collections, together with GenBank accessions for all available *Heliococcus* species and Pseudococcidae species recorded in Australia. Analysis is based on unique 28S-D2 sequences (~600 bp); the number of taxa represented by each leaf is indicated (*n*). Label colors denote *H. summervillei* morphotypes: type A (red) and type B (green). Subfamily names are included for clarity.

branch (70% BS), below the support threshold defined in our study (80% BS). However, in the 18S tree, one type A specimen (A2) aligns more closely with *Heliococcus adenostomae* McKenzie than any other *H. summervillei* representatives (92% BS). This placement was maintained across multiple reconstruction methods (neighbor-joining, UPGMA, maximum parsimony, Bayesian inference).

The dynamin tree appears to resolve the morphotypes (100% BS) (Fig. 5) but is based on only one type A specimen (A1), as the other (A2) yielded a sequence below the minimum length threshold for analysis (≪150 bp); any apparent resolution may therefore be due to chance. The type B subgroup is polytomous in the dynamin tree, with all three unique sequence variants unable to be fully resolved.

Molecular Phylogenetics of *Tremblaya*

ASVs originating from *T. phenacola* were identified in 27 specimens of *H. summervillei* (two type A, 25 type B) collected in this study. Six unique *T. phenacola* ASVs were identified in *H. summervillei*, distributed strictly by host morphotype: three ASVs in type A, and three different ASVs in type B. No *T. phenacola* ASVs were shared between the two host morphotypes. Read counts indicated one "major" variant (>50,000 average reads) and two "minor" variants (<2,000 average reads) within the three ASVs unique to each morphological type. While both type A specimens shared the same major ASV, the two minor ASVs identified in specimen A2 were not detected in specimen A1, likely due to low overall reads in that specimen (see Supplementary Table S5 for read counts). The major and minor ASVs unique to type B were found in all 25 type B specimens. *Wolbachia* was detected in the outgroup specimen *C. longulus* but not in any *H. summervillei* specimens. No other notable bacteria were identified.

Forty (40) additional sequences were retrieved from GenBank for the bacterial P-endosymbionts of other mealybug taxa: 37 sequences from *Ca.* Tremblaya and 3 sequences from the outgroup

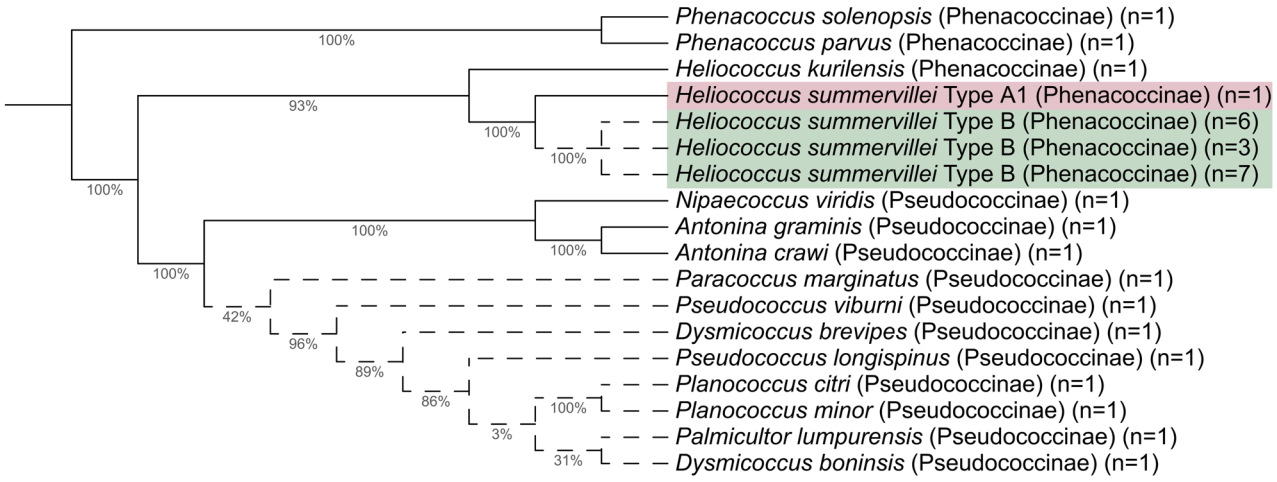

Fig. 5. Maximum-likelihood cladogram of *Heliococcus summervillei* specimens from the QUT and QDPC collections, together with GenBank accessions for all available *Heliococcus* species and Pseudococcidae species recorded in Australia. Analysis is based on unique dynamin sequences (~200 bp); the number of taxa represented by each leaf is indicated (*n*). Label colors denote *H. summervillei* morphotypes: type A (red) and type B (green). Subfamily names are included for clarity.

genus *Ca.* Brownia. A slight base compositional bias was detected in the outgroup sequences (*B. rhizoecola*, $n=3$), which were relatively AT-rich (χ^2 test, df = 3, $P<0.05$) at approximately 46% to 48% GC content. Most sequences ($n>40$) had a small number of short indels at consistent positions throughout the aligned region (1 to 8 bp, approx. 6 indels per sequence). Neutrality tests returned negative Tajima values ($D_{16S} = -1.94$, $P=0.05$) and low R_2 values ($R_2 = 0.05$, $P=0.06$) for the total population, but any significance was lost when looking at the type B subpopulation alone ($D_{16S} = 1.84$, $P=0.06$; $R_2 = 0.21$, $P=0.99$).

Cophylogenetic analysis was limited by sample size, with few specimens yielding usable sequences across both the host and endosymbiont datasets ($n=3$). We determined formal cophylogenetics to be underpowered and of limited interpretive value, and host–symbiont congruence was therefore assessed by visual comparison of the gene trees. The P-endosymbiont phylogeny mirrored host insect taxonomy, with distinct clades (≥98% to 100% BS) corresponding to mealybug lineages (Fig. 6): *T. phenacola* in Phenacoccinae, *Candidatus* Tremblaya princeps (*T. princeps* hereafter) in Pseudococcinae, and *B. rhizoecola* in Rhizoecidae. Interspecies-level relationships in the *T. phenacola* clade were well supported (≥91% BS). Sequences derived from *Phenacoccus* hosts appeared polyphyletic, with five of seven sequences grouping together at the base of the clade (100% BS); the remaining two sequences (from *Phenacoccus azaleae* Kuwana and *Phenacoccus aceris* Signoret) emerged as closest relatives to one another (100% BS), albeit deeper in the tree, nearer to the monophyletic group derived from *Coccura* species ($n=4$, 100% BS) than their more closely related hosts in *Phenacoccus*. In contrast, many relationships within the *T. princeps* clade were not well resolved, with 15 of 18 sequences falling into a group preceded by an unreliable internal branch (38% BS); however, two sequences derived from the host genus *Planococcus* (*Planococcus minor* Maskell and *Planococcus citri* Risso) did separate out from the rest of the clade with high support (100% BS).

Tremblaya phenacola sequences from *Heliococcus* hosts (*Heliococcus kurilensis* Danzig, *Heliococcus clemente* Miller, and *H. summervillei*) ($n=8$) formed a well-supported monophyly (100% BS) within the broader Phenacoccinae-derived clade ($n=25$). Sequences from *H. summervillei* formed a distinct monophyletic subgroup within the *Heliococcus*-associated lineage (100% BS).

In contrast with our host-based molecular analyses, phylogenetic reconstruction of the P-endosymbiont from *H. summervillei* resolved two distinct *T. phenacola* lineages that correspond with host morphotype (100% BS): *T. phenacola* derived from type A (90% BS) and *T. phenacola* derived from type B (92% BS). Low resolution between the type A minor sequence variants (48% BS) was attributed to high sequence similarity (>99%) and a lack of phylogenetically informative characters, with differences (A ↔ G transitions) at only two positions.

Discussion

Host insect morphology and P-endosymbiont molecular phylogenetics support the identification of a new variant (*H. summervillei* type B) associated with the extensive outbreak of dieback in pastures across eastern Australia. The first confirmed specimens of this new variant were detected in Far North Queensland in 2016 (Schutze et al. 2019), shortly after the first reports of pasture dieback in 2012 (QLD DAF 2017).

A wide range of tropical and sub-tropical pasture, turf and wild grasses were observed as hosts of the new variant, including forage sorghum and a sedge. The historical variant (type A) has a similar broad host range within Poaceae and has been reported in multiple countries over the last 100 yr, but with a more restricted geographic footprint (Summerville 1928, Brookes 1978, Ghosh & Ghose 1987, Ben-Dov 1994, Brinon et al. 2004, Schutze et al. 2019, Gibbs 2020, Hauxwell et al. 2022b; G. Evans, *personal communication*, 2020). All contemporary type A specimens identified in this study were collected at or near sites of early dieback records in Queensland, in Atherton (Brookes 1978) and Cooroy (Summerville 1928). This suggests that the ongoing Australian outbreak reflects the rapid expansion of a distinct lineage rather than a shift in host breadth.

The consistent absence of hind-tibial translucent pores is a stable and easily observed character in the type B variant,

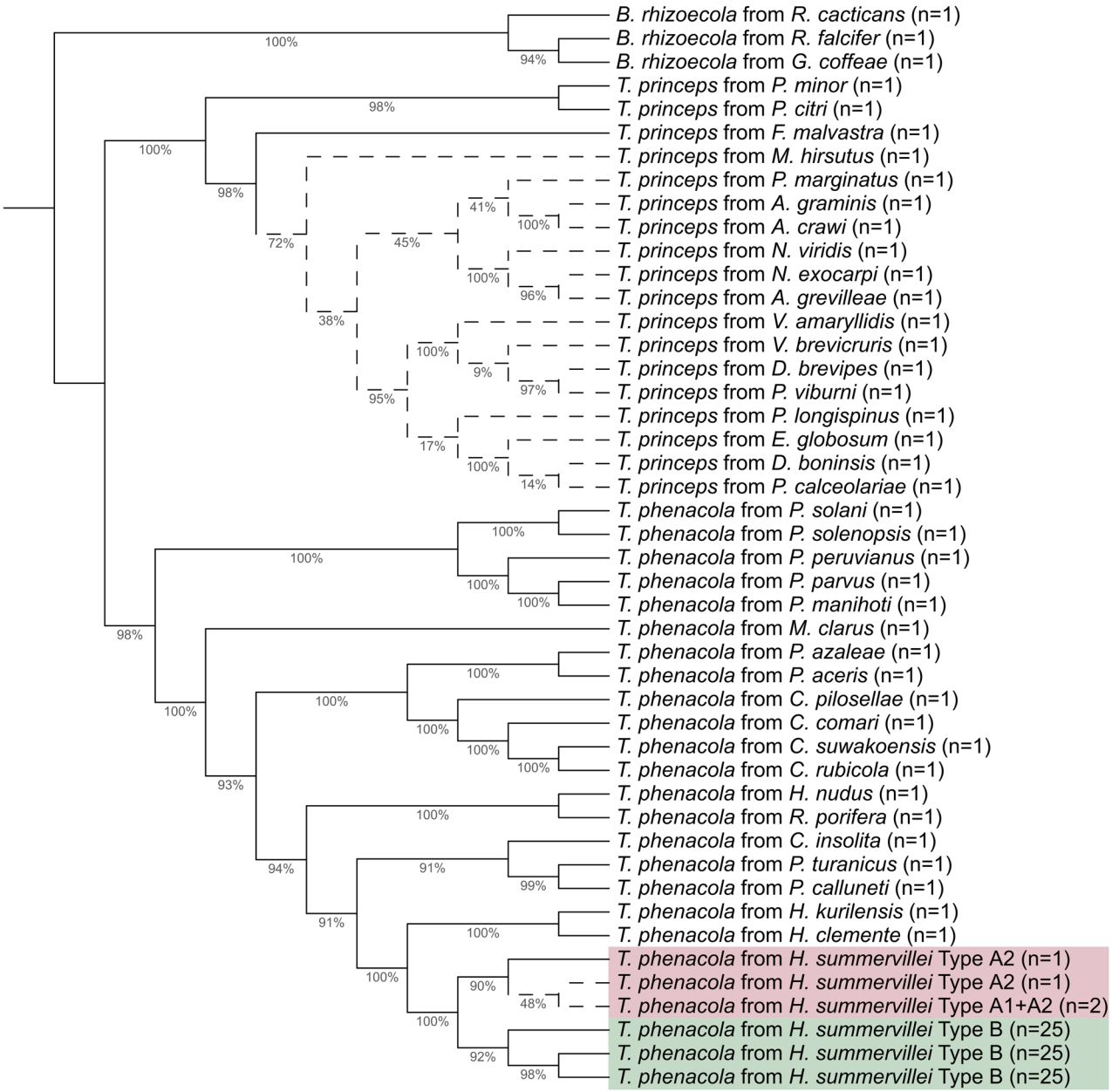

Fig. 6. Maximum-likelihood cladogram of the primary endosymbiont *Tremblaya phenacola* derived from *Heliococcus summervillei* specimens in the QUT collection, together with GenBank accessions for all available *Tremblaya* sequences from *Heliococcus* hosts and P-endosymbiont sequences from Pseudococcidae species recorded in Australia. Analysis is based on unique 16S V3–V4 sequences (~420 bp); the number of taxa represented by each leaf is indicated (*n*). Label colors denote *H. summervillei* morphotypes: type A (red) and type B (green).

independent of environmental factors such as host plant or collection locality. This suggests that it is a reliable and practicable diagnostic trait for distinguishing the new variant. Translucent pores are widely used in contemporary Pseudococcidae taxonomy and employed as a major decision point across multiple genera (Miller & Miller 2002, Miftakhurohmah et al. 2022, Ahmed et al. 2025), demonstrating that pore distribution is considered stable, phylogenetically informative, and taxonomically reliable. However, several characters traditionally used in taxonomic identification are known to vary with environmental conditions and demonstrate intraspecies morphological variability in the adult females of many Pseudococcidae (Cox 1983, Hardy et al. 2008), including *H. summervillei* (Brookes 1978). Pore characters should therefore be interpreted alongside additional taxonomic evidence and not in isolation (Ahmed et al. 2025).

The functional significance of translucent pores in mealybugs remains uncertain and is not within the scope of this paper. They may play a role in pheromone emission (Marsh 1975, Williams 1985, Watson and Kubiriba 2005) but evidence across taxa is mixed, with both sexual and parthenogenetic species exhibiting variable pore presence (McKenzie 1967, Cox & Williams 1981, Waterworth et al. 2012). Both Australian variants of *H. summervillei* are primarily

sexually reproductive (Hauxwell et al. 2022b), though the historical *H. summervillei* variant has been observed to be capable of asexual reproduction (Summerville 1928), and both may undergo seasonal cycles of parthenogenesis as in aphid species (Murano et al. 2018). However, any relationship to the presence or absence of translucent pores is unclear.

We found no evidence of genetic structure within the type B variant population across our sample range, with insect and symbiont sequences showing no association with geography, host plant, collection date, or life stage. The new variant appears to be genetically homogeneous at the markers used here (18S, 28S-D2, dynamin, 16S), consistent with a recent expansion rather than multiple introductions. Additional markers or genome sequencing may reveal further variation not captured in this study.

Patterns of non-neutral evolution in the new variant were also indicated in neutrality tests. Combined with the low genetic variation across the Australian population, the observed patterns are broadly consistent with a recent reduction in genetic diversity (caused by a bottleneck or founder event, for example) followed by rapid population expansion. Similar patterns of rapid range expansion have been documented in other agroecosystem pests, including the papaya mealybug *Paracoccus marginatus* Williams & Granara de Willink (Yadav et al. 2025) and the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Huang et al. 2024). A significant reduction in genetic diversity can paradoxically contribute to invasion success through factors like genetic purging and adaptive phenotypic plasticity (Estoup et al. 2016, Dogantzis et al. 2024). Such mechanisms may explain the rapid spread and apparent hypervirulence of *H. summervillei* in Australia in recent years.

Genomic and mitochondrial markers had variable success in amplifying and resolving taxa to species in subfamily Pseudococcinae and to variants within *H. summervillei*. Although COI has been successfully used in other mealybug taxonomic studies (Gullan et al. 2003, Malausa et al. 2011, Park et al. 2011, Abd-Rabou et al. 2012, Beltra et al. 2012, Wang et al. 2016), multiple COI primers—both universal and Pseudococcidae-specific—failed to amplify in *H. summervillei*. Successful amplification of 18S and 28S-D2 markers from the same samples suggests that there may be substantial divergence or primer mismatch in the mitochondrial genome of *H. summervillei*. Furthermore, "universal" primers are known to fail for many mealybug species, and several features of the COI gene (eg high AT content, high abundance of point mutations, high levels of homoplasy between species) inhibit the design of robust species-specific primers (Malausa et al. 2011, Wang et al. 2015).

Amplification of dynamin was only occasionally successful, both before and after PCR optimization. Analyses were further limited by the few dynamin or dynamin-like sequences available for comparison, and dynamin was largely unable to resolve relationships within Pseudococcinae. Additionally, sequences retrieved from GenBank contained a 100 bp gap that was absent from sequences that we generated. The accessioned sequences appear to have been submitted as mRNA transcripts, so the corresponding DNA sequence for an intron spanning the observed gap has likely been lost following reverse translation to generate the coding DNA sequences used in our analyses. This corresponds with the presence of a single short intron (65 to 85 bp) in the dynamin gene that is observed in Eriococcidae (Hardy 2007).

Although we were able to successfully amplify 18S and 28S-D2, they were also unable to resolve many species-level relationships within Pseudococcinae. This is consistent with previous studies (Downie & Gullan 2004, Hardy et al. 2008, Hodgson 2012, Kaydan et al. 2015, Choi & Lee 2022). Despite their prevalent use in mealybugs, 18S and 28S-D2 have been criticized for lacking sufficient variation to resolve closely related species (Malausa et al. 2011, Park et al. 2011, Wang et al. 2016). The separation of *H. summervillei* specimen A2 from its morphological match in specimen A1 and sistering with *H. adenostomae* in our 18S data may be a result of limited phylogenetic signal due to low variation across the marker region.

Despite poor amplification and limited resolution of variants within *H. summervillei*, the unique sequence variants in all host marker datasets were strictly partitioned by host morphotype. It is important to note that because the historical type A material was only examined morphologically, any distinction between specimens of types A and B in our molecular data reflects only contemporary variation rather than a historical-contemporary lineage split, and the low variability of the markers used here means that such partitioning should be interpreted cautiously and is not in itself evidence of reproductive isolation or species-level divergence. Nevertheless, this finding suggests possible genetic differences between the morphological variants and warrants further investigation into their genomes.

Mealybug lineages share a long co-evolutionary history with their distinct P-endosymbionts (Thao et al. 2002, Downie & Gullan 2005, Gruwell et al. 2010, Lin et al. 2019, Michalik et al. 2019) and hosts of subfamily Phenacoccinae and their *T. phenacola* counterparts were well resolved in our study. However, relationships between *T. princeps* were largely unresolved. This may be a result of the alignment trimming required for our phylogenetic analyses to match the length of the accessioned sequences for *T. princeps* (contiguous 16S-23S operon) to the short sequences we generated for *T. phenacola* (16S V3–V4 region only). Longer sequences may be needed to confirm host–symbiont congruence in subfamily Pseudococcinae, as in other studies (Thao et al. 2002, Downie & Gullan 2005).

Insects can harbor multiple variants of a single endosymbiont, resulting in ASV patterns that may not necessarily reflect differences in the host. However, the clear and consistent separation of *T. phenacola* ASVs by *H. summervillei* type suggests that P-endosymbiont lineages are tightly associated with host morphotype in this system. The absence of shared P-endosymbiont lineages is consistent with a lack of maternal exchange between the host morphotypes. Notably, all contemporary type A specimens identified in this study were collected in mixed populations with the new type B variant. Although our data do not provide direct evidence regarding hybridization (or lack thereof) between the *H. summervillei* morphotypes, the co-occurrence of individuals matching the historical morphology (type A) with distinct *T. phenacola* ASVs amongst larger cohorts of consistently different morphology (type B) with their own unique *T. phenacola* ASVs suggests that the two types may be maintaining separate maternal lineages.

A full taxonomic review of *Heliococcus* is required to resolve the relationships presented in this study. We recognize that the absence of shared P-endosymbionts alone, even in a

mixed population of morphologically distinct hosts, is not sufficient to infer reproductive isolation. Furthermore, host-based molecular phylogenetics failed to resolve the two types. Resolution of the P-endosymbiont phylogeny but not the host phylogenies may reflect monomorphism across the selected host-based molecular markers and/or greater sensitivity of symbiont-based markers. The differences detected here might also result from the faster generation times, and thus faster evolutionary rates, of bacterial endosymbionts compared to their insect hosts (Moran et al. 1995, Wernegreen 2002). Higher-resolution host genomic data, especially in comparison with international specimens, are required to evaluate gene flow in *H. summervillei* and confirm whether the distinct morphotypes and partitioning in our symbiont data reflect deeper evolutionary structure. This could also help determine if the type B population in Australia is the result of a recent introduction.

Though our data do not suggest any geographic origin for the new variant of *H. summervillei*, recent detections of a similar variant in Mexico and the US suggest it is highly mobile (Aca-Martinez et al. 2025, Biles et al. 2025, Powell & Hauxwell 2026). While the formal taxonomic status of the new type B variant is to be determined, it continues to spread across eastern Australia—and possibly the Americas—with significant implications for international biosecurity and a need for rapid and reliable identification.

Our results show that the absence of hind-tibial translucent pores is a consistent character difference in the new variant of *H. summervillei*. We propose this morphological character, in combination with independent molecular data from the maternally inherited P-endosymbiont *T. phenacola*, to support the identification of variants within *H. summervillei*.

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Author Contributions

Ynna Hernandez-Europa (Conceptualization [equal], Data curation [equal], Formal analysis [lead], Investigation [equal], Methodology [lead], Resources [equal], Software [lead], Validation [lead], Visualization [lead], Writing—original draft [lead], Writing—review & editing [equal]), Genevieve Dickson (Conceptualization [equal], Data curation [equal], Formal analysis [supporting], Investigation [supporting], Methodology [supporting], Project administration [equal], Resources [equal], Validation [equal], Writing—original draft [equal], Writing—review & editing [equal]), Muhammad Z. Ahmed (Investigation [equal], Methodology [equal], Writing—review & editing [equal]), Mark Schutze (Investigation [equal], Methodology [equal], Writing—review & editing [equal]), Boyd Tarlinton (Data curation [supporting], Formal analysis [supporting], Investigation [supporting], Methodology [supporting], Software [supporting], Validation [supporting], Visualization [supporting], Writing—original draft [supporting]), Brett Williams (Supervision [supporting], Validation [equal], Writing—review & editing [equal]), and Caroline Hauxwell (Conceptualization [lead], Funding acquisition [lead], Investigation [equal], Methodology [equal], Project administration [lead], Supervision [lead], Validation [equal], Writing—review & editing [equal])

Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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Conflicts of Interest

None declared.

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