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SHORT COMMUNICATION

Metagenomic ecosystem monitoring of soft scale insects and mealybug communities

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ABSTRACT

Soft scale insects and mealybugs are phloem-feeding Hemipterans that are considered major pests in agriculture and horticulture throughout the world. However, correct taxonomic identification in the field can be difficult, making it hard for growers to implement control strategies. In viticulture, soft scale insects are a major issue due to their ability to secrete honeydew, which facilitates the development of sooty mould, and their propensity for being transmission vectors of several viral diseases of grapevine. To facilitate the rapid identification and quantification of vineyard-associated insects a metagenomic-based bioinformatic pipeline (MitoMonitor) was developed for generalised ecosystem monitoring, which automated the assembly and classification of insect mitochondrial genomes from shotgun sequencing data using the Barcode of Life Database API. The proof-of-concept application of MitoMonitor on metagenomic data obtained from eight samples from South Australian vineyards led to the identification of *Parthenolecanium corni* (European fruit scale)—which was thought to be absent in Australian vineyards—as the dominant coccoid species across the samples, with less frequent, and also lower abundance of *Pseudococcus viburni* (obscure mealybug) and *Pseudo. longispinus* (long-tailed mealybug). In addition, parisitoidism by *Coccophagus scutellaris* (Aphelinidae) wasps was also detected. The discovery of *Parth. corni* as a member of scale communities in these samples has significant implications for the development of effective control strategies for this important group of pests in affected areas.

KEYWORDS: grapevine, scale, mealybug, metagenomics, DNA barcoding

INTRODUCTION

Worldwide, the family Coccidae (Coccomorpha) contains more than 1100 species classified as 163 genera, although only 80 species have been described as occurring in Australia (García Morales *et al.*, 2016). Likewise, of the 2143 described Pseudococcidae (Coccomorpha), only 210 have been detected as contemporary Australian populations (García Morales *et al.*, 2016; Moir, 2021).

Several coccids have been shown to affect the grapevine *Vitis vinifera*, including various species within the genera *Ceroplastes*, *Coccus*, *Cryptinglisia*, *Eulecanium*, *Mesolecanium*, *Neopulvinaria*, *Neolecanium*, *Parasaissetia*, *Parthenolecanium*, *Pseudokermes*, *Pulvinaria*, *Saissetia* and *Trijuba* (Ben-Dov, 1977; Ben-Dov and Hodgson, 1997; Rakimov *et al.*, 2013; Rakimov *et al.*, 2015). Among these, *Parth. persicae* (European peach scale), *Parth. pruinosum* (frosted scale), and *Coccus hesperidum* (brown soft scale) are frequently encountered in Australian vineyards, while *Co. longulus* (long brown scale), *Parasaissetia nigra* (black scale), and *Saissetia* species have also been observed at a lower frequency (Rakimov *et al.*, 2013).

Recent surveys conducted in Australia to assess disease pressure and the distribution of Coccids indicate that soft scale insects are rapidly becoming a significant pest of grapevines (Essling, 2018; Venus, 2017). The distribution of soft scale insects and mealybugs appears to be uneven across different grape-growing regions, with Western Australia and Queensland showing less frequent infestations than other states (Rakimov *et al.*, 2013).

While severe infestations of soft scale insects and mealybugs can have direct detrimental effects on vine growth through the depletion of nutrients (Schulze-Sylvester *et al.*, 2021), the main negative impacts of infestation manifest themselves through secondary effects. Firstly, the secretion of sugar-rich honeydew as a by-product of soft scale insect and mealybug feeding facilitates the growth of sooty mould throughout the vine, which can lead to quality reductions and/or total loss of the grape crop (Ben-Dov and Hodgson, 1997).

In addition to promoting sooty mould, soft scale insects and mealybugs also represent known or suspected vectors of grapevine ampelo- and viti-viruses, including grapevine virus A (GVA) (Hommay *et al.*, 2008) and grapevine leafroll-associated viruses (GLRaV) 1 and 3 (Bahder *et al.*, 2013; Engelbrecht and Kasdorf, 1990; Sforza *et al.*, 2003). Infection by these viruses can have negative effects on yield and fruit quality, including reduced accumulation of sugars, increased acidity and irregular flavour profiles (Goheen and Cook, 1959; Naidu *et al.*, 2014). The mobile nymph stages of both types of insects are thought to transmit these viruses between adjacent vines, although recent studies indicate that passive aerial dispersal of the nymphs can promote long-distance dispersal throughout a vineyard (Barrass *et al.*, 1994; Hommay *et al.*, 2019).

Studies have demonstrated the existence of several natural enemies of coccids in Australia, including parasitoid wasps, beetles, moth larvae, and lacewings (Rakimov *et al.*, 2013; Rakimov *et al.*, 2015). Therefore, chemical control strategies for coccids are only recommended in severe infestation cases to avoid harming the beneficial parasitoids that inhabit the vineyards (Frank, 2012; Rakimov *et al.*, 2015b).

Ecosystem monitoring utilising molecular and/or next generation sequencing methodologies have been used to successfully characterise beetle species richness (Crampton-Platt *et al.*, 2015) and airborne fungal diversity (Banchi *et al.*, 2018), and to track pollinator populations (Harper *et al.*, 2023). However, successful species classification generally requires reliable and well populated genetic databases to first be developed to carry out high-throughput monitoring (Crampton-Platt *et al.*, 2015). Targeted ecosystem monitoring was previously carried out by Rakimov *et al.* (2013), utilising a COX1 amplicon to identify individual soft scale insects that infested grapevines across several Australian vineyards. The results of this work led to the conclusion that *Parth. persicae* was the dominant soft scale insect present, with secondary infestations commonly observed by *Parth. pruinosum*.

In order to facilitate the rapid identification and quantification of vineyard-associated insects, an untargeted ecosystem monitoring protocol was developed and its utility was demonstrated by determining the composition of insect populations associated with soft scale insect infestation. Shotgun metagenomic sequencing was employed on population scrapings from vines in four wine regions of South Australia subject to soft scale insect infestation. To enable the high-throughput analysis of the large datasets produced by this approach, a rapid pipeline for assembling and classifying the insect mitochondrial genomes present was established, taking advantage of the Barcode of Life Database (BOLD) API (Ratnasingham and Hebert, 2007).

MATERIALS AND METHODS

1. Collection and DNA extraction and sequencing

In 2022, samples were collected from eight different vineyards in South Australia (Table 1) by taking scrapings containing multiple individuals of soft scale insects from infested vines. The choice of vines for sampling was based on visible soft scale insect infestation; a row within each vineyard was randomly selected and scrapings were taken every 2–3 bays alternating between the left and right sides of the row. The scrapings were sampled from several parts of the vine (trunk, shoots, backs of leaves and bunches) to ensure different life stages could be captured. All the scales from one vine were put in the same tube in ethanol to preserve tissue. DNA isolation was carried out by grinding scrapings taken from one plant per vineyard using a Geno/Grinder 2010 (SPEX), followed by extraction using the DNEasy Plant Kit (Qiagen).

Sequencing libraries were prepared using the Illumina DNA library kit and sequenced on an Illumina NovaSeq 6000 using 2 x 150bp chemistry (Ramaciotti Centre for Genomics, University of New South Wales, Sydney, Australia). Quality

TABLE 1. Assembly statistics for each metagenomic sample.

Sample	Location	Contigs	Total length (kb)	Longest contig	N_{50}	Full mitochondrial genomes	Unique insect taxa
7361	Langhorne Creek	452	425.6	44283	955	4	6
7372	McLaren Vale	305	212.5	10753	747	0	3
7373	McLaren Vale	963	787.4	12427	1023	1	2
7374	Langhorne Creek	910	735.4	15563	1078	1	1
7375	Adelaide Hills	873	717.9	15198	1124	1	2
7376	Adelaide Hills	1015	786.6	15297	900	1	1
7377	Barossa Valley	940	689.8	6523	861	1	2
7378	Barossa Valley	981	763.3	15429	911	1	2

control was carried out on short read data using fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and ngsReports (Ward *et al.*, 2020b).

All sequencing data can be obtained from NCBI under the BioProject accession PRJNA986325.

2. Genome assembly and analysis

Mitochondrial genome assembly was first carried out by mapping raw sequence reads to all available and complete insect mitochondrial genomes (n = 12880) in the NCBI GenBank using BWA-mem (Li, 2013) and sorted using SAMtools (Li *et al.*, 2009); all unmapped reads were removed. Read IDs of mitochondrial mapped reads were used to extract putative mitochondrial reads from the original *fastq* files using seqtk subseq (Li, 2012). Putative mitochondrial reads were then assembled using megahit under default settings. Contigs were remapped back to the RefSeq insect mitochondrial genome database using minimap2 (Li, 2018) with the 'splice' alignment preset. Contigs that were successfully mapped to the insect mitochondrial database were extracted using seqkit subseq (Shen *et al.*, 2016). Each mitochondrial contig was then annotated independently using MITOS (Bernt *et al.*, 2013) with the insect mitochondrial genetic code and the MITOS reference database.

COX1 sequences were extracted from each of the contig annotations and provided as a seed sequence to NOVOplasty (Dierckxsens *et al.*, 2017) to carry out circularised assembly of the mitochondrial genome using the raw short reads. Mitochondrial genes, rRNAs, and tRNAs were then annotated within each circularised genome using MITOS (Bernt *et al.*, 2013) with the insect mitochondrial genetic code and MITOS reference database.

3. Species assignment and phylogenetics

Taxa identification was carried out using two methods. First, COX1 sequences were extracted from all putative mitochondrial contigs and queried against the 'COX1' BOLD database in a highly parallelised manner using the BOLD.R (Mudalige, 2021) and futures parallelisation (Bengtsson, 2020) R packages to pass sequences to the BOLD API (Ratnasingham and Hebert, 2007). Second, mitochondrial contigs were queried against the RefSeq Insect mitochondrial genome database from above using BLASTn (Camacho *et al.*, 2009).

COX1 sequences identified as *Parthenolecanium corni* using the BOLD database were extracted using geaR (Ward *et al.*, 2020a) and aligned to the two COX1 amplicon loci available for this genus using MAFFT (Katoh and Standley, 2013) and maximum likelihood phylogenies were constructed using the best fit model argument in IQ-TREE (Nguyen *et al.*, 2015).

To provide further evidence that *Parthenolecanium sp.* from the Langhorne Creek scraping were *Parth. corni,* the individuals were visually assessed for taxonomic characteristics, including dorsal tubular duct density and tubular duct presence on the dorsum of the anal cleft, which according to Gill (1988), differentiates *Parth. pruinosum* from *Parth. corni*.

4. MitoMonitor pipeline

The MitoMonitor pipeline was written utilising the snakemake workflow framework and can be accessed at github.com/AWRI/MitoMonitor.

RESULTS AND DISCUSSION

1. Highly parallelised, automated assembly of insect mitochondrial genomes from metagenomic samples using MitoMonitor

Ecosystem monitoring using high throughput genomic methods is a powerful tool to deconvolute the composition of mixed population samples (Banchi *et al.*, 2018; Crampton-Platt *et al.*, 2015; Harper *et al.*, 2023). The transformation of shotgun sequencing data into estimated species and abundance requires a multi-stage procedure of genome assembly, annotation and species assignment. To facilitate the automated and reproducible implementation of these bioinformatic workflows, the MitoMonitor pipeline was developed. MitoMonitor is a snakemake implementation that extracts putative mitochondrial reads from short read data and assembles them utilising a metagenomic assembly method in megahit. Putative mitochondrial contigs are then compared to a database of all the insect mitochondrial genomes available on NCBI to remove nuclear and non-

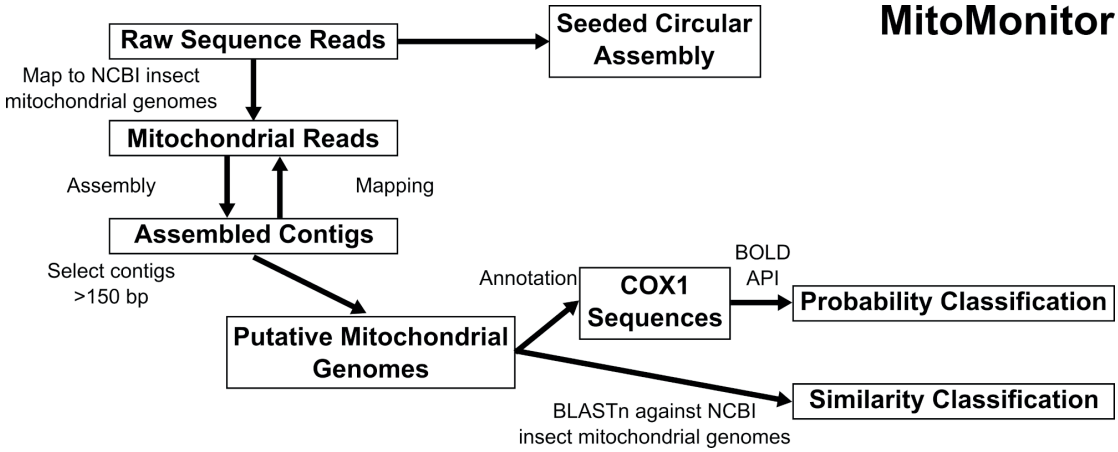


FIGURE 1. Workflow schematic of the MitoMonitor pipeline implemented in snakemake.

insect mitochondrial fragments. Insect mitochondrial contigs are automatically annotated using the invertebrate genetic code, circularised and classified to taxonomic origin utilising information present in databases from both NCBI and BOLD (Figure 1).

2. Application of MitoMonitor to investigate insect communities associated with soft scale infestation of grapevine

To investigate the composition of insect populations associated with soft scale insect infestation, shotgun metagenomic sequencing was employed on population scrapings from areas of soft scale insect infestation from vines across four wine regions within South Australia, which was then passed through the MitoMonitor pipeline. Individual grapevines were surveyed for soft scale insect infestation across eight vineyards in Langhorne Creek, Adelaide Hills, Barossa Valley and McLaren Vale (Table 1), with an average of 28.9 ± 14 Gb of sequencing data produced across the samples.

MitoMonitor confidently classified an average of 2.5 ± 0.78 M reads as having a mitochondrial origin, which were retained for subsequent assembly and analysis. Assembled putative mitochondrial contigs ranged in size from 0.22 to 44.2 kb, and an average of 804 ± 270 mitochondrial contigs were recovered per metagenomic assembly (Table 1). Mitochondrial annotation identified a COX1 coding sequence present in 4.9 % of all mitochondrial contigs, suggesting many contigs are likely fragments derived from the same species (Table 1).

Classification of COX1 against BOLD identified seven Insect taxa across all samples, confidently (≥ 0.95 probability) assigning six to species level. COX1 sequences classified to any insect lineages were then utilised as seed sequences for circularised mitochondrial genome assembly, resulting in circularised complete assemblies for four unique taxa (Table 1). Across the eight vineyard samples, complete mitochondrial genomes, containing 12 protein-coding genes and 2 rRNA arrays, were recovered for *Parth. corni* (15.5 kb), *Pseudo. viburni* (15.2 kb), *Anatrachyntis badia* (Lepidoptera) (15.4 kb) and *Corticarina gibbosa* (Coleoptera) (16.1 kb). Whereas partial mitochondrial genomes were recovered for *Coccophagus scutellaris* (Hymenoptera) (6.5 kb), and *Pseudo. longispinus* (0.88 kb).

When considering individual samples, multiple Hemipteran pest species were identified within the same scraping, suggesting that soft scale insect and mealybugs can co-occur in close proximity to each other (Figure 2). Only DNA from a single insect species, predicted to be *Parth. corni*, was identified across all 8 locations (Figure 2), with all 8 samples sharing 100 % DNA identity across the mitochondrial COX1 locus. Three of the sampled sites were also predicted to contain DNA from *Pseudococcus* species, with two samples being predicted to contain *Pseudo. viburni* (7361, Langhorne Creek; 7372, McLaren Vale), while the third was predicted to contain mitochondrial DNA from *Pseudo. longispinus* (7373, McLaren Vale) (Figure 2).

Previous studies have highlighted the presence of several natural enemies of coccids in Australia, including parasitoid wasps, beetles, moth larvae, and lacewings (Rakimov *et al.*, 2013; Rakimov *et al.*, 2015). Mitochondrial genome fragments were recovered for two distinct Hymenopteran taxa from three different locations. The parasitoid wasp *Coccophagus scutellaris* was identified in the samples from the Barossa Valley, both of which contained *Parth. corni* as the only other predicted insect taxon. A single mitochondrial fragment with best BLAST hits against multiple Chalcidoid wasp mitochondrial genomes was also identified in the 7361 Langhorne Creek sample, although the exact species associated with this sequence remains unknown due to gaps in the available databases that link taxonomic assignments to appropriate genetic information.

The MitoMonitor ecosystem monitoring process is therefore capable of determining the abundance of specific soft scale insect and mealybug species, while detecting insect species that can act as potential biocontrol agents for these pests. As such, this pipeline could be used as an important biodiversity monitoring tool for assessing the impact of various biocontrol strategies, although a complete description of all potential species is limited by the completeness of reference databases.

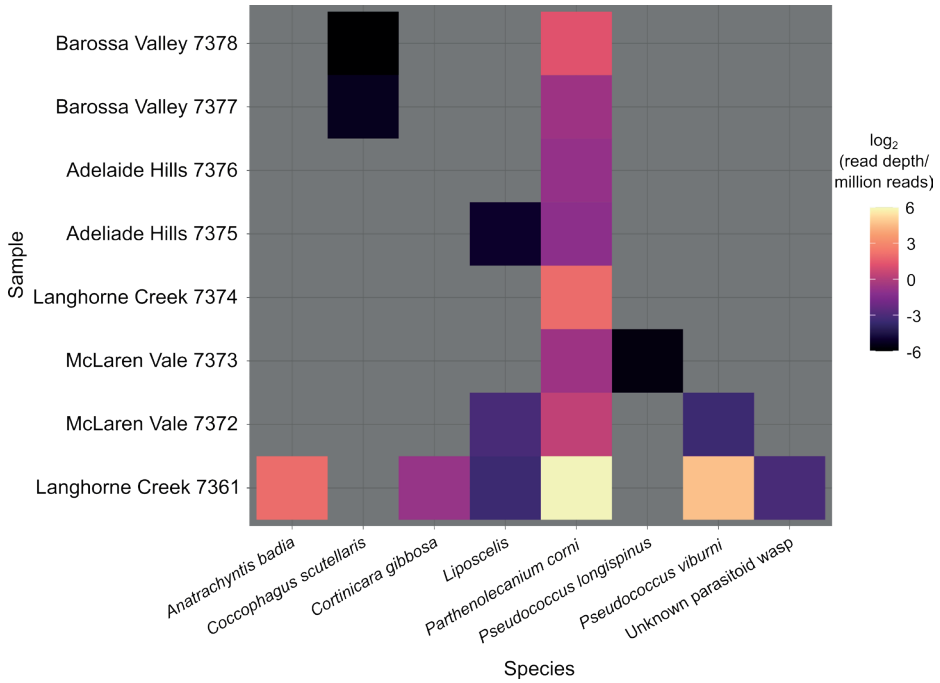


FIGURE 2. Abundance estimates of insect species present in Australian vineyard samples.

Insect abundance estimates based on shotgun sequencing reads mapped against assembled mitochondrial fragments that were classified as insect species, either using the BOLD API or by BLASTn against insect NCBI GenBank mitochondrial genomes. Species abundance is presented as the log2 of total read depth per million reads in each sample. A full table of these values can be found in Table S1.

Therefore, collaboration between taxonomists and molecular biologists is required to increase the power of databases and broaden the analytical power of the methodology.

In addition to mitochondrial genomes from mealybugs, soft scale insects and their potential parasitoids, complete mitochondrial genomes were recovered for the insect species *Anatrachyntis badia* (Florida Pink Scavenger) and *Corticarina gibbosa* (a species of Minute Brown Scavenger Beetle) in sample 7361 from Langhorne Creek, as well as a partial mitochondrial genome from an unknown species of *Liposcelis* (bark louse) in scrapings taken from the Adelaide Hills (7375), Langhorne Creek (7361) and McLaren Vale (7372). This would suggest that these species may also be living within or be associated with colonies of soft scale insect and mealybug; however, due to the limited number of samples, the full scope of ecological diversity across the vineyards is likely to have been underestimated.

3. Evidence for *Parthenolecanium* corni in South Australian vineyards

While *Parth. corni* was described in the Australian taxonomic record from a single set of samples in 1976 (Belbin *et al.*, 2021), since then its presence has not been recorded and it was not previously considered to be an active pest species in Australia. Instead, all subsequent taxonomic studies on the distribution of soft scale insects in Australian vineyards have identified *Parth. persicae* and *Parth. pruinosum* as the only species of *Parthenolecanium* observed (Rakimov *et al.*, 2013). Given the ubiquitous detection of *Parth. corni* in the metagenomic samples, additional phylogenetic analysis was carried out on the COX1 sequence against datasets derived from BOLD and NCBI to confirm the MitoMonitor classification.

Phylogenetic reconstruction utilising all available sequences for the *Parthenolecanium* genus across the COX1-5P region revealed that the *Parth. corni* COX1 7361 haplotype was monophyletic, with all other publicly available COX1 sequences for *Parth. corni* and a maximum genetic distance of 0.9 % across the accessions (Figure 3). Furthermore, the *Parth. corni* 7361 haplotype generated by MitoMonitor was identical to sequences obtained from Australian (GBMNA27795-19), Chinese (GBMNC41505-2), and Chilean (GBMIN67815-17, GBMIN67813-17, GBMHH24788-19, and GBMHH24786-19) populations of *Parth. corni* (haplotype H2, Table 2). The two *Parth. persicae* haplotypes present in BOLD were separated by a single base difference. However, both haplotypes shared a genetic distance of 9.1 % from the *Parth. corni* 7361 COX1 haplotype (Table 2). In comparison, the single publicly available *Parth. pruinosum* COX1-5P, which was also identical to several sequences from isolates of *Parth. corni*, differed by 0.18 % from the 7361 COX1 haplotype.

While the COX1-5P fragment is the *de facto* standard for molecular-based phylogenetic reconstruction in insects, technical issues necessitated the use of an alternate marker from within the central region of the COX1 mitochondrial gene (COX1-central) in an earlier study by (Rakimov *et al.*, 2013). It was therefore not possible to compare the COX1-central data with the curated sequences within BOLD (Ratnasingham and Hebert, 2007).

TABLE 2. Genetic distance (%) between the Parthenolecanium COX1 haplotypes.

Parth. corni 7361	*Parth. corni* H1	*Parth. corni* H2	*Parth. corni/ pruinosum*	*Parth. persicae* H1	*Parth. persicae* H2	*Parth. persicae*	*Parth. pruinosum*	*Parth. corni/ pruinosum**
COX1-5P	0.94	0	0.18	9.1	9.1			
COX1-central						9.1	1.8	0

*Haplotype from "unusual" Parth. pruinosum isolated from Gumeracha South Australia.

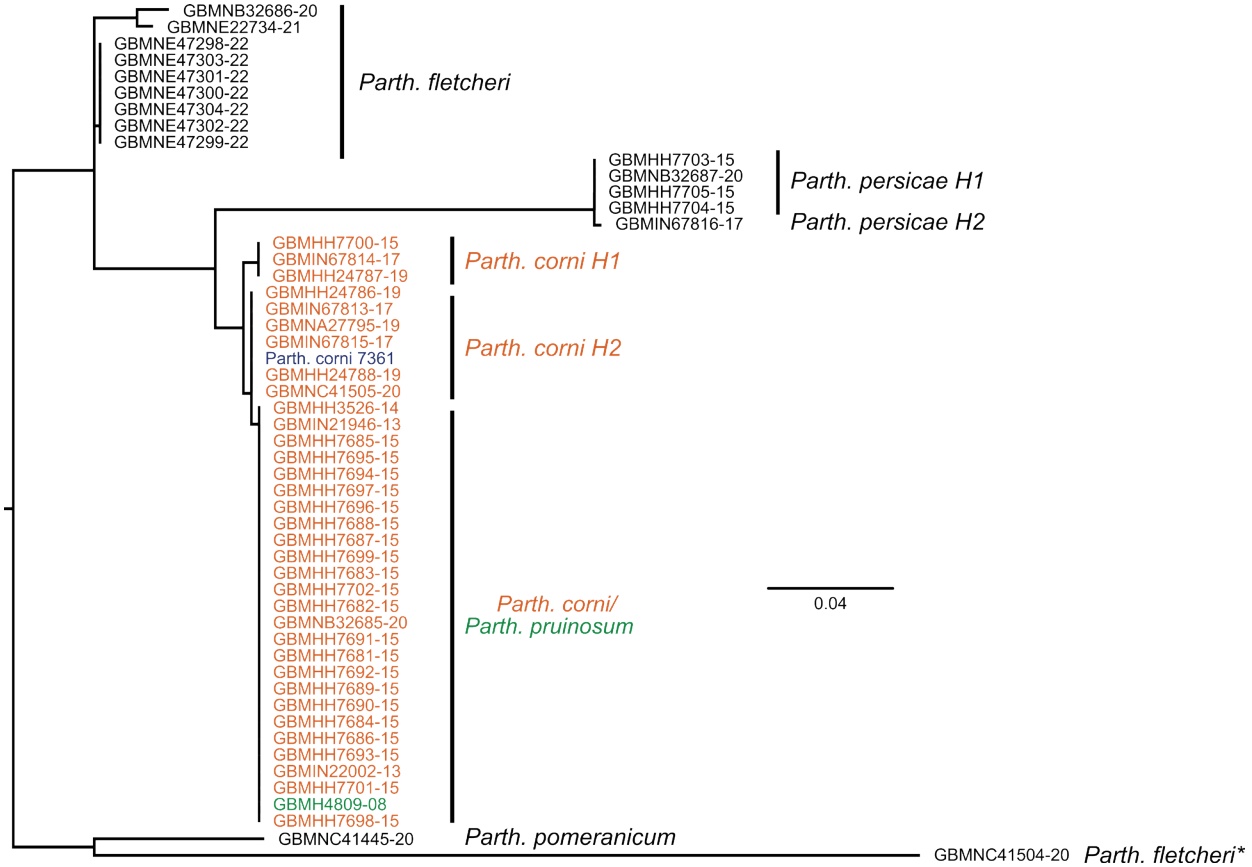


FIGURE 3. Phylogenetic reconstruction of the COX1-5P locus.

Phylogenetic reconstruction of the *Parth. corni* 7361 haplotype against publicly available *Parthenolecanium* datasets across the COX1-5P universal locus sourced from Barcode of Life Database (BOLD). Publicly available data are denoted by their BOLD accessions.

As MitoMonitor provided complete mitochondrial genomes for the predicted *Parth. corni* samples in this study, it was possible to construct a second phylogeny that encompassed sequences form the COX1-central locus produced in Rakimov *et al.* (2013), in addition to a single, independent COX1-central sequence from *Parth. corni* (NCBI:AB439534.1) (Figure 4). As seen for COX1-5P, the *Parth. corni* 7361 COX1 haplotype was highly divergent (9.1 %) from the sequences associated with *Parth. persicae* (Rakimov *et al.*, 2013), clearly distinguishing the two species at the molecular level.

However, unlike the COX1-5P region, which could not differentiate *Parth. corni* and *Parth. pruinosum*, the 7361 haplotype was shown to be identical to the *Parth. corni* AB439534.1 accession, but distinct (1.8 % difference) from the majority of *Parth. pruinosum* samples (Table 2). The exception to this clear species delineation was the presence of sequences from two isolates from Gumeracha, South Australia (KC784922.1 and KC789423.1), which has previously been flagged as representing taxonomically-divergent *Parth. pruinosum* (Rakimov *et al.*, 2013).

In addition to providing information from both COX1 loci (5P and central), the availability of a complete mitochondrial genome for the *Parth. sp.* identified in this study allowed the two phylogenies to be directly compared. By combining the associated metadata information from both topologies it is likely that the *Parth. corni* species designation given to the 7361 haplotype by the BOLD classification (which includes vouchered museum specimens and sequences from the Centre for Biodiversity Genomics) is likely to be correct. Furthermore, it also indicates that both Gumeracha sequences are likely to represent *Parth. corni*, rather than "an unusual" representation of *Parth. pruinosum* (Rakimov *et al.*, 2013).

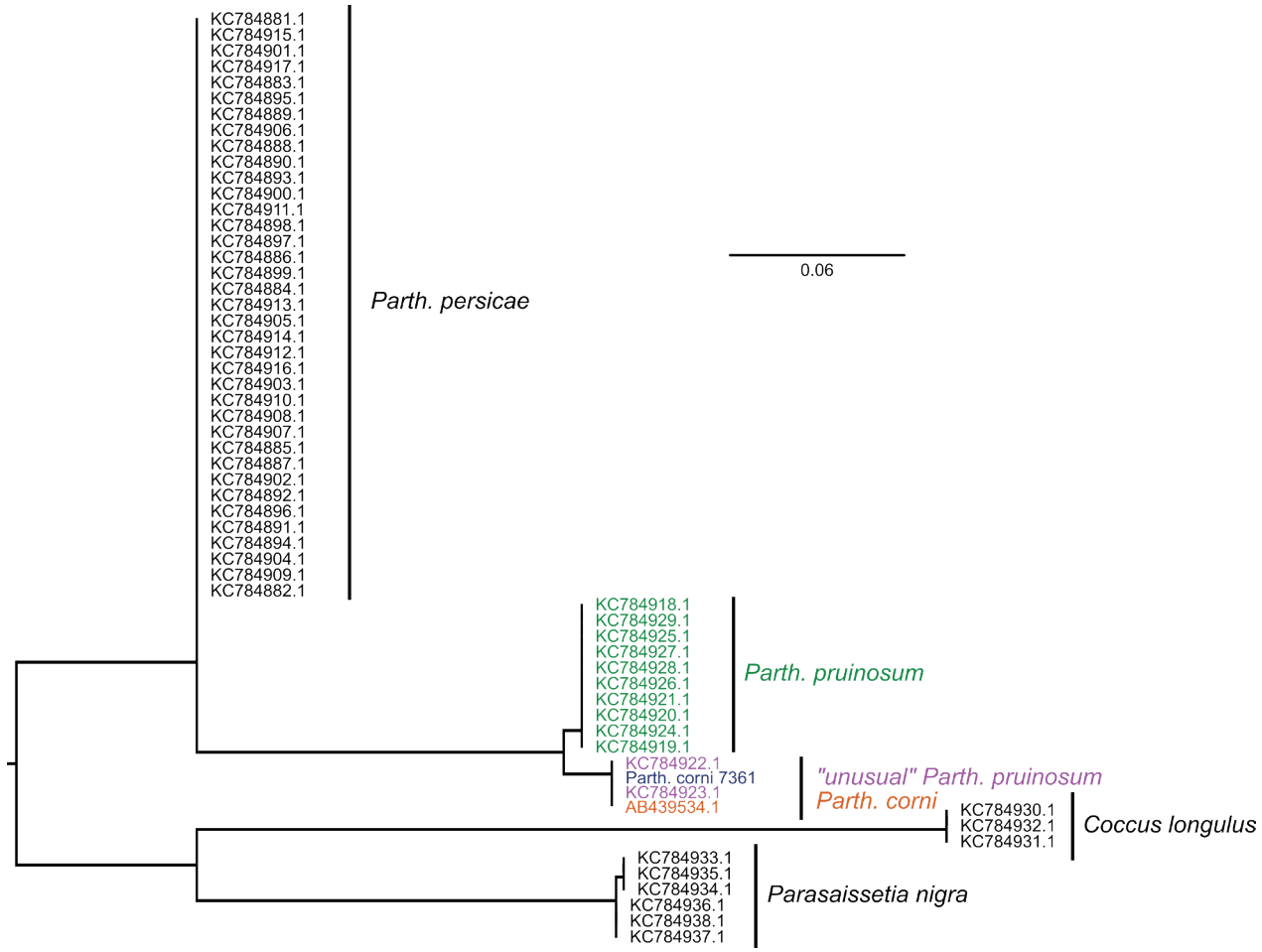


FIGURE 4. Phylogenetic reconstruction of the COX1-central locus.

Phylogenetic reconstruction of the *Parth. corni* 7361 haplotype against publicly available *Parthenolecanium* datasets utilising the COX1-central locus (Rakimov *et al.*, 2013). Samples of *Parth. pruinosum* that displayed unusual taxonomic and lifecycle traits from Rakimov *et al.* (2013) are highlighted in pink. Publicly available data are denoted by their NCBI GenBank accessions.

While the failure to observe *Parth. corni* as a historical pest within Australian vineyards may be due to a recent biological incursion, it may also be a case of misidentification due to complications within the *Parthenolecanium* taxonomic record, as several studies question the validity of current species limits (Danzig, 1997; Gill, 1988; Nakahara, 1981). Specifically, Gill (1988) stated that *Parth. pruinosum* was indistinguishable in the field from *Parth. corni* if the powdery wax was absent, which is generally the case when dealing with dead and/or dried individuals. However, a lack of tubular ducts on the dorsum, including on the anal cleft, can be used as a taxonomic character to delineate *Parth. corni* and *Parth. pruinosum*. Accordingly, the distinct lack of tubular ducts along the dorsum of individual soft scale insects isolated from the South Australian vineyard scrapings (Figure S1), further supports the notion that the sequence data obtained from the metagenomic datasets were derived from *Parth. corni*.

The discovery of *Parth. corni* as a common component of soft scale communities within these South Australian vineyards is consistent with recent observations of multivoltine reproduction within soft scale populations in many Australian viticultural regions (Venus, 2017). First described in California (Coquillett) *Parth. pruinosum* is generally described as univoltine (García Morales *et al.*, 2016; Gill, 1988; Ueda *et al.*, 2008), as is *Parth. persicae*, the other major soft scale insect species previously reported in Australian vineyards (García Morales *et al.*, 2016; Gill, 1988). In contrast, *Parth. corni* is frequently described as multivoltine (García Morales *et al.*, 2016), especially in warmer climates (Canard, 1960; Gill, 1988).

Whether the presence of *Parth. corni* is due to misidentification, incursion, or a combination of both, it has significant implications for the control of soft scale insects within Australian viticulture: current agrochemical applications, which target soft scale insect nymphs, are based on the control of strictly univoltine species (*Parth. persicae* and *Parth. pruinosum*), in which nymphs are present at a single defined time each year. The multivoltine nature of *Parth. corni* dictates that a spread of life stages exists throughout the year, likely rendering univoltine-based control strategies far less effective, and which could potentially explain the increasing prevalence of serious soft scale insect infestations in Australian vineyards.

CONCLUSION

Soft scale insects are a major pest in global viticulture due to their ability to facilitate the development of sooty mould and their propensity for being transmission vectors of grapevine viral diseases. The development of the metagenomic-based ecosystem monitoring approach described in this study has resulted in a proof-of-concept to detect and quantify major pest species, together with key parasitoids and other associated insect species. The discovery of *Parth. corni* as a significant member of infesting soft scale insects in South Australia corroborates empirical observations of soft scale insects with multivoltine lifestyles; it thus has significant implications for the development of effective control strategies for this important group of pests in these areas.

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