Australian Government
Wine Australia

AGRICULTURE VICTORIA

Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (*Xylella*) in Australia and New Zealand by understanding its potential vectors



FINAL REPORT TO WINE AUSTRALIA

Project Number: **WA2001 & 4-EWI2BY6**

Principal Investigator: **Mark Blacket**

Research Organisation: **Agriculture Victoria Research (DEECA)**

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Authors

Mark Blacket [(1)], Cait Selleck [(1)], Duncan Jaroslow [(1)], Francesco Martoni [(1)], Lea Rako [(1)], Narelle Nancarrow [(1)], Pragya Kant [(1)], Piotr Trebicki [(1,2)], and Jessica Hoskins [(3)]

[(1)] Agriculture Victoria (DEECA)
[(2)] Macquarie University
[(3)] NSW DPI

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Abstract

Xylella and its primary vectors are absent from Australia. To understand which Australian insects might be able to vector *Xylella* this project focused on five activities: multi-year insect field surveillance in southeastern Australian crops, examination of Australian insect reference collections, genetic analysis of insect diversity, genetic analysis of bacterial communities in insects, and feeding physiology experiments on plants. Five types of spittlebug insect were found to be widespread in Australia feeding on multiple plant families, possibly making them vector risks. One, *Bathyllus*, exhibited feeding behaviour indicating it would likely be able to spread this plant pathogen should *Xylella* arrive.

Executive Summary

Spittlebugs and froghoppers (Hemiptera: Cercopoidea), and some leafhoppers (Hemiptera: Cicadellidae) are xylem-feeding insects which worldwide can cause economic damage through vectoring plant pathogenic bacteria such as *Xylella fastidiosa*. Australia does not have *X. fastidiosa* nor its main spittlebug and sharpshooter vectors, but it is home to at least forty species of native and endemic spittlebug and froghopper (superfamily Cercopoidea), which could potentially vector bacteria.

The main objectives of this project were to provide the Australian plant industry with robust tools and knowledge to effectively respond to a *Xylella* incursion, or mitigate the potential spread and severity of *Xylella,* if this pathogen were to arrive in Australia. This project focussed on facilitating improved understanding of the biology and population dynamics of potential insect vectors of *Xylella* in and around host crops in multiple regions across southeastern Australia.

The project focused on five main activities: (1) multi-year insect field surveillance in southeastern Australian crops, (2) examination of Australian insect reference collections, (3) genetic analysis of insect diversity, (4) genetic analysis of bacterial communities within insects, and (5) feeding physiology experiments on key crop and *Xylella*-susceptible plants.

(1) Project field surveillance was conducted using multiple collection methods over three years in commercial crops (including grape, citrus, cherry and olive) in southeastern Australia, revealing variation in the abundance of insect communities in different geographic locations and on different plant taxa (with >20,000 insect specimens collected). Many leafhoppers were collected, but only two species of potential *Xylella* vectors, both spittlebugs, were detected, with no other potential native vectors (i.e., sharpshooters) found. The pattern of insect abundance in and around crops suggested that effective surveillance of potential *Xylella* vectors should be conducted during late spring and early summer, preferably using sweep nets or light trapping to capture spittlebugs.

(2) We investigated spittlebug specimens in reference collections across Australian museums and state agricultural departments. Specimen metadata (e.g., date, location, host plant, collection method) from these collections was used to examine spatiotemporal patterns, common collection methods, and plants associated with Australia's spittlebug community. This assessment, of more than 5000 specimens, revealed that the most common genera in Australia are *Anyllis, Bathyllus, Chaetophyes*, *Pectinariophyes,* and *Philagra*, which represent more than three-quarters of the Australian reference specimens analysed. These genera are geographically distributed across multiple Australian states and found on numerous plant families. The genera *Bathyllus, Anyllis* and *Philagra* may have the greatest potential for vectoring of *X. fastidiosa*, if it was introduced to Australia, due to their broad geographic distributions, relatively wide putative host ranges, and high abundances during spring and summer. *Bathyllus* was found to have the widest host range of any recorded Australian spittlebug, with *Bathyllus albicinctus*, the only species in the genus, recorded on 44 plant genera spread across 27 plant families.

(3) The insect genetic analysis conducted here generated 135 new COI DNA sequences for 22 Australian spittlebug taxa, across three families and 10 genera, including some scientifically undescribed species. We also assessed genetic diversity within *Bathyllus albicinctus,* which was shown to be a single species, despite substantial morphological variation between individuals. This new dataset forms the first comprehensive work on the genetic diversity of the Australian spittlebugs and froghoppers and, together with the high-resolution morphological images of each taxon that we have generated, now provides an invaluable tool for improving diagnostic identification of the Australian spittlebugs.

(4) Diagnostic testing for presence / absence of plant pathogenic bacteria (*Xylella, Ralstonia*) was conducted on spittlebug adults and nymphs collected from Victoria. Standard molecular assays from the recently updated *Xylella* National Diagnostic Protocol (NDP) were used on field-collected specimens in the first instance of the NDP assays being applied to insect extracts, with no cross-reactivity observed, validating these for use on insects. Furthermore, bacterial communities (microbiomes) present in nymphs of two common spittlebug species were assessed using high throughput sequencing (HTS) DNA metabarcoding of the bacterial DNA. This technique was applied to two native species, *Bathyllus albicinctus* and *Philagra parva,* and revealed significant divergence in the composition of microbiomes harboured by insect nymphs feeding on different plants, even when these plants were within a few meters of each other.

(5) Detailed studies investigating the feeding physiology of the common native Australian spittlebug, *Bathyllus albicinctus*, to assess its ability to serve as a potential *Xylella* vector were conducted. Across multiple experiments, *Westringia,* olives, and, to a lesser extent grapevines and oranges, were found to potentially be able to support reproductively viable *B. albicinctus* populations. We also monitored feeding behaviour of *B. albicinctus* on susceptible plants using the electrical penetration graph (EPG) technique. These experiments showed that *B. albicinctus* adults continuously feed on grapevine, olive and orange plants and the feeding patterns obtained match those previously described for exotic xylem feeding spittlebugs known to vector *Xylella*. In addition, Xe (xylem egestion) patterns were observed in insects feeding on all of these susceptible plants, suggesting that *B. albicinctus* should be considered a likely vector of *Xylella* if this devastating bacterial disease was to be introduced in Australia.

Project outcomes were formally communicated at more than ten industry publications / conferences / workshops, including the Hemipteran Plant Interactions Symposium (HPIS) in Melbourne in December 2022 to a local and international audience. At the HPIS, formal and informal meetings took place with collaborators from Australia, New Zealand and Europe.

Many staff contributed to the research conducted in this project. The majority of project research was conducted through Agriculture Victoria (DEECA), with some tasks undertaken by NSW DPI and Macquarie University.

Background

Xylella fastidiosa is a destructive plant pathogenic bacterium, with multiple subspecies affecting over 650 plant species including grapes, citrus, cherries, olives, and native Australian plants, including wattles and coastal rosemary (*Westringia*). It is xylem limited and transmitted by Cicadellinae leafhoppers (sharpshooters) and Cercopoidea (spittlebugs and froghoppers, referred to as 'spittlebugs'). *Xylella* and its exotic vectors have been identified by the Plant Health Committee as the number one National Priority Plant Pest for Australia in 2019, by New Zealand MPI as an 'unwanted organism', and by the European Commission as one of the most dangerous plant bacteria worldwide.

Australian plant industries need tools and knowledge that can be effectively implemented to eradicate *Xylella fastidiosa* or prevent and suppress its spread. The only known control strategies that can be implemented to either eliminate *X. fastidiosa,* or reduce its spread, based on sensitive and specific diagnostics, are the removal of the source of infection and the control of the vector. However, knowledge on the distribution, biology and population dynamics of potential insect vectors in Australia and New Zealand is lacking. This may put industries at risk and prevent effective eradication or suppression of the spread of *X. fastidiosa* during and after an incursion.

The objectives of this project were to provide the Australian plant industry with robust tools and knowledge to effectively respond to a *Xylella* incursion, or to mitigate the potential spread and severity of *Xylella* if this pathogen is established in Australia. These objectives will be achieved through improved understanding of the biology and population dynamics of potential vectors of *Xylella* in and around host crops in multiple regions and crops across southeastern Australia. Knowledge of potential vectors shared between Australia and New Zealand will enable both countries greater capacity to respond to an outbreak.

Project Aims

The main aims of the project were to: (*from* Trebicki 2020)

i. Identify effective vector surveillance methods.
ii. Identify potential vectors of *X. fastidiosa* in Australian plant industries at risk (e.g., grape, citrus, cherry and olive).
iii. Document population dynamics of potential vectors (obligate xylem feeders on selected plant species).
iv. Determine the critical times of the year when potential vector populations peak and the spread of *X. fastidiosa* could be most rapid.
v. Understand the range of plant hosts utilised by potential vector species.
vi. Understand the biology and feeding behaviour of potential vectors on selected plants at risk, in order to confirm potential vector status.

Methods

The following activities were planned to support the project aims: (*from* Trebicki 2020).

Vector monitoring sites established in southeastern Australia:

- The project team will establish vector surveillance sites in southeastern Australia.
- Crops that will be monitored include grape, citrus, cherry and olive.
- Monitoring will be done within crops and in some adjacent areas that may contain natural plant hosts.

Vector monitoring and surveillance strategies confirmed:

- At least two different vector surveillance methods will be implemented in the field (e.g. yellow sticky trap and sweep net) to assess the most accurate method for monitoring xylem feeding insects.

Data on potential vectors of *X. fastidiosa*:

- Using the selected surveillance methods (e.g. yellow sticky trap and sweep net) for specific plant industries, the prevalence and incidence of xylem feeders will be monitored fortnightly throughout the year and across several years at study sites in southeastern Australia.
- Xylem feeding insects will be identified to species level and population dynamics described for specific plant industries.
- Previously uncharacterised insect species, that may be potential vectors, will be characterised morphologically and genetically.
- The distribution, abundance, and habitat for the most likely vectors will be investigated.

Plant cultures propagated and maintained:

- Selected plant species identified for vector host range studies will be established and maintained under controlled conditions in chamber and glasshouse facilities at Horsham as a source for subsequent experiments.
- The plant hosts will include grapevines and two other plant species.

Methods to study vectors' host ranges:

- The most suitable methods to study vector biology under controlled conditions will be identified via literature reviews and discussions with international colleagues, assessed in the field and deployed to suit host plant and species of potential *Xylella* vector.
- Colonies of the most abundant xylem feeders suspected of being potential vectors of *X. fastidiosa* for specific plant industries will be established under controlled conditions (plant growth chambers and glasshouses) at Horsham and used for host range and biological studies.

Outreach efforts and project meetings:

- Engagement with growers, advisers and industry through annual meetings, workshops and media releases.
- Project six monthly coordination meetings to discuss ongoing experiments, results and other research activities, in combination with visits to field trial sites and/or the laboratory.
- Linking of this project to relevant activities in other research projects in Australia (*Xylella* diagnostic project), New Zealand (*Xylella fastidiosa* and its vectors in the native estate project), Spain and USA as required.
- A *Xylella* workshop and symposium to be held during the International Hemipteran Plant Interaction Symposium (planned for Melbourne, Dec 2021).
- Close research relationships Trans-Tasman, as well as with collaborative labs in Spain, France, UK and USA. Exchange students and visits to collaborative labs will ensure implementation of the latest and most suitable methods to deliver the best outcome for growers and the industry.

Project Activities (Workplan)

Table 1. Activity Workplan, established at the start of the project (*from* Trebicki 2020).

	OUTPUTS	ACTIVITIES
Surveillance methods	Data on potential vectors of *X. fastidiosa*	Using the selected surveillance methods (e.g. yellow sticky trap and sweep net) for specific plant industries at particular locations, prevalence and incidence of xylem feeders will be monitored on a fortnightly basis across sites in South Australia, Victoria and New South Wales. Xylem feeding insects will be identified to species level and population dynamics described for specific plant industries.
Feeding behaviour & physiology	Data on the potential vectors' host ranges	Informed by surveillance study, potential vector host range will be described. Studies will be conducted under controlled conditions in Horsham. This study will describe the vector's relationship with the host plant and its ability to vector xylem-limited pathogens based on host suitability.
	Plant cultures propagated and maintained	Selected plant species identified for vector host range studies will be established and maintained under controlled conditions as a source for subsequent experiments.
Host plant preferences & survival of potential vectors	Colonies of the most abundant xylem feeders established and maintained under controlled environment conditions	Colonies of the most abundant xylem feeders for specific plant industries will be established under controlled conditions (plant growth chambers and glasshouses) at Horsham. Those suspected of being potential vectors of *X. fastidiosa* will be used for host range and biological studies.
Communication	Outreach efforts and project meetings	Engagement with growers, advisers and industry.

Additional Activities

Complementing the above planned activities, several additional ones were performed in the last year of the project (Table 2). These extra studies primarily involved generating ecological and molecular data relevant to potential vector surveillance, feeding behaviour and physiology:

1. Through investigating spittlebug specimens in reference collections across Australian museums and state agricultural departments. Specimen collection data from these collections was used to examine spatiotemporal patterns, common collection methods, and plants associated with Australia's spittlebug community.
2. Through obtaining DNA barcode reference sequences of freshly collected spittlebugs and froghoppers to assess diversity of this potential *Xylella* vector group in Australia and species delimitation of some widespread species.
3. Through performing 16S high throughput sequencing (HTS) metabarcoding to characterize the bacteria present (microbiome) in two of the most widespread spittlebug species.
4. Through performing diagnostic testing of potential insect vector DNA, to confirm the absence of exotic *Xylella* in spittlebug specimens collected from Victoria, Australia.

Table 2. Additional activities conducted in the project.

	OUTPUTS	**ACTIVITIES**
Surveillance methods	Review of historical specimen collection data	Specimen collection data from these collections was used to examine spatiotemporal patterns, common collection methods, and plants associated with Australia's spittlebug community.
	Reference DNA barcodes of Cercopoidea	Assess diversity of this relatively understudied group of potential *Xylella* vectors in Australia and species delimitation of some widespread morphospecies.
Feeding behaviour & physiology	16S metabarcoding of insect DNA	Characterize the bacteria present (microbiome) in two of the most widespread spittlebug species, compared with host plant and collection location.
	Diagnostic testing for *Xylella* species in insect DNA	Confirm the absence of exotic *Xylella* species in spittlebug specimens collected from Victoria, Australia.

Results/Discussion

Complete methods, results, and discussion of the project findings have been compiled as a series of comprehensive Technical Reports (Table 3). These are provided as attachments to this Final Report. Detailed summaries of these project components are provided here.

Table 3. List of project research activity components provided as Technical Reports (attachments).

	COMPONENT	AUTHORS	REPORT
Surveillance methods	1. Southeastern Australian surveillance trapping for potential *Xylella* vectors around commercial host plants	Duncan Jaroslow, Narelle Nancarrow, Piotr Trebicki, Cait Selleck, & Mark Blacket	Technical Report (AgVic, 17 pages) Attachment 1
		Jess Hoskins, & Mark Stevens	Technical Report (NSW DPI, 9 pages) Attachment 2
	2. Australian Cercopoidea Reference Collection Specimens	Cait Selleck, Duncan Jaroslow, Francesco Martoni & Mark Blacket	Technical Report (AgVic, 68 pages) Attachment 3
	3. Biodiversity and COI barcoding of the Australian spittlebugs (Hemiptera: Cercopoidea)	Francesco Martoni, Lea Rako, Duncan Jaroslow, Cait Selleck, Pragya Kant, Jess Hoskins, & Mark Blacket	Technical Report (AgVic, 23 pages) Attachment 4
Feeding behaviour & physiology	4. Diversity and composition of the bacterial communities (microbiome) in two common spittlebugs, *Bathyllus albicinctus* and *Philagra parva*	Francesco Martoni, Lea Rako, Duncan Jaroslow, Cait Selleck, Pragya Kant, Narelle Nancarrow, & Mark Blacket	Technical Report (AgVic, 32 pages) Attachment 5
	5. Diagnostic testing of potential insect vectors for the presence of *Xylella* species	Pragya Kant, Fiona Constable, & Mark Blacket	Technical Report (AgVic, 17 pages) Attachment 6
	6. Feeding behaviour of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa*	Piotr Trebicki, Clara Lago, Cait Selleck, Francesco Martoni, & Mark Blacket	Technical Report (AgVic, 16 pages) Attachment 7
Host plant preferences & survival of potential vectors	7. Host plant preferences and survival of the native Australian spittlebug, *Bathyllus albicinctus*	Duncan Jaroslow, Lea Rako, Narelle Nancarrow, Piotr Trebicki, Cait Selleck, Francesco Martoni, & Mark Blacket	Technical Report (AgVic, 25 pages) Attachment 8
			Total = 207 pages

(1) Southeastern Australian surveillance trapping for potential *Xylella* vectors around commercial host plants

(from Technical Reports, Attachments 1 and 2)

- Field Surveillance in Victoria

The main aim of this surveillance activity was:

1) To compare the abundance and temporal variation of Cercopoidea and Cicadellidae between crops and collection techniques at multiple sites in Victoria.

The capacity of *Xylella fastidiosa* to establish, and thus impact Australian agricultural commodities, may depend upon the ubiquity of these insects in Australian commercial crops. Investigating the abundance of these insects around key growing areas should provide insights into the vulnerability of Australian crop growers, and effective methods for monitoring of disease-vectoring insects.

The Cercopoidea (spittlebugs and froghoppers) and Cicadellinae (sharpshooters), belonging to the insect suborder Auchenorrhyncha (Hemiptera), have been implicated in the spread of *X. fastidiosa* in North American and Europe. In Australia, the cercopoid insect *Bathyllus albicinctus* (Cercopoidea: Aphrophoridae) has been identified (through this project) as having a relatively high potential risk of vectoring *X. fastidiosa*, due to its recorded association with a diverse group of plant genera and families. For these reasons, it was hypothesised that cicadellines and cercopoids, including the spittlebug *B. albicinctus*, would be likely potential candidates for spreading *X. fastidiosa*, should this pathogen arrive in Australia.

Our study surveyed the invertebrate communities at two commercial farms: Grampians Olive Estate (GO) and Norton Estate (NE), both located in the Wimmera shire of Victoria. Four sampling methods were used to survey the Auchenorrhyncha at these farms, at least fortnightly, for approximately three years. Many cicadellid specimens were caught (Figure 1), demonstrating the effectiveness of the collection methods, however the xylem-feeding cicadelline sharpshooters were not detected around these commercial farms. Differences in *B. albicinctus* abundances were observed between field locations and sampling methods. The results of this study provide insights into the seasonal abundance of *B. albicinctus* and sampling methods best suited to catching these insects.

The surveys conducted at GO or NE sites to broadly document patterns in the abundance of potential *X. fastidiosa* vectors revealed that:

a) The use of sweep nets and visual assessment of crops were best for catching *B. albicinctus*. While pan traps and sticky mat traps were more effective for catching Cicadellidae.

b) *B. albicinctus* adults were only caught between October and January each year (Figure 2).

c) Nymphal (immature) *B. albicinctus* were observed feeding on both crop plants (olives) and surrounding vegetation in the field (Figure 3).

d) Cercopoidea, other than *B. albicinctus*, were not caught at GO or NE.

These experiments demonstrated that future surveillance of potential *X. fastidiosa* vectors should be conducted during late spring and early summer, preferably using a sweep net for *B. albicinctus* and a sweep net and/or sticky mat traps for Cicadellidae (noting that no known xylem-feeding members of the Cicadellidae were caught during this survey).

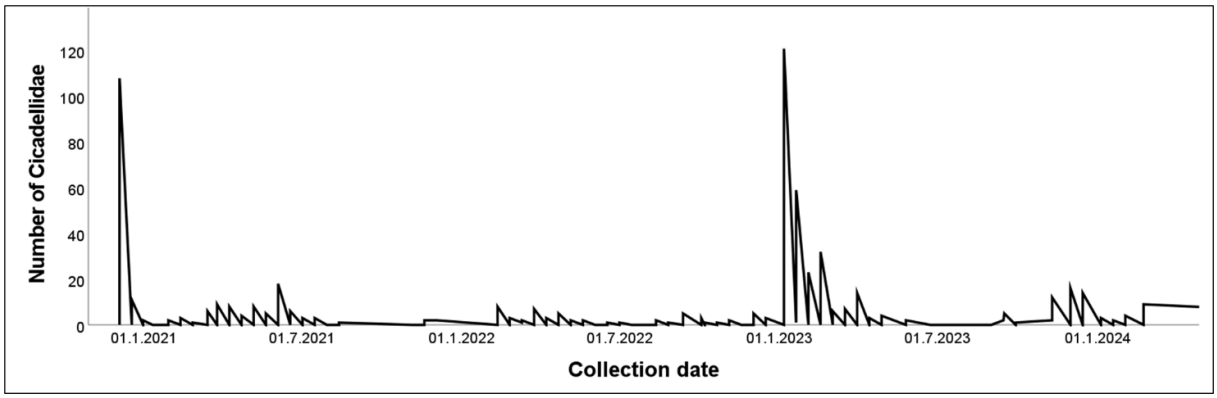


Figure 1. Cicadellidae abundance throughout the study period.

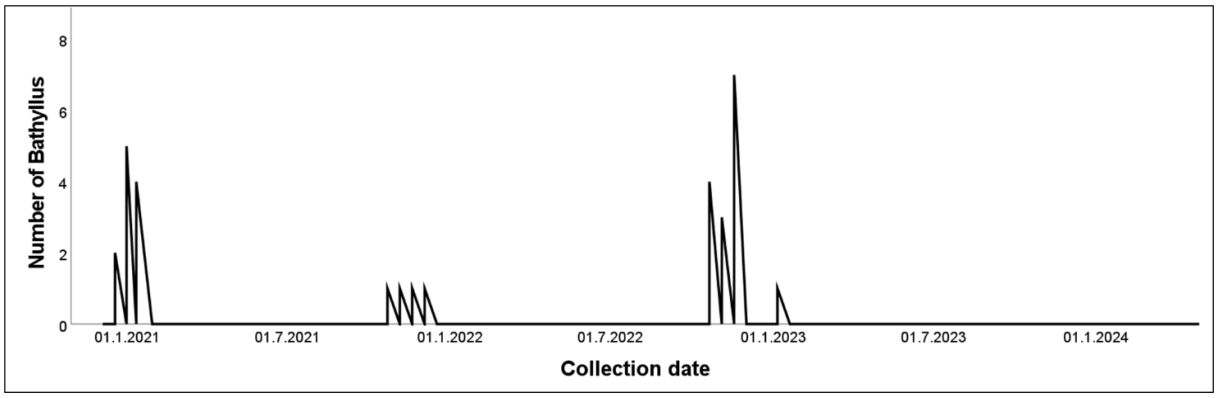


Figure 2. *Bathyllus albicinctus* abundance throughout the study period.

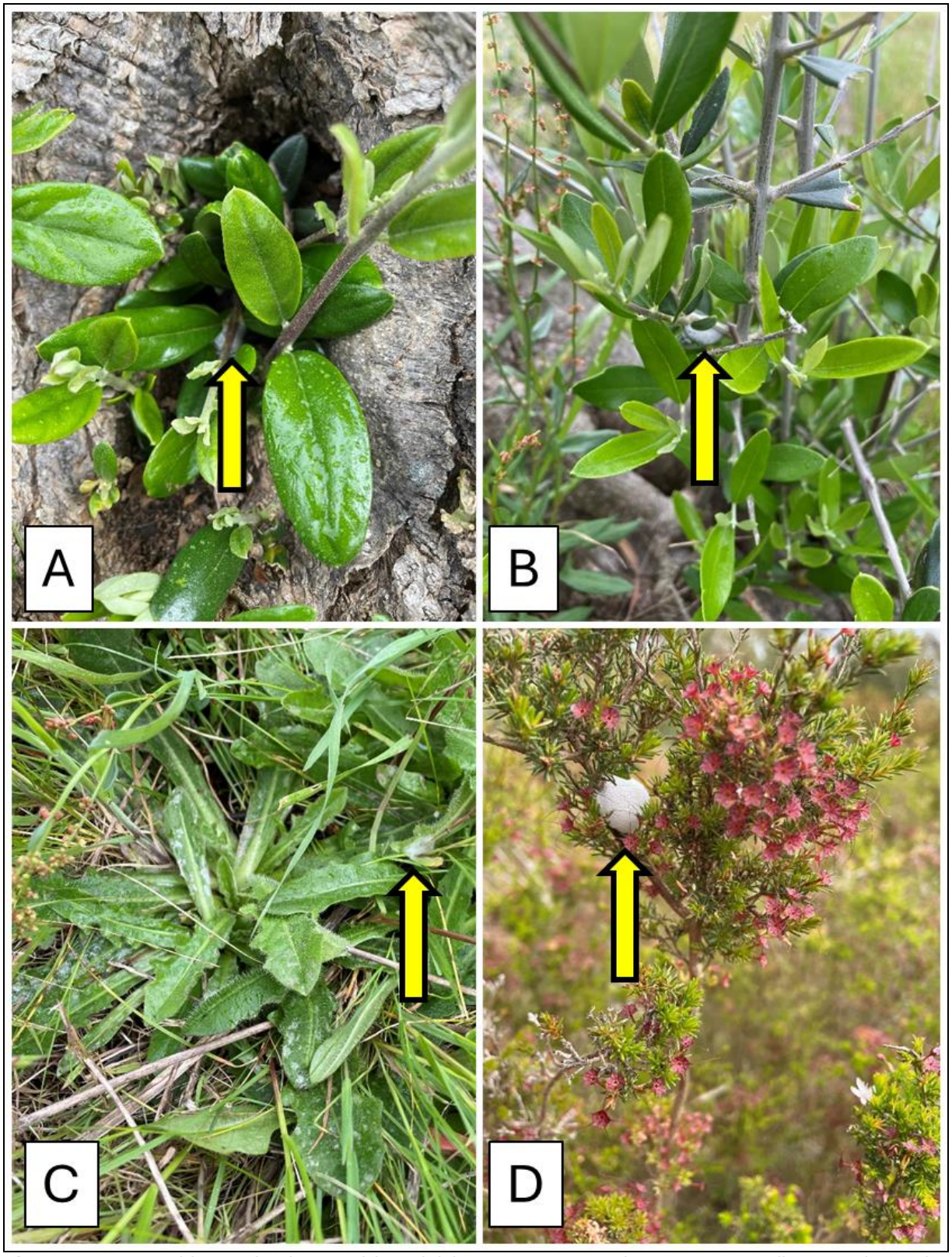


Figure 3. Cercopoid nymphs (covered in spittle) present at GO site, Oct 2023. Olive tree (A & B), flatweed (C) and heather (*Erica* sp.) (D). Presence of spittle is indicated by arrows.

- Field Surveillance in NSW

The main aim of this surveillance activity was:

2) To compare the abundance and temporal variation of Cercopoidea and Cicadellidae between crops and collection techniques at multiple sites in NSW.

This study investigated the presence and distribution of potential native leafhopper and spittlebug vectors of *Xylella fastidiosa* in horticultural crops and native vegetation in the Riverina area of NSW, over three years of trapping and sweep netting sampling at ten locations (grapes x 4 sites, citrus x 2 sites, cherries x 2 sites, and olives x 2 sites; Table 5). More than 20,000 leafhopper, treehopper, and spittlebug specimens were collected from crops, with all species identified regardless of potential vector status (Table 6).

We found only three individuals of one spittlebug species, *Pectinariophyes stalii* in the crop sites, with no other potential native vectors, such as members of Cicadellini found. The low abundance of *P. stalii*, and the absence of other species from groups recognised as containing xylem-feeders indicates that should *Xylella fastidiosa* enter Australia, there is likely a low risk of spread within the Riverina area, and any spread that does occur would probably be slow. However, further investigation of the distribution of potential native vectors in other areas of NSW is required to better inform biosecurity response plans.

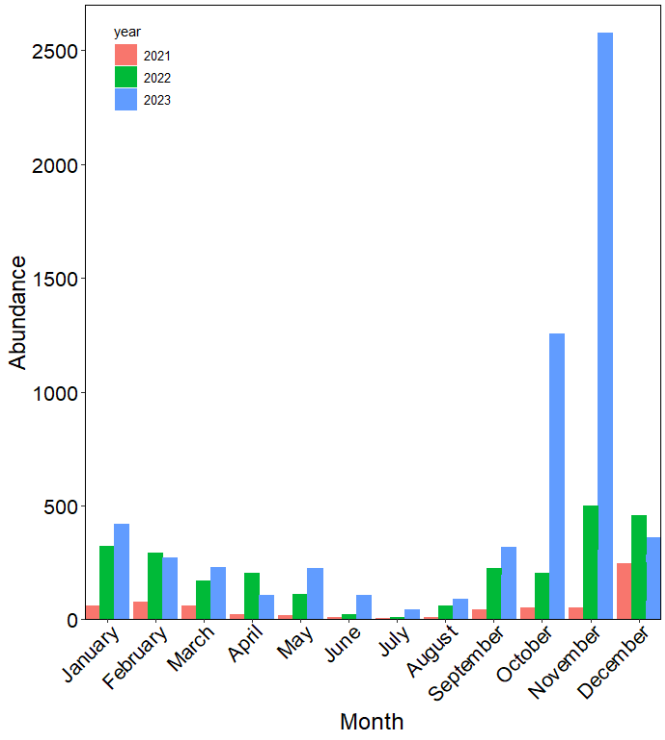


Figure 4: Abundance for each full year sampled. Large numbers in October and November of 2023 are primarily attributed to one grass feeding non-vector leafhopper species, *Anzygina* species.

Table 5: Site sampling locations, crop type and establishment and end dates.

Site	Location	GPS Co-ordinates	Crop	Establishment	End
YAI	Yanco	34.6149, 146.4156	Citrus (oranges)	17/11/2020	24/04/2023
GRC	Griffith	34.1919, 146.0445	Citrus (oranges)	23/11/2021	4/12/2023
GRG	Griffith	34.1915, 146.0450	Grapes	23/11/2021	4/12/2023
LIL	Leeton	34.3421, 146.2528	Grapes	13/12/2021	22/12/2023
YAR	Yenda	34.1402, 146.1056	Grapes	20/12/2021	28/12/2023
TOO	Leeton	34.3324, 146.2301	Grapes	20/01/2022	8/02/2024
RAP	Hanwood	34.2447, 146.0222	Cherries	9/03/2022	27/03/2024
GIA	Bilbul	34.1628, 146.0648	Cherries	9/03/2022	27/03/2024
MOR	Widgelli	34.2206, 146.1211	Olives	17/03/2022	27/03/2024
BRU	Ganmain	34.5324, 147.0448	Olives	27/10/2022	27/03/2024

Table 6: Leafhopper, treehopper, and spittlebug species and abundance in crops in the Riverina area of NSW. Species highlighted in blue represent known vectors of other viruses and bacteria.

Membracoidea-Cicadellidae		Cherry	Citrus	Grape	Olive	Total
Deltocephalinae						
Athysanini	*Arawa pulchra* Knight	7	12	49	28	96
	Limotettix incertus Evans	7	29	44	12	92
Chiasmini	*Chiasmus varicolor* (Kirkaldy)		1			1
	Exitianus plebeius (Kirkaldy)	4	58	66	15	143
Deltocephalini	*Horouta austrina* (Kirkaldy)			11	40	51
	Horouta jahmoi Fletcher				8	8
	Horouta perparvus			2	1	3
	Hourouta spp. complex[1]	1	1	4	34	40
	Maiestas spp. complex[2]	80	123	150	79	432
Eupelicini	*Paradorydium* cf. *pseudolyricen* Kirkaldy				10	10
Goniagnathini	*Goniagnathus osmelaki* Fletcher			2		2
	Goniagnathus venenensis Fletcher				1	1
Macrostelini	*Balclutha incisa* (Matsumura)	23	63	101	2	189
	Balclutha rubrostriata (Melichar)		1			1
	Balclutha saltuella (Kirschbaum)	9	4	8		21
	Cicadulina bimaculata (Evans)			2		2
	Nesoclutha phryne (Kirkaldy)	33	70	53	54	210
	Orosius complex[3]	767	399	784	1650	3600
	Orosius sp.1		1		23	24
Paralimnini	*Soractellus* nr *brunneus* Evans		1	8	2	11
Scaphoideini	*Scaphoideus foshoi* Fletcher & Semararo	1		1		2
Penthimiini	Penthimiini sp.1				1	1
Iassinae						
Batracomorphini	*Batracomorphus angustatus* (Osborn)	225	172	129	88	614
Idiocerinae						
	Idiocerinae sp.1			1		1
Macropsinae						
Macropsini	Macropsini sp.1			1		1

Megopthalminae						
Agalliini	*Austroagallia torrida* Evans	302	66	229	65	662
Typhlocybinae						
Empoascini	*Austroasca malvae* (Evans)			1		1
	Austroasca viridigrisea (Paoli)	90	67	57	30	244
Erythroneurini	*Anzygina* spp. complex[4]	4749	2530	4420	1604	13303
	Empoascanara cf. *peregrina* Dworakowska	1				1
	Zygina cf. *evansi* (Ross)				1	1
Xestocephalinae						
Xestocephalini	*Xestocephalus sidnicus* Kirkaldy				3	3
	Xestocephalus tasmaniensis Evans	226	5	96	10	337
Membracoidea-Membracidae						
Centrotinae						
Terentiini	*Pogonotypellus australis* (Goding)				38	38
Cercopoidea-Machaerotidae						
Enderleiniinae						
Enderleiniini	*Pectinariophyes stalii* (Spångberg)	1	1	1		3
Total		**6526**	**3604**	**6220**	**3799**	**20149**

[1]*Horouta austrina* (Kirkaldy), Horouta perparvus (Kirkaldy), *Horouta jahmoi* Fletcher
[2]*M. veta* (Knight*), M. knighti* Webb & Viraktamath
[3]*O. orientalis* (Matsumura), *O. argentatus* (Evans), *O. canberrensis* (Evans)
[4]*A. zealandica* (Myers), *A. honiloa* (Kirkaldy)

(2) Australian Cercopoidea Reference Collection Specimens

(from Technical Report, Attachment 3)

The two main aims were to inform Xylella vector surveillance activities by:

1) Compiling cercopoid specimen records from Australian invertebrate reference collections.
2) Analysing temporal and spatial trends of specimen records, determine common collection methods and examine Australian Cercopoidea plant associations.

Outside Australia, the insect group known as spittlebugs (superfamily Cercopoidea) is attributed with vectoring the lethal plant bacterium *Xylella fastidiosa* between host plants during feeding. Identifying high and low risk *X. fastidiosa* vectors in Australia is increasingly relevant to Australian agricultural security, with *X. fastidiosa* presenting a growing threat to global agriculture. Surveillance relies on effective and consistent collection methods for vector insects to screen for bacterial pathogens. If *X. fastidiosa* were to arrive in Australia, the effectiveness of surveillance and management could be restricted by a limited understanding of Australia's native cercopoid abundance, phenology, distribution, and diet.

Our study explored these key knowledge gaps. We investigated cercopoid specimens in reference collections across Australian museums and state agricultural departments. Specimens present in these collections were used to better understand the spatiotemporal patterns, common collection methods, and plants associated with Australia's cercopoid community. Specimen information including collection date, location, host plant, and insect identity (at genus level) were obtained from a total of 6350 preserved cercopoid specimens, with 5555 (87.5%) of these records deemed appropriate for further analysis. Our assessment revealed that:

a. Effective adult insect collection methods differ between cercopoid families (Table 7). For example, 'light trapping' is noted as the most common collection method overall, however for Aphrophoridae the most common collection method is 'hand collecting', followed by beating / sweeping / sieving / vacuuming (activities are that often performed together).

b. Overall Cercopoidea showed a strong pattern of seasonal abundance (Figure 5), however this varied greatly with state / territory. For example, *Anyllis* were only collected in South Australia from January to March but were collected from Queensland in all months except June.

c. The five most abundant cercopoid genera, *Anyllis*, *Bathyllus*, *Chaetophyes*, *Pectinariophyes*, and *Philagra* (Figure 7), represent more than 75% of all specimens analysed (each genus representing >10% of all specimens), are geographically distributed across multiple Australian states and are found on multiple plant families (Figures 6 & 8).

d. Notably, the genera *Bathyllus, Anyllis* and *Philagra* appear to have the greatest potential for vectoring of *X. fastidiosa* (if it was introduced to Australia) due to their broad geographic distributions, relatively wide putative host ranges, and higher abundances during spring and summer when the risk of spreading *X. fastidiosa* is likely higher. *Bathyllus* was found to have the widest host range of any recorded Australian Cercopoidea. *Bathyllus albicinctus*, the only species in the genus, was recorded on 44 plant genera spread across 27 plant families (Figure 8).

e. The plant genera *Eucalyptus* and *Acacia* may have a high risk of becoming infected with *X. fastidiosa* in the case of an incursion, in part due to the high diversity of Cercopoidea that are associated with these plants (Figure 8).

This information represents a previously unavailable resource, with approximately half of these specimen records not databased within reference collections, and a much higher percentage not available to researchers without prior connections to each reference collection. The records analysed here provide valuable insights into when and where *X. fastidiosa* is likely to establish or spread in Australia in the case of an incursion (based on Australian insect potential vectors), and thus where and how surveillance and management efforts can be most effective.

Table 7. Collection method for cercopoid records, separated by cercopoid family.

	Cercopoid family			
Collection method	**Aphrophoridae**	**Cercopidae**	**Machaerotidae**	**Grand Total**
Light	267	350	1228	1845
Hand	451	73	86	610
Beat/Sweep/Sieve/Vac	325	89	22	436
Flight trap	150	38	26	214
Ground trap	63	12	5	80
Pyrethrum	5	0	6	11
Reared	2	5	1	8
Trap	3	1	0	4
Total	1266	568	1374	3208

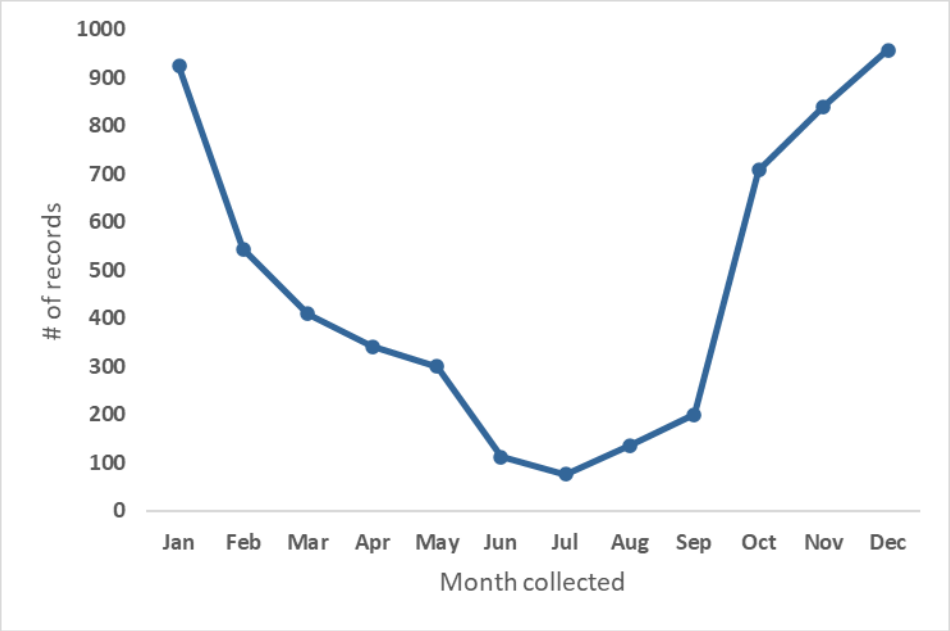


Figure 5. Count of adult cercopoid records for each month. All records included in the analysis contained information on month of collection.

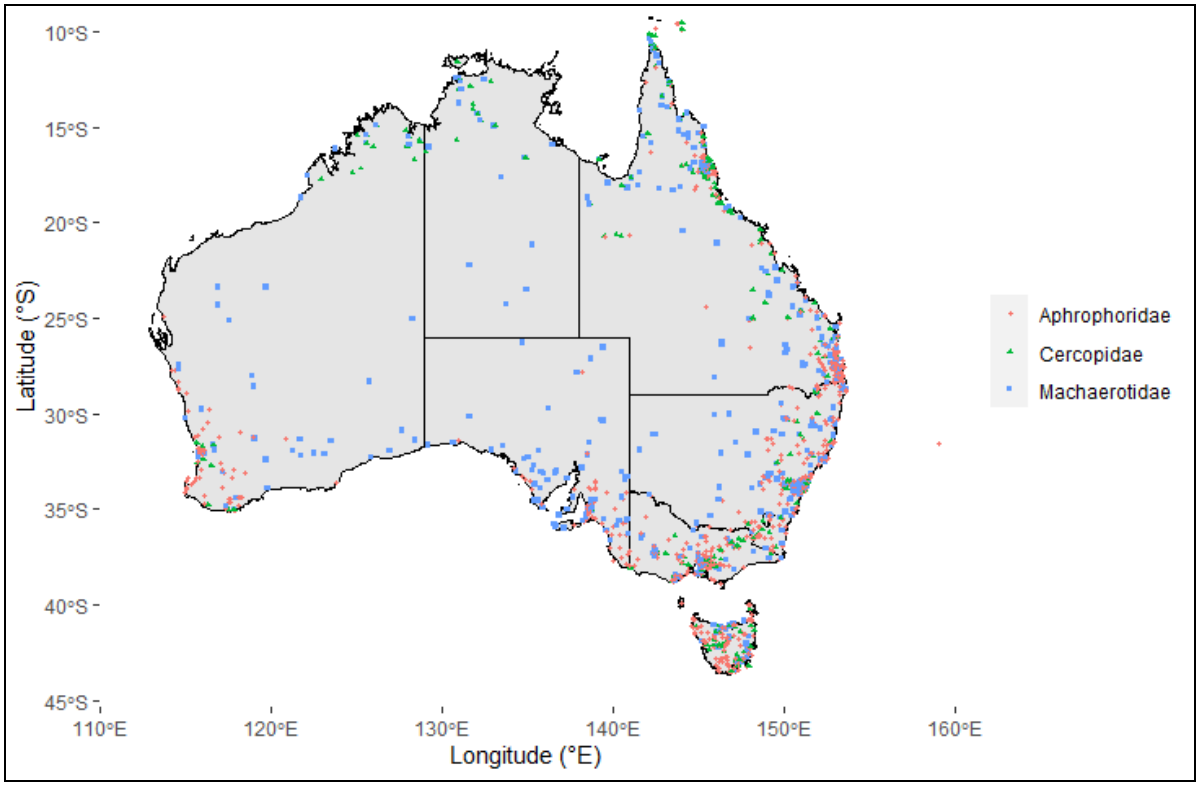


Figure 6. Collection locations of Aphrophoridae (red crosses), Cercopidae (green triangles) and Machaerotidae (blue squares) recorded across mainland Australia and the surrounding islands.

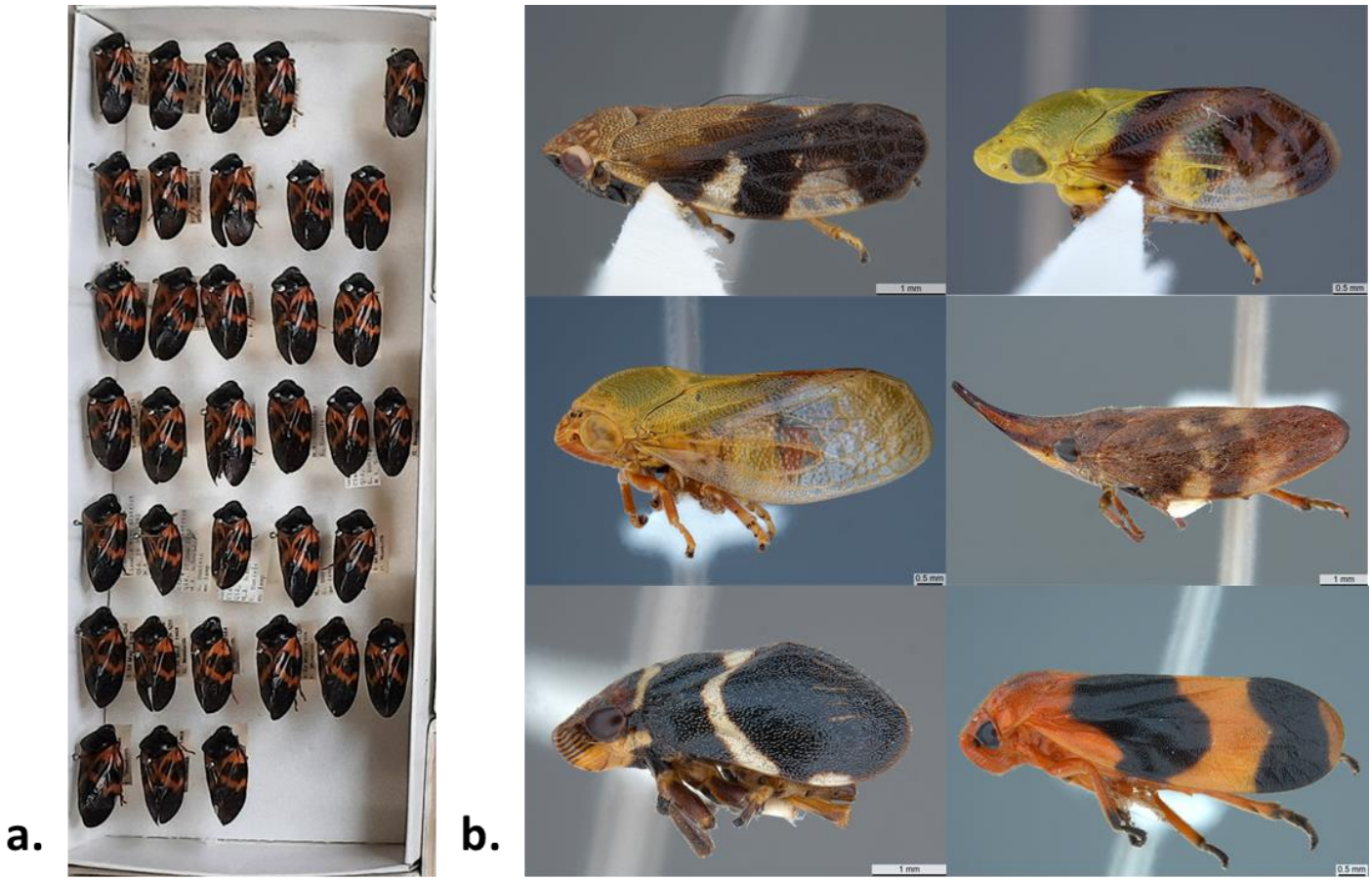


Figure 7. Examples of Cercopoidea from reference collections. **a.** A unit tray of *Megastethodon* specimens housed in the QLD Mus collection. **b.** The top six most abundant genera recorded from the specimen reference collections visited (genus images from left to right, and top to bottom: *Anyllis*, *Chaetophyes*, *Pectinariophyes*, *Philagra*, *Bathyllus*, *Euryaulax*).

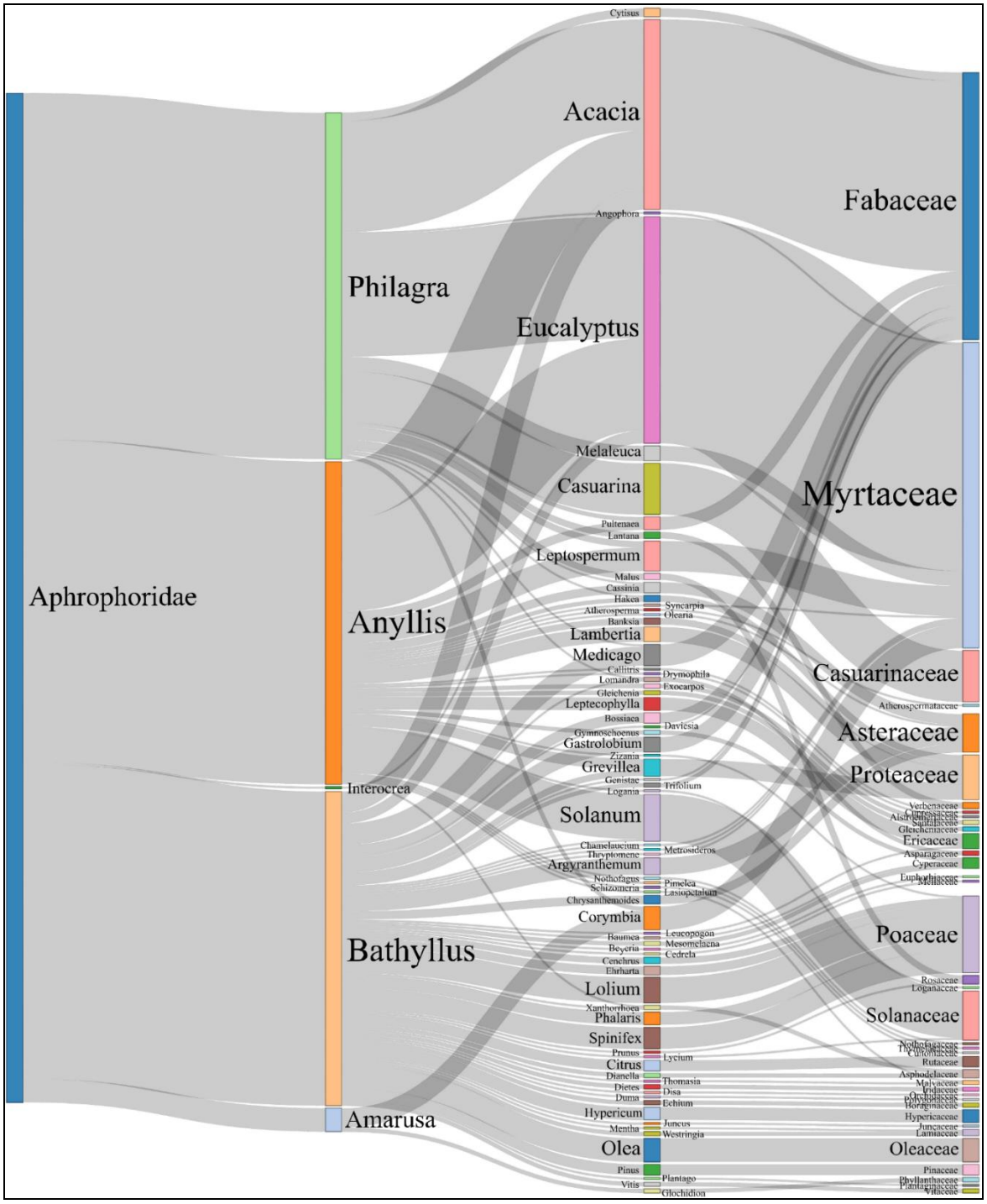


Figure 8. Plant genera associated with genera of the Aphrophoridae from 472 insect records. The thickness of grey lines is relative to the proportion of records flowing from (left to right): insect family to insect genus, insect genus to plant genus, and plant genus to plant family.

(3) Biodiversity and COI barcoding of the Australian spittlebugs (Hemiptera: Cercopoidea)

(from Technical Report, Attachment 4)

The main aim of this surveillance activity was:

1) To explore the biodiversity and assess genetic diversity within the Australian Cercopoidea superfamily, by extracting DNA and sequencing a fragment of the COI gene (i.e., DNA barcoding) from specimens freshly caught from the field through an array of collection methods, to determine how many species were collected.

Spittlebugs and froghoppers (Hemiptera: Auchenorrhyncha: Cercopoidea) are xylem-feeding insects, some of which can cause economic damage worldwide through vectoring plant pathogenic bacteria such as *Xylella fastidiosa*. Australia does not have *Xylella fastidiosa* nor its primary vector, the spittlebug *Philaenus spumarius*, but it is home to many described and undescribed species of spittlebugs.

Australian cercopoid diversity has been relatively scarcely studied, with the main literature assessment (mostly morphology-based), dating back more than a decade ago. In fact, only a few Australian cercopoid species had been previously characterised using DNA. Such a paucity of DNA records across the main publicly available repositories (e.g., BOLD, NCBI), strongly impacts the application of molecular diagnostic methods and a prompt and accurate DNA-based identification of both native and exotic species, which may not be able to be differentiated from the native ones due to a lack of DNA sequences.

Better understanding of genetic diversity across the superfamily in Australia will allow faster and more precise molecular diagnostic techniques, including DNA barcoding and DNA metabarcoding, to be applied. Furthermore, DNA sequences of the COI gene from multiple individuals per species may also allow exploration of species delimitation across taxa, revealing cryptic taxonomic diversity that may hide undescribed species, among the more than 40 currently described species.

Here we generated 135 new COI sequences for 22 Australian cercopoid taxa, across three families and 10 genera (Figure 9). We also assessed genetic diversity within some of the most widespread Australian species, including *Bathyllus albicinctus* which was shown to be a single species (Figures 9 & 10). This new dataset forms the first comprehensive work on the genetic diversity of the Australian Cercopoidea, and together with the high-resolution morphological images of each taxon that we have provided, will be invaluable tool for improving diagnostic identification of the Australian spittlebugs.

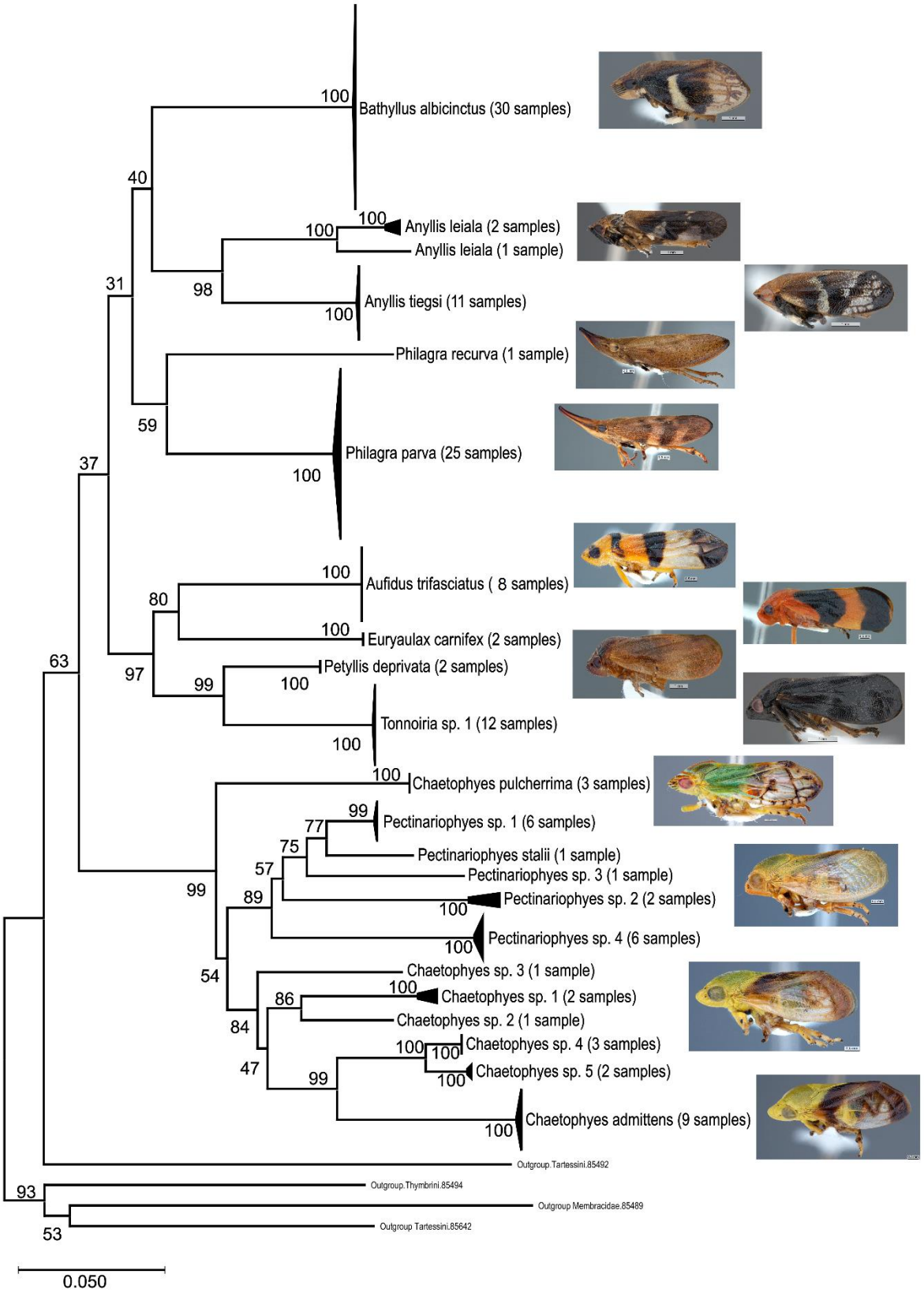


Figure 9: Neighbour-Joining COI tree generated using a total of 135 sequences obtained in this work. The tree separates 20 taxa at 3% minimum divergence and 22 taxa at 1.5% minimum divergence.

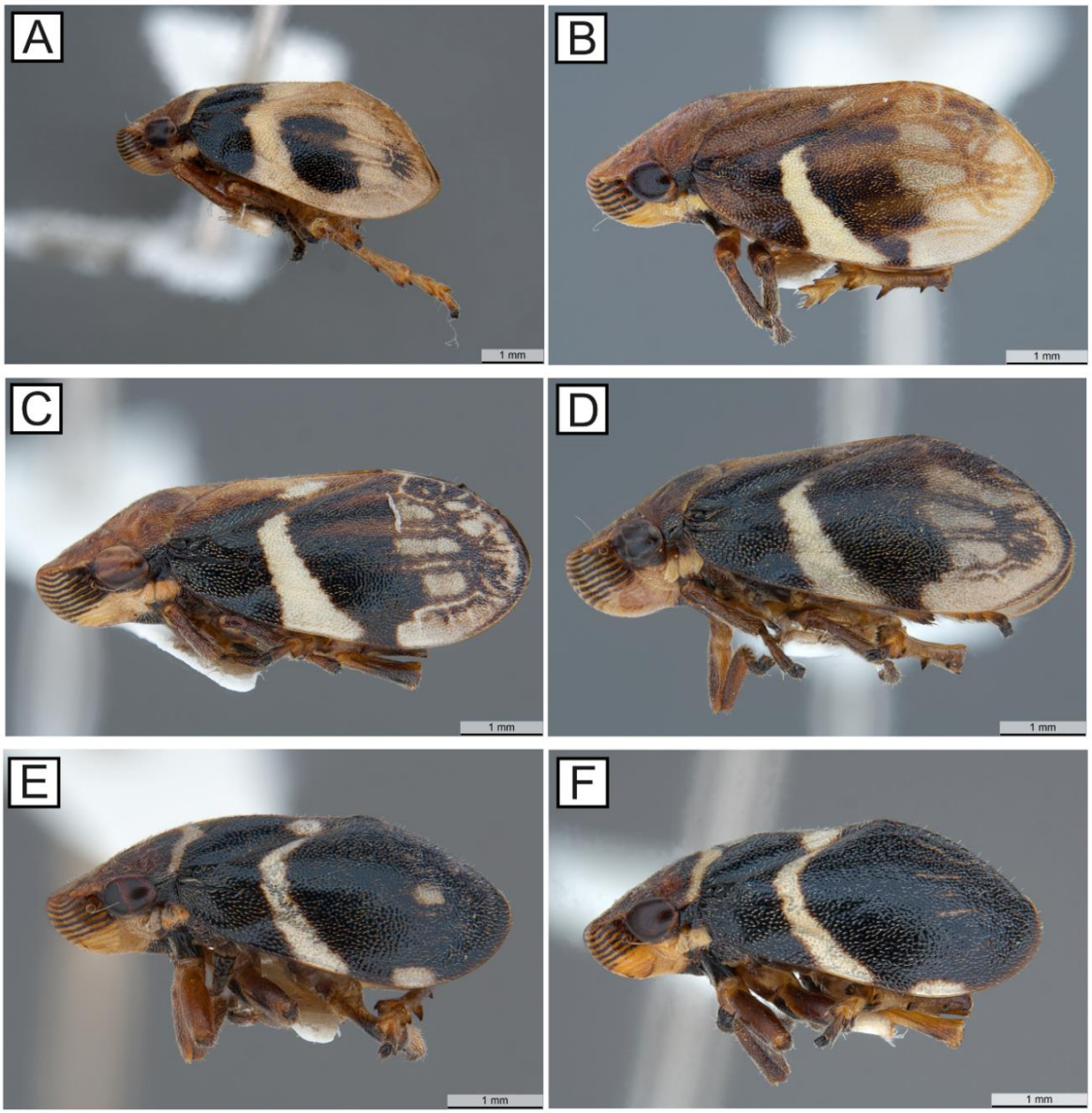


Figure 10: Habitus lateral photos showing examples of the morphological variation across the 30 specimens of *Bathyllus albicinctus* analysed for this study, grouped in six morphotypes (A-F). Note, A, E and F are males; B, C and D are females.

(4) Diversity and composition of the bacterial communities (microbiome) in two common spittlebug species, *Bathyllus albicinctus* and *Philagra parva*

(from Technical Report, Attachment 5)

The main aims of this feeding behaviour & physiology research were:

1) To explore bacterial diversity (microbiome) of two widespread Australian spittlebug species and compare their obligate symbionts with those of other spittlebugs recorded worldwide and with those spittlebug species known to vector *Xylella*.

2) To assess whether factors such as geographic location and host plant associations contributed to shaping the bacterial communities associated with each insect population.

Spittlebugs and froghoppers (Hemiptera: Auchenorrhyncha: Cercopoidea) are xylem-feeding insects, with some known to cause serious economic damage worldwide through vectoring plant pathogenic bacteria, such as *Xylella fastidiosa*. Australia does not have *Xylella fastidiosa,* nor its primary vector, the exotic spittlebug *Philaenus spumarius*, but it is home to many described and undescribed spittlebug species. Amongst the more than 40 described species of spittlebugs present in Australia, *Bathyllus albicinctus* and *Philagra parva* (belonging to the family Aphrophoridae) are two of the most widely distributed, being present in almost all Australian states and territories.

Should *B. albicinctus* or *P. parva* be able to vector *X. fastidiosa*, they would be widespread biosecurity risks in case of an incursion of the pathogenic bacterium into Australia. Unfortunately, not much is known about these spittlebugs. Identification of *B. albicinctus* and *P. parva* is challenging with adults displaying high morphological diversity, which could possibly indicate cryptic taxonomic diversity, while nymphs appear morphologically similar other Aphrophoridae species and are found on a wide variety of host plants.

Here, we collected and analysed more than eighty Aphrophoridae spittlebug nymphs, from twelve host plants, across five locations in the state of Victoria (Australia) to assess diversity and composition of the bacterial communities (microbiome) harboured by *B. albicinctus* and *P. parva*. The aim of this study was to assess which factors contribute to shaping the bacterial communities harboured by these widespread spittlebug species, including geographic location, insect phylogenetics and host plant associations. We analysed the insect genetic variation using COI barcoding and assessed the bacterial diversity using 16S high throughput sequencing (HTS) metabarcoding, comparing the bacterial communities across a variety of host plants.

This study revealed very low insect genetic divergence within the insects, but a significant divergence in the diversity of the bacterial communities harboured by both *B. albicinctus* and *P. parva* nymphs feeding on different plants, even when these plants were within a few meters of each other (Figures 11 & 12). Such diverse bacterial communities may play an important role in facilitating vectoring and transmission of plant pathogenic bacteria, while the insect-plant relationships highlight the risk of transmission to many plant hosts. These results highlight the importance of understanding insect-plant-bacteria interactions, especially in a biosecurity context.

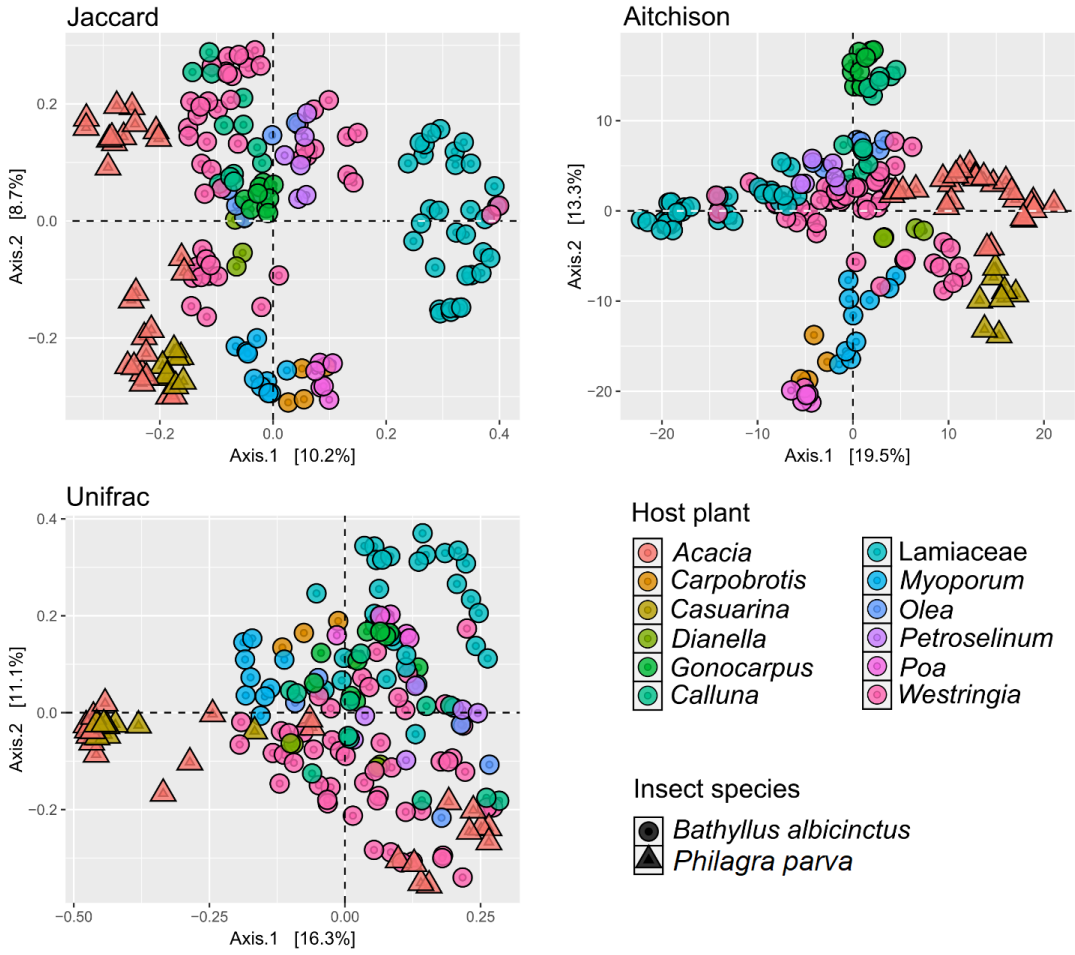


Figure 11: PCoA of the bacterial β-diversity recorded from all samples analysed for this work. The plot shows differences in the β-diversity harboured by insects belonging to different species (*Bathyllus albicinctus* and *Philagra parva*) as well as by populations of the same species feeding on different host plants.

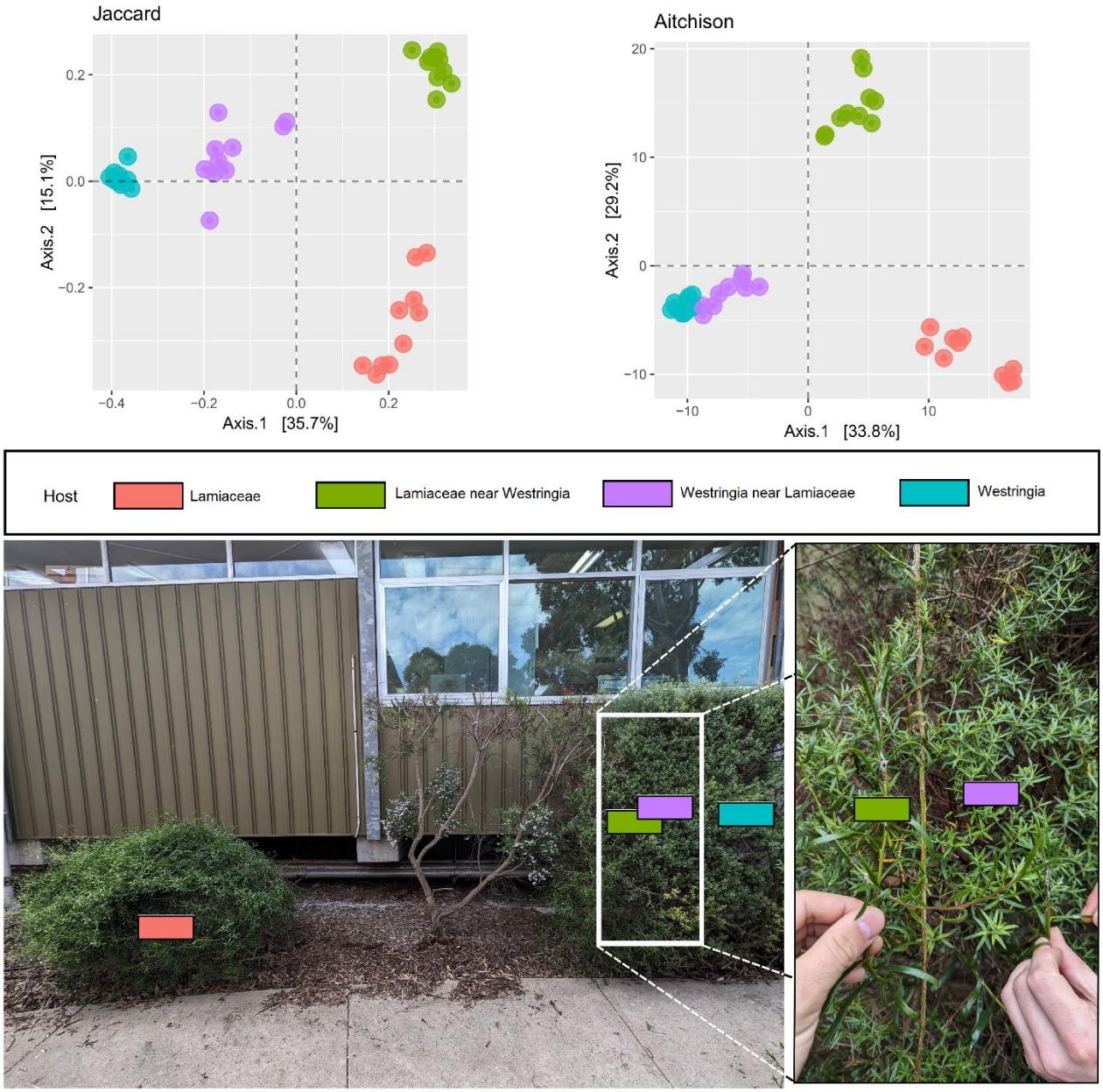


Figure 12: Principal Component Analysis (PCoA) of the bacterial β-diversity recorded from *Bathyllus albicinctus* specimens collected in Horsham on two plants. The PCoA plot shows differences in the β-diversity harboured by insects feeding on different plants at the same location.

(5) Diagnostic testing of potential insect vectors for the presence of *Xylella* species

(from Technical Report, Attachment 6)

The main aims of this feeding behaviour & physiology research were to utilise available molecular assays designed to detect *Xylella* species to:

1) Validate the updated *Xylella* National Diagnostic Protocol (NDP) molecular assays on two widespread potential *Xylella* vectors, *Bathyllus albicinctus* and *Philagra parva* (nymph and adult specimens).
2) Rule out the presence of exotic *Xylella* species in the insects collected in Victoria during field surveillance in this project.

In Australia, the presence of diverse native spittlebugs raises concerns about their role as potential vectors for *Xylella* species, similar to their counterparts overseas. While Australia currently remains free of *Xylella fastidiosa*, the wide geographic ranges and polyphagous feeding habits of some spittlebugs, such as those in the genus *Bathyllus* and *Philagra*, could potentially facilitate the spread of this bacterium if it were introduced to Australia. Research and monitoring are essential to understand the possible vector capacity of Australian spittlebugs. Identifying and studying these native species to develop proactive measures to prevent and manage possible *Xylella* incursions, will help ensure protection of Australia's agricultural and native flora health.

In this study spittlebug adults and nymphs were collected from Victoria and tested for the presence of *Xylella* species using molecular assays from the recently updated *Xylella* National Diagnostic Protocol (NDP). This is the first instance of the NDP assays being applied to insect extracts, with no cross-reactivity observed, validating the use of these assays on insects. This work enhances *Xylella* preparedness through validation of reliable molecular tests which can be applied to potential *Xylella* insect vectors in Australia.

The key findings of this study were:

1. Application of the standard *Xylella* NDP assays designed to detect exotic *Xylella* species in a qPCR, as well as using endpoint PCR assays, as described in the recently updated *Xylella* (draft) NDP (Figure 13).
2. Applying these key diagnostic assays on a range of spittlebug insect DNA samples, including multiple species and life stages, has validated and refined the updated NDP for use on insects.
3. Importantly, this testing confirmed the specificity of the *Xylella* diagnostic assays, as no cross-reactivity was observed.

4. *Bathyllus albicinctus* and *Philagra parva* spittlebug adults (n=20) and nymphs (n=87) collected from multiple sites and host plants in Victoria were tested and all found to be negative for exotic *Xylella* species (Figure 13).

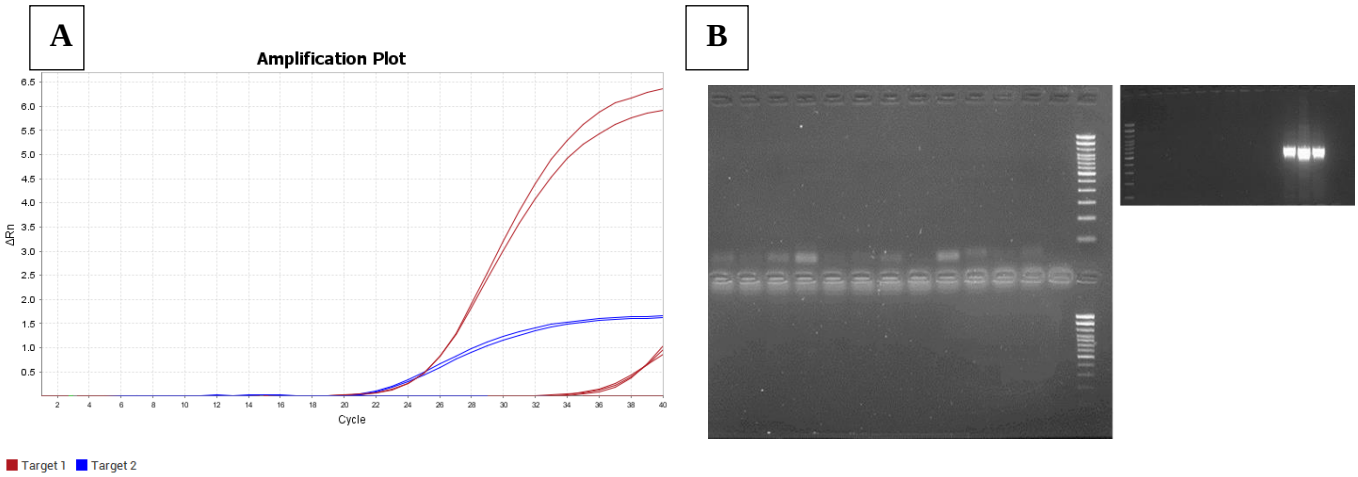


Figure 13. Adult samples (*B. albicinctus* from Victoria) testing for *Xylella* species. (A) Results of the duplex qPCR for *Xylella fastidiosa* (Red) and *Xylella taiwanensis* (Blue) were negative. The early red and blue amplification plots were positive controls for *X*. *fastidiosa* and *X*. *taiwanensis*, respectively. The late amplification after 34 cycles was threshold and flat lines were considered as negatives. (B) The gel picture of generic endpoint PCR) were reconfirmed negative. The three bright bands of 650 bp (right) are positive controls from *X. fastidiosa*, *X. taiwanensis* and ICMP 15179 *X. fastidiosa* spiked in insect DNA.

(6) Feeding behaviour of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa*

(from Technical Report, Attachment 7)

The main aim of this feeding behaviour & physiology research was:

1) To assess the feeding behaviour of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa.*

The movement of plant material across national borders enables the rapid spread of pests and diseases worldwide. *Xylella fastidiosa* (*Xylella*) is one of the most harmful plant pathogenic bacteria worldwide and is at the top of Australia's exotic National Priority Plant Pests (NPPP) list. *Xylella* is transmitted by xylem-feeding insects, and accordingly any xylem feeder could act as a potential vector. Fortunately, *Xylella*, as well as known exotic insect vectors are not currently present in Australia.

The present study was conducted to investigate feeding physiology of a common native Australian spittlebug, *Bathyllus albicinctus*, to assess its ability to serve as a potential *Xylella* vector. We monitored the feeding behaviour of *B. albicinctus* on grapevines, olives and oranges using the electrical penetration graph (EPG) technique (Figure 14). Performing 4-hour continuous recordings on the three host plants, we identified all stages of feeding previously described for spittlebugs. Our results showed that *B. albicinctus* continuously feed on these three plant species and the feeding patterns obtained match those previously described for exotic xylem feeding spittlebugs known to vector *Xylella*. In addition, Xe (xylem egestion) patterns were observed in insects feeding on all of these susceptible plants (Figure 15), suggesting that *B. albicinctus* should be considered a likely vector of *Xylella* if this devastating bacterial disease was to be introduced in Australia.

An additional project activity involved conducting an EPG training course, at AgriBio in December 2022, associated with an international Hemipteran-Plant Interactions symposium. This course included lectures on EPG background and application, as well as hands-on practical training using Faraday cages, wiring and mounting insects and plants, monitored signal recording (data acquisition), signal analysis, data processing, and statistical analyses. Additional feeding behaviour studies were conducted during this course, using nymphs collected from *Acacia decurrens* and EPG tested on capsicum, confirming the *B. albicinctus* feeding behaviour patterns we had previously observed.



Figure 14. Electrical Penetration Graph (EPG) setup with the electrode terminal coupled to a live *Bathyllus albicinctus* adult.

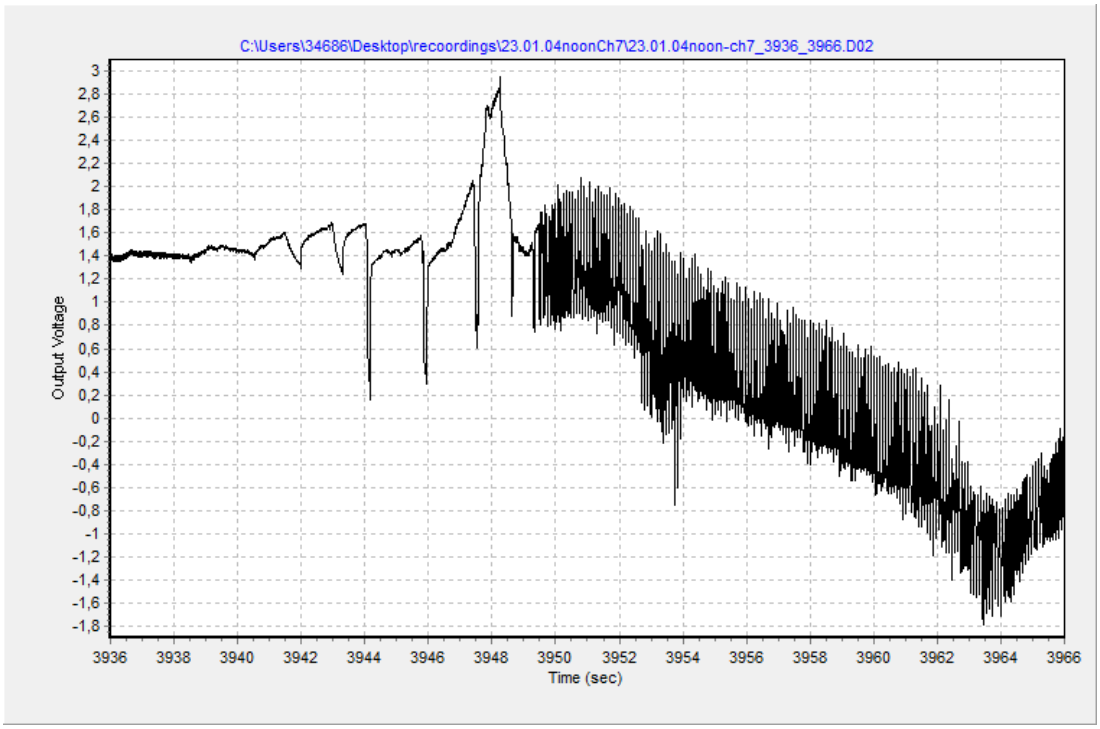


Figure 15. Xylem egestion (Xe) observed in *B. albicinctus* feeding on grapevine.

(7) Host plant preferences and survival of the native Australian spittlebug, *Bathyllus albicinctus*

(from Technical Report, Attachment 8)

The three main aims of the host plant preferences and potential vectors survival research were:

1) To assess longevity of *B. albicinctus* nymphs maintained on *Westringia*, a known host plant.
2) To compare survival of *B. albicinctus* adults on a range of common potential host plants.
3) To compare maturation and longevity of *B. albicinctus* nymphs on specific host plants known to be susceptible to *Xylella*.

The xylem feeder *Bathyllus albicinctus* (Aphrophoridae: Cercopoidea: Hemiptera) has been identified as one of the most abundant and widely distributed Cercopoidea insects in Australia and might be a likely vector of the exotic *Xylella fastidiosa* if this were to arrive in Australia. Previous work (this project) has indicated that *B. albicinctus* populations seasonally reach high levels, and are associated with a wide range of plants. However, it is unknown if all of these associated plants are equally viable hosts. Due to the variety of plant records and distribution across the southern half of Australia, it has been predicted that *B. albicinctus* is likely to be able to successfully feed and survive on a broad range of plants, from diverse taxonomic groups.

In the present study, we investigated the feeding physiology and longevity of *B. albicinctus* by monitoring nymphal (immature) and adult insect development in three independent experiments:

1. The first experiment involved monitoring the maturation of size-segregated *B. albicinctus* nymphs on *Westringia* (coastal rosemary; Lamiaceae), a previously documented viable host, under laboratory conditions. The main goal was to document the development from immature nymphs to fully developed adults and determine the persistence of *B. albicinctus* adults on this host. It was expected that larger nymphs would reach maturity and likely perish first, being further along in their development. However, size appeared strongly related to sex, with large female insects found to generally survive longer than smaller males in the same cohort. Interestingly, some adult *B. albicinctus* females were shown to survive on *Westringia* for up to three months after maturation (Figure 16).

2. The second experiment included adult *B. albicinctus* of mixed ages contained in cages with four single species of plant, or with a mixture of these plants, in a screenhouse (Figure 17). The goal was to determine if *B. albicinctus* had a preference in host plant, and if adult survival differed between host plants. *B. albicinctus* males survived in cages with capsicum, basil, and *Westringia* (Figure 18). Contrastingly, female insects only survived the study period on *Westringia*. Notably, neither sex survived the study period when only supplied with barley (Figure 18).

3. The third experiment assessed development and longevity of *B. albicinctus* nymphs on three nursery-grown (olive, grapevine, orange) and one field-transplanted (thistle) plant species susceptible to *X. fastidiosa*. This was done by monitoring survival and maturation of *B. albicinctus* nymphs and comparing these between host plant groups. The host suitability and seasonal timing of insect development differed between plant species, with olives appearing suitable for allowing *B. albicinctus* to survive (Figure 19).

These three studies have greatly increased our understanding of *B. albicinctus* plant host preferences, insect development, and longevity (Figures 16 to 20) which provides vital background knowledge relevant to monitor for and prevent establishment and potential spread of *Xylella* if it were to arrive in Australia.

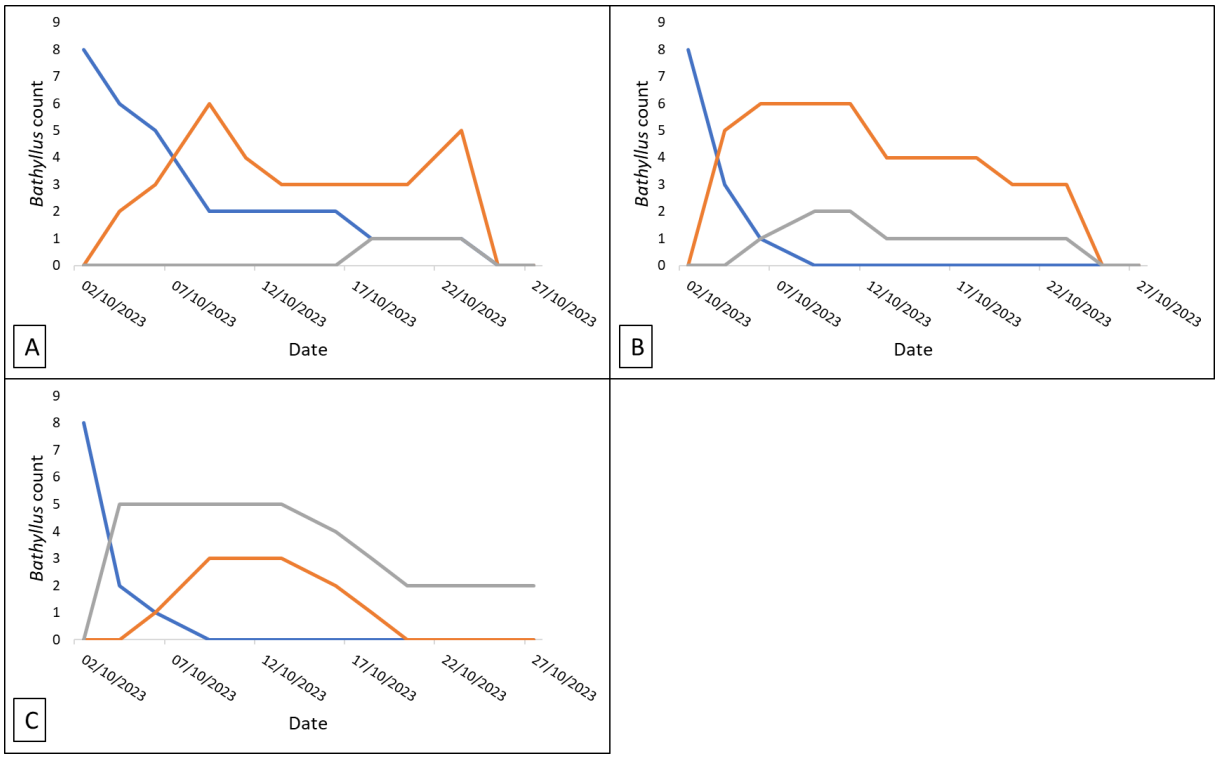


Figure 16. Line graph visualising the changing number of *Bathyllus albicinctus* nymphs, adult males, and adult females in Experiment 1. Changes over initial three weeks (Oct 2023) are represented for the (A) Small Cohort, (B) Medium Cohort, and (C) Large Cohort. Lines are colour coded by insect life stages; nymph (blue line), adult male (orange line), and adult female (grey line). Note: the two adult females in the large cohort persisted for three months until mid-January 2024, which is not plotted.

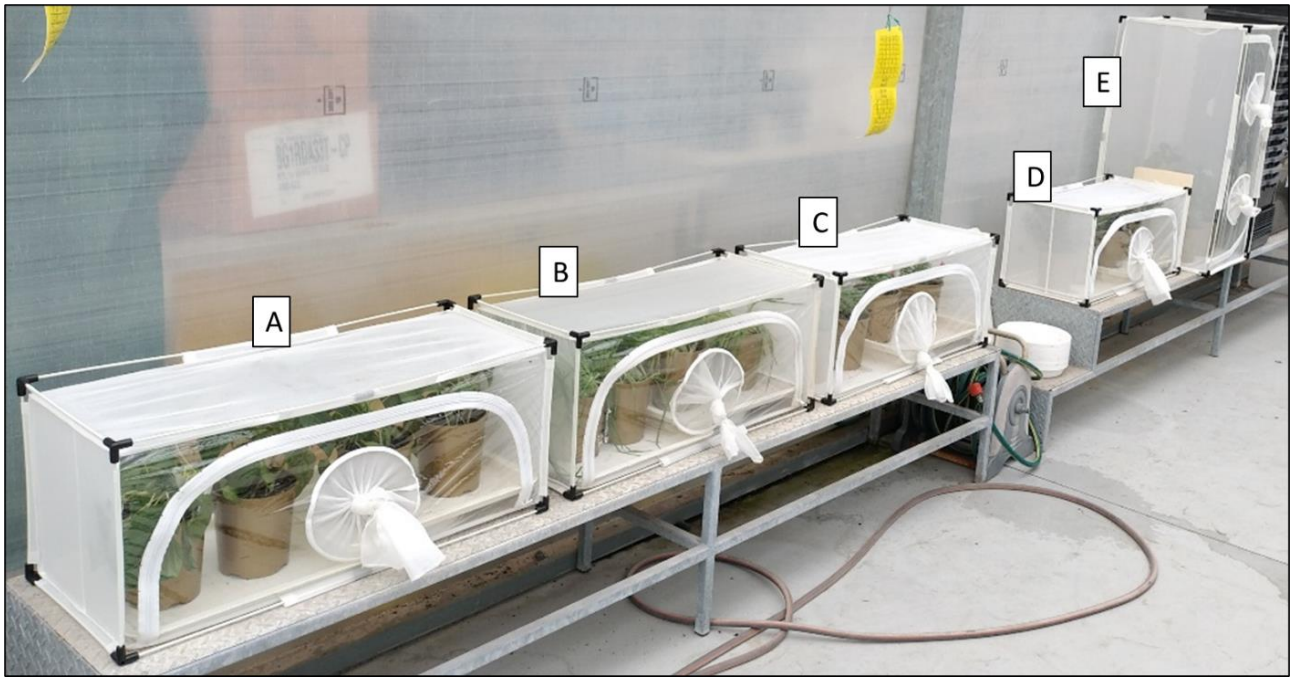


Figure 17. Plants with *Bathyllus albicinctus* in BugDorm cages within screenhouse; capsicum (A), barley (B), basil (C), coastal rosemary (D), mix (E).

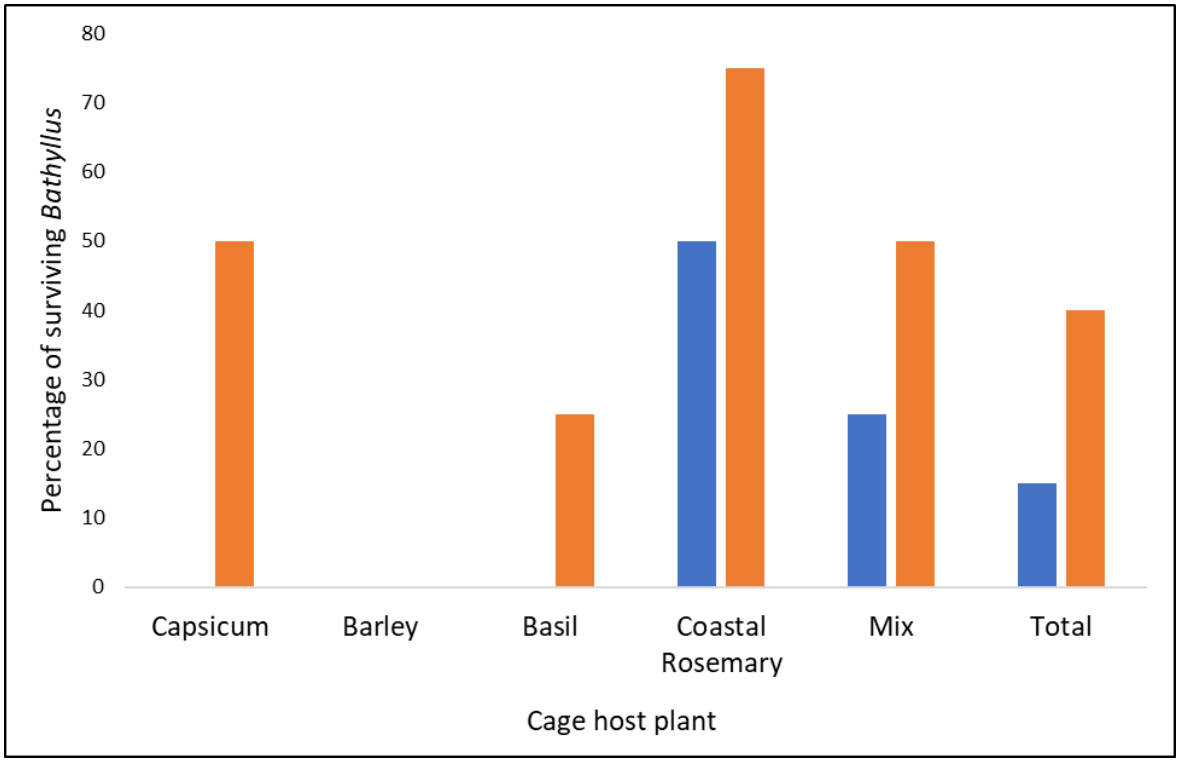


Figure 18. Proportion of *Bathyllus albicinctus* that survived 50 days, across host plant species, and adult male (orange) and female (blue) insects.

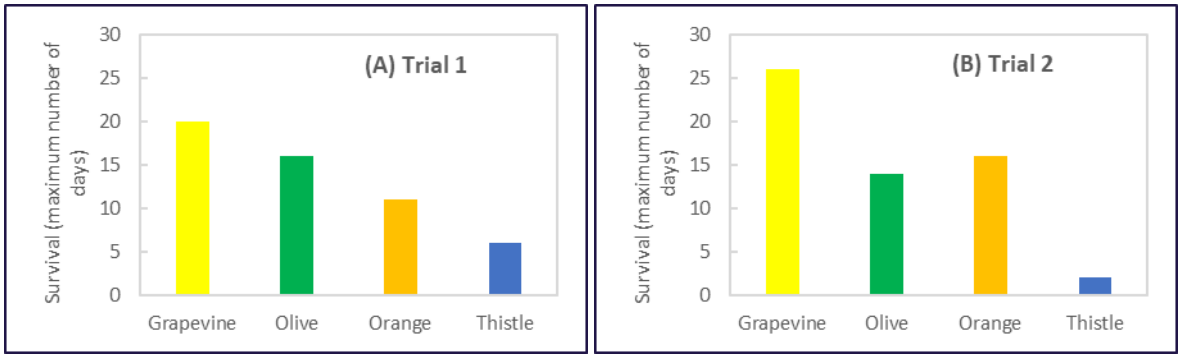


Figure 19. The maximum number of days that *Bathyllus albicinctus* survived on each host type in (A) Trial 1 and (B) Trial 2.

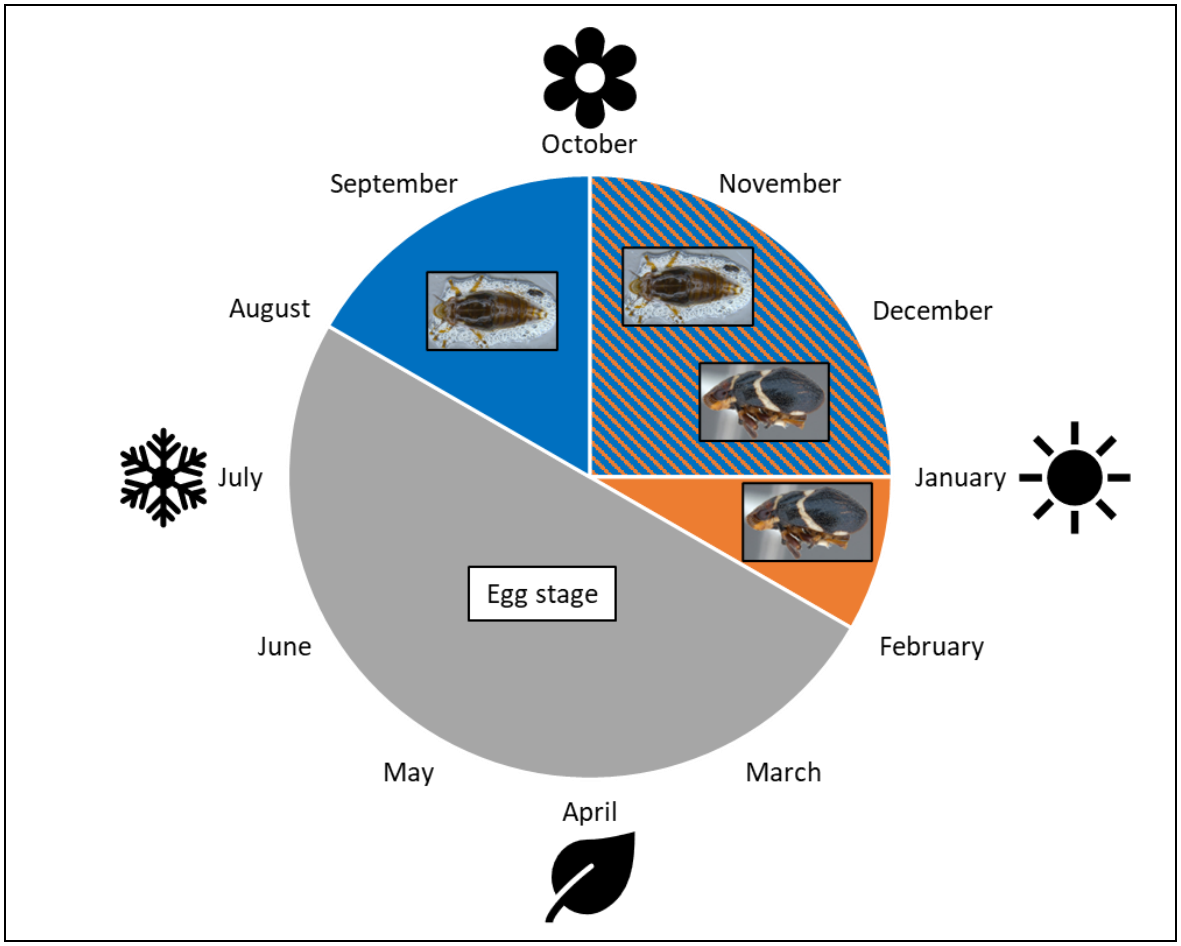


Figure 20. Pie-chart visualising the estimated seasonal phenology of *Bathyllus albicinctus* life-stages across one calendar year in southeastern Australia. Colours indicate the calendar months when mature *B. albicinctus* adults (orange), immature stages (blue), or both (orange and blue stripes), are likely to be encountered, and when insects can be inferred to be in an egg stage (green). Although eggs were not directly observed, it may be inferred that immature *B. albicinctus* are in an egg stage during the months when neither nymph nor adults are present.

Outcome / Conclusions

The knowledge gained through this project on the occurrence of potential *Xylella* vectors in Australia will support biosecurity and protect Australian plant industries by enabling them to effectively respond to an incursion of *X. fastidiosa* or manage the spread and severity if this serious pathogen is established. This project met all identified project activity outcomes (Table 1 & 2). The key outcomes of this project are outlined in the Technical Reports (Attachments) and are summarised below:

Surveillance methods

Patterns of insect abundance:

- Both *Bathyllus albicinctus* and Cicadellidae abundance were highest from late spring to early summer, and hence when sampling efforts are most likely to succeed.
- Cercopoid genera varied greatly in distribution throughout Australia and in seasonality. For each state, the season of first collection and peak collection month is relatively similar between genera. Each cercopoid family was recorded in all eight main Australian states and territories, with the exception of Aphrophoridae in the Northern Territory. The five most represented genera were each collected in at least six states/territories. Cercopids, *Petyllis* and *Tonnoiria,* are notable in that although the number of records is relatively low, these genera were collected in seven and six of the Australian states/territories, respectively.
- In three years of sampling in the Riverina region (New South Wales), only one cercopoid species of potential vector, *Pectinariophyes stalii,* was found in very low numbers in the crops, and none in areas of native vegetation surrounding the crops. This finding indicates that should *Xylella* arrive in Australia there is probably a low risk of spread to and within the Riverina area due to a lack of species from groups generally regarded as xylem-feeders.
- The majority of cercopoid genera in insect reference collections each accounted for less than 3% of the total number of Cercopoidea. However, in contrast, the five most abundant genera each represented more than 10% of the cercopoid records. These common Cercopoidea genera were: *Anyllis*, *Pectinariophyes*, *Bathyllus*, *Chaetophyes* and *Philagra*. *Chaetophyes* and *Pectinariophyes*, both in family Machaerotidae, were also the most widespread genera, being found in all eight of the main Australian states and territories. As the five most widespread and most often caught Cercopoidea, found on the ubiquitous *Eucalyptus*, *Acacia*, and even *Solanum* plants, these genera should be considered for future *Xylella* vector studies, noting that it is currently unknown if particular species of these genera are also widespread across Australia, or if they have more limited distributions.

Effective collection techniques:

- For Cicadellidae, sticky traps were the most successful sampling technique, although a substantial number of these were mouldy, which makes it difficult to accurately identify insects to species.
- The effectiveness of common collection methods varied between cercopoid taxa and should be considered when targeting surveillance efforts toward specific genera. Aphrophoridae were most often recorded with active search methods such as beating, sweeping, sieving, vacuuming, and hand collecting. However, Aphrophoridae were also collected with light trapping at a high rate, and light trapping was by-far the most commonly recorded collection method for both Cercopidae and Machaerotidae. This information should prove especially useful for biosecurity officers when planning field collecting for potential *Xylella* vectors.
- The common and widespread species *B. albicinctus* was found to be present at both Victorian commercial farm sites, but in lower abundance than (non-Xylem feeding) Cicadellidae. Statistical analysis indicated that the effectiveness of sampling techniques was not the same between these taxa. For *B. albicinctus*, sweep netting was the most successful sampling technique. Future sampling of *B. albicinctus* should consider using sweep nets and avoid sticky traps or pan traps.
- Additional field collections and molecular characterisation should be conducted to assess the Cercopoid diversity across Australia. These should focus on the use of light trapping and sweep netting, which proved more effective than trap-based collections. The aim of this research should be to collect additional species and make the Cercopoid database more comprehensive, increasing the accuracy of its use for diagnostic identification of potential vectors.

Diversity of potential vectors:

- Successful collection of additional species / population would enable further DNA-based assessment to better understand population-level and species-level genetic diversity across Australian states. COI-based genetic assessment not only provides additional insight into the biodiversity of the Australian Cercopoidea, but can determine the presence of new / undescribed native species. Characterisation of such “hidden” diversity is of paramount importance in case of the detection of a suspected exotic, since an unknown native species may be misidentified as exotic and lead to an expensive biosecurity response.
- Further molecular work including multi-gene phylogenetic assessments and microbiome analysis can provide a powerful insight in the similarities between the Australian spittlebugs and their exotic counterpart. The generation of a large dataset of Australian Cercopoid species freshly collected can allow additional analyses such as deeper phylogenetic work (using multiple markers in addition to COI) and microbiome analysis that would not be

possible using older specimens preserved in collections. Assessing the microbiome diversity of multiple Australian native species allows to compare these with that of exotic species capable of vectoring *Xylella,* and could highlight which native species could be more at risk of transmission of the same bacteria.

Feeding behaviour & physiology

Host plant feeding and associated bacteria:

- Eleven cercopoid genera were associated with more than one plant family. The aphrophorid genera were found on the widest variety of plants, with *Bathyllus* found on 44 plant genera across 27 plant families. However, *Interocrea*, also in Aphrophoridae, was found on only one plant genus, *Eucalyptus*. The machaerotid genera were recorded on the fewest plant families (nine plant families). This suggests that Aphrophoridae have the highest potential to transmit *Xylella fastidiosa* to a wide range of plant families, including agriculturally relevant plants (grasses, olives, pine trees, citrus, grapes etc.).
- *Bathyllus albicinctus* was experimentally demonstrated to be able to feed on three plant types – grapevine, olive, and orange – rapidly accessing the xylem within 25 minutes. Xylem was ingested for hours at a time and insects exhibited xylem egestion (Xe) feeding behaviour. Considering that this has been correlated with transmission of *Xylella fastidiosa* in exotic spittlebugs, these behavioural findings together with other aspects of the biology of *B. albicinctus*, including large seasonal peaks of populations which can feed on a wide range of plant species, suggest that *B. albicinctus* should be considered a likely vector of *Xylella* if this devastating bacterial disease was to be introduced in Australia.
- 16S microbiome analysis is important for greater understanding of feeding behaviour in native spittlebug species. The 16S bacterial metabarcoding analysis performed here was extremely effective in highlighting bacterial biodiversity patterns across different species and populations. The 16S fragments generated here, despite the limitations due to their relatively short length, still enabled a bacterial biodiversity assessment capable of providing a valuable insight into the environmental factors shaping bacterial communities.
- Comparison of the main primary symbionts across multiple native species and similarities with exotic pests. The analysis conducted here showed that both native spittlebug species examined harboured the “traditional” primary symbionts of spittlebugs (*Candidatus* Sulcia and *Ca.* Zinderia), as opposed to the *Sodalis*-like species harboured by *Philaenus.* It would be valuable for future studies to aim to determine if this is the case for other Australian native species or not.

Bacterial diagnostics:

- The application of National Diagnostic Protocol (NDP) assays for insect testing significantly enhances our surveillance capabilities for exotic *Xylella* pathogens. By applying these key assays to insect samples, including multiple spittlebug species and lifestages, we validated and refined the NDP for use on insects. The testing confirmed the specificity of the assays, with no cross-reactivity observed. This successful validation ensures that these assays can be reliably used to detect *Xylella* in insect vectors.

Host plant preferences & survival of potential vectors

- Experiment 1: We have identified variation in the maturation timing of *B. albicinctus* nymphs, feeding on *Westringia*, and compared the longevity of resulting adults between insect size cohorts. Nymphs matured into adults in October, similar to observations from previous field studies in Victoria. Although most insects perished within October 2023, two adult females persisted until January 2024. This demonstrates that the *B. albicinctus* life span, and therefore its opportunity to potentially transmit *X. fastidiosa,* is highly variable between nymph size, sex, and season when insects are abundant.
- Experiment 2: We identified variation in the survival of *B. albicinctus* adults between a range of host plant species. Insects were most likely to survive on coastal rosemary (*Westringia*). Insect reproduction would only be possible where *Westringia* was available, as females did not survive elsewhere. Even without the ability to reproduce, the other host plants may still be susceptible to *X. fastidiosa* if infected *B. albicinctus* insects attempted to feed on these plants. This highlights the significance *Westringia* may have as a potential reservoir source of *B. albicinctus* insects, and potentially *X. fastidiosa* if it were to arrive in Australia.
- Experiment 3: The timing of insect collection was important to insect longevity, with October-collected insects surviving up to 20 days and December-collected insects surviving up to 25 days. Feeding by *B. albicinctus* on grapevine, olive, oranges, and sow thistles indicated that these plants might be susceptible to *X. fastidiosa* infection by *B. albicinctus*. *B. albicinctus* feeding on olives were the most likely to reach adulthood, indicating that olive orchards could act as a source of *B. albicinctus*. On sow thistle, however, *B. albicinctus* did not reach adulthood or survive longer than 10 days, possibly due to the impact of transplanting on thistle health and suitability as a host. The low survival and low maturation count on Experiment 3 host plants was contrasting to Experiment 1, in which all nymphs reached adulthood on an alternative host plant, *Westringia*.

Recommendations

The project findings led to the following recommendations for future research (outlined in the attached Technical Reports) and summarised below:

Surveillance methods

Patterns of insect abundance:

- The results from surveying Cercopoidea and Cicadellidae in commercial crops revealed variation in the abundance of both insect communities. This variation was found to be associated with both geographic location and host plants. Future research could support control efforts by investigating the environmental conditions and factors that lead to differences in insect abundance between the field sites, and thereby indicate when or where high populations of Cercopoidea and Cicadellidae can be expected.
- Further work is necessary to identify and delimit distributional boundaries of potential xylem-feeding leafhoppers and spittlebugs in NSW. While the Riverina area seems relatively safe from the spread of *X. fastidiosa* , there is limited knowledge of potential vectors in the horticultural areas closer to coastal regions in NSW. Knowledge of potential native vectors in these areas is required to develop a suitable biosecurity plan to limit the spread of *X. fastidiosa* should it arrive in Australia.
- Commercial crops in Victoria would be at risk of *X. fastidiosa* infection from Cercopoidea, namely due to the presence of *Bathyllus albicinctus* and its potential to possibly vector *X. fastidiosa*. While Cicadellidae were also collected in large numbers, they were all assumed to be phloem-feeders, due to an absence of xylem-feeding Cicadellidae species in these commercial fruit orchards. Large fluctuations in *B. albicinctus* abundance between seasons, however, suggests that the risk of *X. fastidiosa* transmission from *B. albicinctus* would also fluctuate with season. The absence of xylem-feeders other than *B. albicinctus* from Victorian crops also elevates its importance as a potential vector, should *X. fastidiosa* arrive in Australia. As such, southeastern Australian crops may be best protected if control efforts are targeted towards *B. albicinctus*.
- As *B. albicinctus* the only potential vector detected in field crop surveillance in Victoria, a local biological control agent targeting *B. albicinctus* during its few months of high abundance could reduce the chance of *X. fastidiosa* transmission. Therefore, identifying and evaluating a biological control targeting *B. albicinctus* could provide another management tool to stymie the establishment of *X fastidiosa* in Victorian crops, as is being investigated for *Philaenus spumarius* (Cercopoidea: Aphrophoridae), Europe's primary *X. fastidiosa* vector (Molinatto et al. 2020: McCaw et al. 2024).

- State / territory of collection was a good predictor of peak cercopoid collection period, suggesting insect abundance varies with geographic region and, by extension, climate conditions. Future work could focus on key climatic measurements that can predict areas in which Australian Cercopoidea are most likely to develop and thrive. This will greatly assist the management of *X. fastidiosa* if it arrives, notably predicting vectors, locations, and seasons suitable for transmitting *X. fastidiosa*.

Effective collection techniques:

- Sweep netting was the most effective sampling technique for *Bathyllus albicinctus*, while sticky traps caught the most Cicadellidae generally. Despite success with *B. albicinctus*, sweep netting may not be ideal for other cercopoid species. Some traps were relatively better than others, for catching Cicadellidae and Cercopoidea, and generally rely on visual inspection of trap contents. The development of genetic primers specific to Cercopoidea or Cicadellidae would allow for more rapid and sensitive surveillance of putative *X. fastidiosa* vectors, rather than relying solely on visual inspection of field-vegetation or collected traps.

Diversity of potential vectors:

- Based on the historical reference specimen collection data, some genera appear to have wide environmental tolerances while others are more restricted. When teasing the data apart further into species-level trends, it becomes clear that certain genus-level distribution trends are driven by one common species which masks the trends of other congeneric species; for example, *Philagra parva* is distributed over six states, while the other three described species have only been collected in NSW and QLD. Reassuringly, however, genus-level trends did not significantly change as more data was added; the trends present when analysing ~3,500 records (i.e., earlier analyses, not shown) were solidified, not altered, when analysing the complete ~5,600 records.
- The field collection conducted during the past year enabled us to collect 22 taxa, of which at least five are possibly indicative of new / undescribed Australian species. Future works should build on this expertise and further explore the native diversity to improve the genetic characterisation of the Australian Cercopoidea
- Both COI identification (for the insects) and 16S profiling (for the insect microbiome) should be extended to the entirety of the Australian cercopoid fauna. This will enable to compare the Australian native species to the exotic ones and highlight which native insects could be at risk of vectoring *Xylella* in case of an incursion.
- New Zealand is likely an optimal place to compare known *Xylella* vectors with potential vectors. Both the Australian *Bathyllus albicinctus*, and the European *Philaenus spumarius*, are currently present in New Zealand. As the most effective European vector of *Xylella*

fastidiosa, *P. spumarius* is a good yardstick against which to measure *B. albicinctus* as a potential vector. This represents a unique opportunity to develop collaborative relationships with our New Zealand neighbours, to explore this further.

Feeding behaviour & physiology

Host plant feeding and associated bacteria:

- While *Xylella fastidiosa* transmission by native cercopoid vector insects in case of a *Xylella* incursion seems probable considering the polyphagous nature of many Australian spittlebugs and the apparent lack of host plant specificity of *X. fastidiosa*, this should be further tested, to assess if transmission is like that of European spittlebugs which are known vectors. The most feasible option is to develop colony-maintenance protocols for Australian spittlebugs and send live insects overseas (e.g., Europe) for *Xylella*-transmission tests.
- Mechanically, feeding in at least one Australian spittlebug species appears to be very similar to feeding in known European vectors. Preliminary feeding experiments involving the Electrical Penetration Graph (EPG) technique have discovered that the egestion feeding period, in which *Xylella fastidiosa* is transmitted by European spittlebugs, lasts longer in *B. albicinctus* adults than in European spittlebugs. This experiment is described and discussed in the *B. albicinctus* feeding behaviour Technical Report (Jaroslow et al 2024). *B. albicinctus* have high vectoring potential based on life history traits (e.g., wide geographic distribution, broad host range, high abundance in urban and natural areas alike), and this insect could therefore act as a 'super-spreader' of *X. fastidiosa* due to feeding behaviour. Considering these factors combined, there is a very high likelihood that *B. albicinctus* is a comparable, if not superior, vector to *P. spumarius*. As such, investigating the population dynamics and actual vectoring potential of *B. albicinctus* would greatly improve Australia's preparedness for a *X. fastidiosa* incursion.
- Future research could involve experimental EPG studies to determine if *B. albicinctus* can transmit *Xylella fastidiosa* to susceptible host plants. Note, these studies would need to be conducted overseas, as *Xylella fastidiosa* is exotic to Australia.
- Additional work could investigate developing more reliable and feasible methods of rearing *B. albicinctus* to maintain colonies for testing *X. fastidiosa* transmission potential, as well as whether *B. albicinctus* displays preferential feeding behaviours on different host plants, including potential sub-optimal hosts.
- Broader assessment of Australian native spittlebug microbiome should focus on a large-scale assessment comparing different species from different locations and host plant associations. This would provide an important dataset to assess and characterise the bacterial diversity already present in Australia.

- Assessment of the main primary symbionts across multiple native species and similarities with exotic pests. Due to the fact *Philaenus spumarius,* the main exotic vector of *Xylella fastidiosa*, is known to have switched one of its primary symbionts, an assessment should be conducted across Australia to determine if any species native to the country underwent the same symbiont switch. The identification of any such species would suggest the insect could be more likely to vector similar bacteria, including *Xylella.*
- Collaborations with New Zealand researchers to assess the microbiome changes within *Philaenus spumarius* and *Bathyllus albicinctus* in a different country. To assess whether the microbiome composition of *Philaenus spumarius* and *Bathyllus albicinctus* in New Zealand show modifications from that of populations of the same species in their native range.

Bacterial diagnostics:

- Improved diagnostics of *Xylella* has recently been achieved through the implementation of the National Diagnostic Protocol (NDP). This protocol provides a standardized, reliable method for detecting *Xylella* species in various insect vectors. By applying these assays, we could accurately identify the presence of *Xylella* in spittlebugs, sharpshooters, and other potential carriers. The NDP's specificity ensures that even low levels of the pathogen should be detected, thereby enabling early intervention and more effective management of the disease. This advancement significantly strengthens our biosecurity measures and preparedness, reducing the risk of *Xylella* spread through insect vectors.

Host plant preferences & survival of potential vectors

- Many insects have different developmental times depending on their food source. As xylem-feeders with nutritional requirements, it is likely that cercopoid nymphs will develop more quickly on optimal plants versus sub-optimal plants. The ways in which a plant is “optimal” or “sub-optimal”, however, are not yet known for Australian Cercopoidea, and may be different for different taxa. Physical toughness of plant stems, nutritional composition of xylem fluid, daily and seasonal xylem pressure changes, and plant defences are just a small number of variables that may affect nymphal development, which could be further explored.
- The results from the laboratory and screenhouse experiments provide useful insight into the seasonal occurrence of *B. albicinctus*. The length of time adults persisted, however, varied between nymph size cohorts. To generalise these findings to field conditions, these experiments should be repeated under field conditions, to understand *B. albicinctus* development on host plants in the field, compared to laboratory or nursery-grown plants.
- As the genus with most associated plant families recorded, *Bathyllus* is a good candidate to test questions regarding viable host plants. The genus *Bathyllus* is monotypic, with *Bathyllus albicinctus* the only described species. This species has been collected on 27 different plant

families, and has been seen to feed on most of these plant families (including the economically significant gymnosperm *Pinus radiata*). However, it is unknown whether nymphs will feed and produce spittle on plants that are not viable food sources in the long-term. No-choice and choice feeding experiments could be conducted to confirm host plant use of this seemingly polyphagous insect.

- Across the three experiments conducted, *Westringia,* olives, and, to a lesser extent grapevines and oranges, were found to potentially be able to support a reproductively viable *B. albicinctus* population. The success of *B. albicinctus* did not neatly align with the taxonomic relationship of host plants (i.e., plant families) and could differ depending on the seasonality. Environmental conditions (Jarošík 2019; Jaroslow et al 2023), host health and species characteristics (Andersen et al 1992; Taylor et al 2011), or an interplay of these factors (Wong and Daniels 2017), contribute to the suitability and attractiveness of a host to insect herbivores (For review: Awmack and Leather 2002; Knolhoff and Heckel 2014), and this is likely the case for *B. albicinctus*. Further investigations into the relationship between *B. albicinctus* and environmental conditions, such as temperature, would allow for predictions of when and where this insect's activity is greatest and, by association, the risk of potentially spreading diseases such as *X. fastidiosa*.
- Insect survival was associated with screenhouse conditions, specifically the cumulative temperature experienced by *B. albicinctus*, although only *Westringia fruticosa* successfully sustained both female and male *B. albicinctus*. Due to their common use as ornamental and commercial crops in Australia, and documented susceptibility to *X. fastidiosa* elsewhere (EFSA et al 2023; Figure 21), *W. fruticosa* should be considered at high risk of *X. fastidiosa* infection potentially vectored by *B. albicinctus*, should the pathogen arrive in Australia. As such, follow-up research should focus on documenting the development and actual vector capacity of *B. albicinctus* feeding on *W. fruticosa*, or similarly attractive and common host plants, under field conditions. Our knowledge of optimal conditions for *B. albicinctus* populations could be improved by documenting the relationship between *B. albicinctus* abundance, reproductive success, and field conditions.



Figure 21. *Westringia fruticosa* infected with *Xylella fastidiosa* and exhibiting visible symptoms. Note the chlorotic and desiccated vegetation. Photo copyright Donato Boscia, CNR – Institute for Sustainable Plant Protection, UOS, Bari (Italy), European and Mediterranean Plant Protection Organisation.

Appendix 1: Communication

Discussions with project members, growers, industry representatives, biosecurity agencies and scientists (Table 8 & 9) took place throughout the project. Project outcomes were regularly communicated with project members of the Agriculture Victoria "*X. fastidiosa* diagnostic project", collaborators in Australia (DPI NSW), New Zealand and Europe.

Project outcomes were also formally communicated during the Hemipteran Plant Interactions Symposium (HPIS) in Melbourne in December 2022 to a local and international audience. At the HPIS, formal and informal meetings took place with collaborators from Australia, New Zealand and Europe.

Additionally, in June 2022, a project team member (Trebicki) attended meetings and field work in Spain and Portugal. The project team member met with growers, industry representatives, biosecurity agencies and scientists in both countries. Highly relevant concerns were discussed related to the impact of *X. fastidiosa* on growers, including social issues, biosecurity agency responses and encountered difficulties, and difficulties associated with the implementation of surveillance and eradication strategies.

Table 8. Conference and workshop presentations.

	CONFERENCE / WORKSHOP	PRESENTATION	AUTHORS	DATE
1	Trans-Tasman *Xylella* and BMSB collaborative workshop, Hort Innovation, Sydney, Australia.	Providing the tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* in Australia and New Zealand: focus on vectors ecology and biology.	Trębicki P	4 Dec 2018
2	Horticulture Industry Network meeting, Melbourne, Australia. (Invited talk).	Mitigating the potential spread of *Xylella fastidiosa* in Australia: focus on vectors ecology and biology.	Trębicki P	31 Jan 2020
3	National *Xylella* Online Forum, Australia.	Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (*Xylella*) in Australia and New Zealand by understanding its potential vectors.	Trębicki P	6 May 2021

4	National Wine Centre, Adelaide, Australia. B3 Conference 2021, New Zealand	Native insect vectors of *Xylella*, Plant Biosecurity Research Symposium 2022 *Xylella* vectors	Trębicki P Trębicki P	11-12 May 2022 17-18 May 2021
5	International Plant Virus Epidemiology Symposium, Madrid, Spain	Preparedness for *Xylella fastidiosa* in Australia; understand biology, physiology and ecology or potential vectors.	Trębicki P	5-8 June 2022
6	International Hemipteran-Plant Interactions symposium (HPIS)	Potential *Xylella* vectors in Australia: knowns and unknowns	Selleck C, Valenzuela I, Semeraro L, Trebicki P	Dec 2022
7	International Hemipteran-Plant Interactions symposium (HPIS)	Membracoidea and Cercopoidea associated with crops vulnerable to *Xylella fastidiosa* in southern New South Wales	Stevens M, Hoskins J & Trebicki P	Dec 2022
8	ICA CSIC, Madrid, Spain	Opportunities and challenges inflicted on food production by climate change, pests and diseases.	Trębicki P	9 August 2023
9	4th European Conference on *Xylella fastidiosa*, Lyon, France.	Understanding potential vectors of *Xylella fastidiosa* in Australia.	Trębick P, Blacket M, Stevens M, Selleck C, Lago C, Hoskins J, Rodoni B	20 Aug 2023
10	Entomological Society 54th Annual General Meeting and Scientific Conference, Albany, Australia.	The native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa.* Australian	Lago C, Fereres A, Moreno A, Blacket M, Trębicki P	13-15 Nov 2023
11	Entomological Society 54th Annual General Meeting and Scientific Conference, Albany, Australia.	Investigating cercopoid (froghopper and spittlebug) diversity in eastern Australian orchards and vineyards	Selleck C, Trebicki P, Hoskins J, Stevens M, Blacket M	13-15 Nov 2023
12	Entomological Society 54th Annual General Meeting and Scientific Conference, Albany, Australia.	Diversity and abundance of potential *Xylella fastidiosa* vectors and related taxa in *Xylella*-susceptible crops and native vegetation in southern New South Wales, Australia	Hoskins J, Trebicki P, Stevens M	13-15 Nov 2023
13	Annual Diagnostics Workshop, Tweed Heads, NSW.	The distribution of froghoppers in Australia - potential vectors of *Xylella fastidiosa*	Selleck C, Trebicki P, Hoskins J, Stevens M, Blacket M	Mar 2024
14	International Congress of Entomology (ICE), Kyoto, Japan.	Australian Cercopoid Phenology in a Biosecurity Context	Selleck C, Trebicki P, Jaroslow D, Martoni F, Rako L, Blacket M	Aug 2024

Abstract submitted for presentation at the (Aug 2024) International Congress of Entomology (ICE), in Kyoto, Japan:

Australian Cercopoid Phenology in a Biosecurity Context

C. Selleck [(1)], P. Trebicki [(2)], D. Jaroslow [(1)], F. Martoni [(1)], L. Rako [(1)], & M. J. Blacket [(1)]

[(1)] AgriBio, La Trobe, 5 Ring Road, Bundoora Vic. 3083
[(2)] Macquarie University, Macquarie Park NSW 2109

Cercopoid hemipterans, also known as froghoppers or spittlebugs, are relatively understudied, and poorly characterized in Australia. This is surprising considering they are xylem-feeders, meaning they have the potential to transmit xylem-limited diseases. For example, in Europe, the meadow spittlebug (*Philaenus spumarius*) contributes greatly to the spread of *Xylella fastidiosa*, a xylem-limited bacterium with a host-plant list of over 600 species. *X. fastidiosa* does not occur in Australia, but it would devastate Australian fruit industries; overseas it significantly affects economically important plants such as almonds, peaches, citrus, cherries, olives, and grapes, among many others.

There are considerable gaps in what we know of cercopoid diversity in Australia, as well as worldwide diversity. Additionally, Australian native cercopoids have not yet been assessed as potential *Xylella* vectors and could significantly influence disease transmission if *X. fastidiosa* were to reach Australian shores. As part of a multi-year *Xylella* Vectors Project, researchers at Agriculture Victoria and the New South Wales Department of Primary Industries monitored horticultural areas in south-eastern Australia for native xylem-feeders such as cercopoids, resulting in the detection of two cercopoid species. Historical data, however, suggests the presence of at least five cercopoid species in the areas covered, showing that modern field-collecting can miss important information.

We investigated effective collecting methods, plant associations, and cercopoid phenology in Australia as a whole. We collated data from ten Australian agricultural institutes and museum reference collections to explore historical cercopoid diversity. With over 6,000 specimens recorded, we determined the most common collecting methods for each of the three cercopoid families present in Australia, the variety of associated plants recorded for each genus, and our current knowledge gaps in collecting method, distribution, and associated plant data. For instance, only around 10% of the specimens in our analysis were accompanied by associated plant data, but roughly 50% included collection method data. By investigating the possible local vectors of *Xylella fastidiosa*, we hope to improve our preparedness for potential eradication of *Xylella* if that becomes necessary. Our research highlights the value of in-depth knowledge of species diversity in combating agricultural and biosecurity risks and issues. It also reiterates what we as entomologists already know: reference collections are invaluable.

Table 9. Industry Publication (see detailed extract below).

PUBLICATION	TITLE	AUTHORS	DATE
MYRMECIA. News Bulletin of the Australian Entomological Society. Volume 59, Part 4, pages 63-64	*Xylella* Vectors	Cait Selleck	Nov 2023

Xylella vectors

During the quieter winter season, Agriculture Victoria's **Cait Selleck** has been travelling to Australian departments of agriculture and museum reference collections examining pinned specimens and gathering collection data from thousands of cercopoid (spittlebug/froghopper) specimens. This is part of a national project focused on better understanding potential native *Xylella* vectors, being led by Agriculture Victoria's **Mark Blacket** and jointly funded by Wine Australia and the Department of Agriculture, Forestry and Fisheries (DAFF).

Cait headed to the Orange Agricultural Collection in late April, to get some ID tips from **Birgit Löcker** and **Peter Gillespie** and look at their extensive Auchenorrhyncha collection. A lot of specimens in this collection have been gathered and identified by **Murray Fletcher** and are maintained by Birgit and Peter.

After an educational couple of days in Orange, the next trip was to the Western Australia Department of Primary Industries and Regional Development (DPIRD) in South Perth to visit **Melinda Moir** at the end of June. Cait was able to collect data from both the DPIRD invertebrate collection and Melinda's personal Auchenorrhyncha collection, which has added valuable host association and collection method data to our database.

While back in Melbourne in July, Cait and Mark met up with **Simon Hinkley** at the Melbourne Museum to sort the museum specimens. Cait found that this collection added some otherwise absent state records for common species, put a few rare species on the board, and corroborated the state-level data of various specimens from other collections.

Most recently, Cait has been to Brisbane to see **Susan Wright** at the Queensland Museum and **Justin Bartlett** at Biosecurity Queensland (QDAF). The Queensland Museum Collection also houses the University of Queensland invertebrate collection, officially donated to the museum in 2011. As such, it had more cercopoid material than Cait had expected, and she only managed to move cercopoids from the unidentified drawers into the cercopoid drawers and copy down the aphrophorid (true spittlebug) record data in this case. She may return in future to finish the job, as the museum had some interesting specimens she had rarely seen in the more southern collections. The QDAF collection consisted of one drawer before Cait visited, and by the time she was done transferring specimens from the undetermined drawers, the Cercopoidea required a second drawer to accommodate the additional specimens. The QDAF collection bolstered species records for many states, as well as adding additional froghopper and tube spittlebug records for ACT, QLD, and some overseas localities.



Cait Selleck (Agriculture Victoria) and **Justin Bartlett** (Biosecurity Queensland) in the QDAF collection.

Cait's colleague **Francesco Martoni** photographed the Cairns NAQS (Northern Australia Quarantine Strategy) collection while visiting in August, allowing Cait to add another 90 specimens to the database. These specimens were largely collected in QLD.

Both **Simon Grove** at TMAG (Tasmania Museum and Art Gallery) and **Kate Sparks** at the SA DAFF office sent Cait their cercopoid list as all the specimens were databased.

Cait's next visits will be to the South Australia Museum collection at the end of November, and DPIPWE in Tasmania after the new year.

In between collection visits, Cait databased the cercopoid specimens back at our VAIC (Victorian Agricultural Invertebrate Collection), and further identified them as much as she could. This fully databased collection makes it rare in the cercopoid collections space.

In general, many Cercopoidea, and more broadly the Auchenorrhyncha, are un-databased in our collections. Of course, this is a well-known and unavoidable issue in collections worldwide. It is difficult to keep up with databasing as new specimens pile up, and inevitably some groups are left behind. This project and others like it can help to push certain taxonomic groups into focus, which are otherwise cherished by only a few dedicated researchers.

Cait Selleck

Appendix 2: Intellectual Property

The main findings of this project are summarized above and are provided in full in the attached Technical Reports (Attachments 1 to 9). The plan is for most of these project findings to be written up for publication in peer reviewed journals. We therefore request an embargo of two years on releasing the detailed results (i.e., Attachments 1 to 9) publicly, to allow these results to be written up for publication.

Appendix 3: References

Trebicki P (2020). Grant Agreement: "Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (*Xylella*) in Australia and New Zealand by understanding its potential vectors". *Project Activity Work Plan, Aug 2020, Department of Agriculture, Water and the Environment.*

References from Attachment 1:

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Appendix 4: Staff

Many staff contributed to the research conducted in this project (Tabe 10). The majority of this project research was conducted through Agriculture Victoria (DEECA), with some specific tasks being undertaken by NSW DPI and Macquarie University, through subcontracts (see below).

Subcontracts:

1) NSW DPI: Conducting field surveillance in NSW cropping systems, and contributions to project findings.
2) Macquarie University: Completion of EPG studies, and contributions to project findings.

Note, from January 2023 the project leader, Piotr Trebicki, moved to a new position with Macquarie University, Sydney. The project however remained with Agriculture Victoria with Mark Blacket becoming the project leader, working closely with the project team, including NSW DPI and Macquarie University, to deliver project milestones.

Table 10. Project staff (see Figure 22), organisations, and roles.

NAME	ORGANISATION	PROJECT ROLE
Mark Blacket	Agriculture Victoria (DEECA)	Project Leader (2023-2024)
Piotr Trebicki	Agriculture Victoria (DEECA)	Project Leader (2020-2023) *
Cait Selleck	Agriculture Victoria (DEECA)	Entomology research, field collecting
Duncan Jaroslow	Agriculture Victoria (DEECA)	Entomology research, field collecting
Francesco Martoni	Agriculture Victoria (DEECA)	Molecular entomology, field collecting
Lea Rako	Agriculture Victoria (DEECA)	Molecular entomology
Linda Semeraro	Agriculture Victoria (DEECA)	Entomology research
Narelle Nancarrow	Agriculture Victoria (DEECA)	Field and glasshouse trials
Precious Mavende	Agriculture Victoria (DEECA)	Field and glasshouse trials
Pragya Kant	Agriculture Victoria (DEECA)	Bacteriology
Fiona Constable	Agriculture Victoria (DEECA)	Bacteriology
Jessica Hoskins	NSW DPI (subcontract)	NSW Project Leader (2023-2024)
Mark Stevens	NSW DPI (subcontract)	NSW Project Leader (2020-2023)
Birgit Loecker	NSW DPI (subcontract)	NSW DPI Entomology Taxonomist
Michelle Hallam	NSW DPI (subcontract)	NSW DPI Technical Staff (2021-2022)
Glen Warren	NSW DPI (subcontract)	NSW DPI Technical Staff (2022-2023)
Minna Russell	NSW DPI (subcontract)	NSW DPI Technical Staff (2023-2024)
Leanne Johnston	NSW DPI (subcontract)	NSW DPI Technical Staff (2023-2024)
Eva Carreras	(Spain)	Visting Masters Researcher
Clara Lago	(Spain)	Visting PhD Researcher

* Macquarie University from Jan 2023 (subcontract).



Figure 22. Project activities with staff listed L-R. **(A)** Field collecting, Norton Estate, Vic; Selleck, Trębicki, Carreras **(B)** Field collecting, Grampians Olive Estate, Vic; Selleck, Nancarrow, Jaroslow, Kant. **(C)** EPG training course, AgriBio, Vic; Nancarrow, Mavende. **(D)** Glasshouse trails, Horsham, Vic; Selleck, Carreras. **(E)** Light trapping, Albany, WA; Martoni, Hoskins. **(F)** Field collecting, Grampians Olive Estate, Vic; Nancarrow. **(G)** Field collecting, Silvan, Vic; Jaroslow, Martoni, Rako. **(H)** Field collecting, Perth, WA; Blacket. **(I)** Australian Entomological Society Conference, Albany, WA; Hoskins, Trebicki.



Figure 23. NSW DPI project activities with staff listed L-R (A) Sticky traps in olive grove, NSW; Warren, Russell. (B) Sweep netting in native vegetation, NSW; Johnston. (C) Insect Collections, Orange Agricultural Institute, NSW; Hoskins, Loecker.

Appendix 5: List of Attachments (total of 235 pages)

Attachment 1. *Southeastern Australian surveillance trapping for potential Xylella vectors around commercial host plants.* Duncan Jaroslow, Narelle Nancarrow, Piotr Trebicki, Cait Selleck, & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 17 pages]

Attachment 2. *Investigating potential native leafhopper and spittlebug vectors in the Riverina area of NSW.* Jess Hoskins, & Mark Stevens. NSW DPI subcontract final report [Apr 2024, 9 pages]

Attachment 3. *Australian Cercopoidea Reference Collection Specimens.* Cait Selleck, Duncan Jaroslow, Francesco Martoni & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 68 pages]

Attachment 4. *Biodiversity and COI barcoding of the Australian spittlebugs (Hemiptera: Cercopoidea).* Francesco Martoni, Lea Rako, Duncan Jaroslow, Caitlin Selleck, Pragya Kant, Jessica Hoskins, & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 23 pages]

Attachment 5. *Diversity and composition of the bacterial communities (microbiome) in two common spittlebug species.* Francesco Martoni, Lea Rako, Duncan Jaroslow, Caitlin Selleck, Pragya Kant, Narelle Nancarrow, & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 32 pages]

Attachment 6. *Diagnostic testing of potential insect vectors for the presence of Xylella species.* Pragya Kant, Fiona Constable, & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 17 pages]

Attachment 7. *Feeding behaviour of the native Australian spittlebug, Bathyllus albicinctus, a potential vector of exotic Xylella fastidiosa.* Piotr Trebicki, Clara Lago, Cait Selleck, Francesco Martoni, & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 16 pages]

Attachment 8. *Host plant preferences and survival of the native Australian spittlebug, Bathyllus albicinctus.* Duncan Jaroslow, Lea Rako, Narelle Nancarrow, Piotr Trebicki, Cait Selleck, Francesco Martoni, & Mark Blacket. Agriculture Victoria Technical Report [June 2024, 25 pages]

Attachment 9. *Identification of Auchenorrhyncha in Victorian Olive orchards and vineyards (2020-2021).* Cait Selleck & Linda Semeraro. Agriculture Victoria Technical Report [Apr 2022, 28 pages].

Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (Xylella) in Australia and New Zealand by understanding its potential vectors

Technical Report: Southeastern Australian surveillance trapping for potential *Xylella* vectors around commercial host plants

June 2024

Duncan Jaroslow[1], Narelle Nancarrow[3,], Piotr Trebicki[1,2], Cait Selleck[1], & Mark Blacket[1]

[1] Agriculture Victoria, AgriBio, Bundoora, Victoria
[2] Applied BioSciences, Macquarie University, Sydney, NSW
[3] Agriculture Victoria, Grains Innovation Centre, Horsham, Victoria

VICTORIA State Government | Energy, Environment and Climate Action

AGRICULTURE VICTORIA

External Project Code	WA2001 & 4-EWI2BY6
RCT No	AgVic 6162
Project Leader Name	Mark Blacket

Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.



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Executive Summary

The capacity of *Xylella fastidiosa* to establish, and thus impact Australian agricultural commodities, may depend upon the ubiquity of these insects in Australian commercial crops. Investigating the abundance of these insects around key growing areas should provide insights into the vulnerability of Australian crop growers, and effective methods for monitoring of disease-vectoring insects.

The Cercopoidea (spittlebugs and froghoppers) and Cicadellinae (sharpshooters), belonging to the insect suborder Auchenorrhyncha (Hemiptera), have been implicated in the spread of *X. fastidiosa* in North American and Europe. In Australia, the cercopoid insect *Bathyllus albicinctus* (Cercopoidea: Aphrophoridae) has been identified (through this project) as having a relatively high potential risk of vectoring *X. fastidiosa*, due to its recorded association with a diverse group of plant genera and families. For these reasons, it was hypothesised that cicadellines and cercopoids, including the spittlebug *B. albicinctus*, would be likely potential candidates for spreading *X. fastidiosa*, should this pathogen arrive in Australia.

Our study surveyed the invertebrate communities at two commercial farms: Grampians Olive Estate (GO) and Norton Estate (NE), both located in the Wimmera shire of Victoria. Four sampling methods were used to survey the Auchenorrhyncha at these farms, at least fortnightly, for approximately three years. Many cicadellid specimens were caught, demonstrating the effectiveness of the collection methods, however the xylem-feeding cicadelline sharpshooters were not detected around these commercial farms. Differences in *B. albicinctus* abundances were observed between field locations and sampling methods. The results of this study provide insights into the seasonal abundance of *B. albicinctus* and sampling methods best suited to catching these insects.

The surveys conducted at GO or NE sites to broadly document patterns in the abundance of potential *X. fastidiosa* vectors revealed that:

a) The use of sweep nets and visual assessment of crops were best for catching *B. albicinctus*. While pan traps and sticky mat traps were more effective for catching Cicadellidae.

b) *B. albicinctus* adults were only caught between October and January each year.

c) Nymphal (immature) *B. albicinctus* were observed feeding on both crop plants (olives) and surrounding vegetation in the field.

d) Cercopoidea, other than *B. albicinctus*, were not caught at GO or NE.

These experiments demonstrated that future surveillance of potential *X. fastidiosa* vectors should be conducted during late spring and early summer, preferably using a sweep net for *B. albicinctus* and a sweep net and/or sticky mat traps for Cicadellidae (noting that no known xylem-feeding members of the Cicadellidae were caught during this survey).

Introduction

Background

This study investigated the community of insects in Australian commercial crops that are expected to have the highest *X. fastidiosa* vectoring potential, specifically *B. albicinctus* (Cercopoidea) and Cicadellinae (Hemiptera: Auchenorrhyncha) (Redak et al 2004; Daugherty and Almeida 2009; Kapantaidaki et al 2021). Well-established, sampling techniques were employed to provide a better representation of the potential *X. fastidiosa* vectors already present in commercial Australian crops. In addition, this provided an indication of the suitability of these survey methods for collecting these target insects.

By comparing the catches from four common collection techniques, this study provides additional information on which techniques are most effective for capturing Cercopoidea and Cicadellinae. Previous studies have identified some reliable sampling techniques for these insects, such as light trapping and collecting by hand (Selleck et al 2024, Martoni et al. 2024). These techniques, however, are not always feasible to employ in commercial crops, and can be biased towards certain taxa. This can be partially explained by variation in individual insect life history strategies, which may not reflect trends at broader taxonomic levels.

The overall goal of this study was to survey for potential insect vectors of *X. fastidiosa* in commercial Victorian crops, to better understand the risk of *X. fastidiosa* spread if it arrives in Australia. With extensive damage caused by *X. fastidiosa* overseas, Cercopoidea and Cicadellinae are among the most important groups of pests due to their role in the rapid spread of *X. fastidiosa* between locations and host plants. With effective monitoring and understanding of the putative insect vector community, future containment efforts will be better prepared for *X. fastidiosa* incursions.

Project Objectives

A major aim of this project was to support the development of effective vector monitoring, surveillance, and control strategies for Xylella and to understand the feeding behaviour and plant host range of potential vector species.

Study Aims

The main aim of this surveillance activity was:

1) To compare the abundance and temporal variation of Cercopoidea and Cicadellidae between crops and collection techniques at multiple sites in Victoria.

Methodology

Survey site

This study was conducted at two farms located near Horsham in the Wimmera Shire of Victoria.

- One farm, Grampians Olive Estate (GO), consisted primarily of several groves of olive trees, surrounded by native forest. Trees were planted during the 1940's and spaced so that no olive canopies were touching, with fragmented patches of grass covering the ground.
- The second farm, Norton Estate (NE), is a vineyard with grapevines surrounded by grain paddocks, separated by remnant forest. NE is managed without the use of chemical pest control agents.

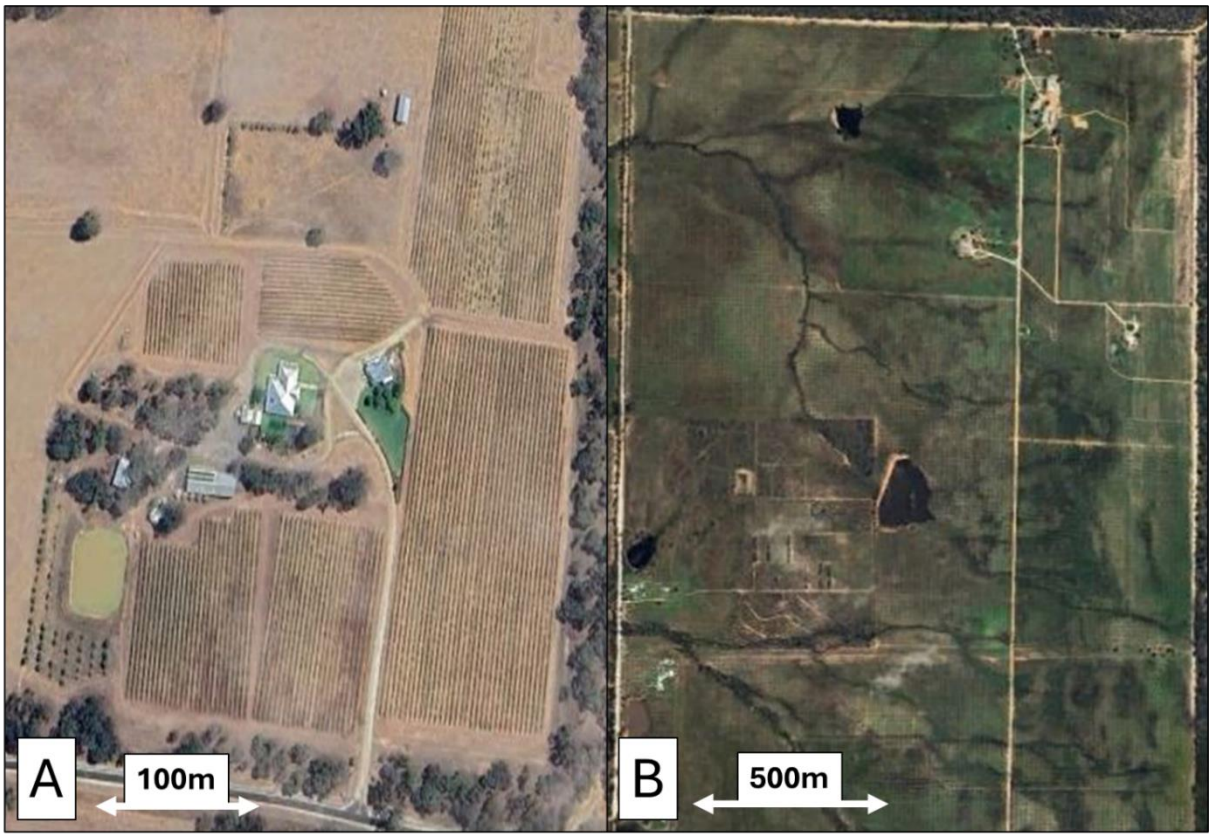


Figure 1. Overhead satellite images of Norton Estate (A) and Grampians Olive Estate (B).

Collection techniques

Insect communities at GO and NE were collected using four different sampling techniques:

1. sticky-mat traps (SMT),
2. yellow pan traps (YPT),
3. sweep net (SN), &
4. visual assessment (VA).

Collections were conducted fortnightly starting on December 4th, 2020, and ending at both farms on February 23rd, 2024.

At each farm, four SMT (10 × 25 cm; Sticky Trap Yellow, Polybutylene, Stikem Special, Bugs for Bugs) and four YPT (active chemical: propylene glycol) were placed every 10-metres along a transect oriented in parallel with crop rows (Figure 2). SMT were attached to a metal rod 50-100 cm above the ground with a YPT on the ground adjacent to the metal rod. Fifty sweep actions using the SN, along with passive VA, were done every 1-metre along the transect running parallel to rows of olives at GO and grapevines at NE.

When collected, SMT were covered with plastic cling-wrap before being closed, and the YPT contents were transferred to a separate jar and labelled appropriately. The propylene glycol in the YPT was replaced and a fresh SMT was attached to the rod. SN or VA collected insects were transferred to a killing jar (containing ethyl acetate). Stored YPT, SMT, SN, and VA collections were transported from Horsham to a laboratory at the Agricultural Biosciences research facility in Bundoora and stored in a freezer (˜20°C) prior to sorting.



Figure 2. Insect traps SMT and YPT at NE (July 2021). Staff: (L-R), Cait Selleck, Piotr Trebicki, Eva Carreras.

Insect counting

Stored collections were inspected under dissection microscope for signs of target insects, which were recorded. Insects recorded included any Cercopoidea or Cicadellidae, with *B. albicinctus* counted separately to other Cercopoidea. Other Auchenorrhyncha were recorded (e.g., Membracidae or Fulgoroidea), although they were omitted from analysis due to a low number of specimens.

Statistical analysis

All statistical analysis was done in SPSS (version 28.0.1.0, IBM®; $\alpha = 0.05$). Differences in the abundance of *B. albicinctus* and Cicadellidae between NE and GO were tested via independent samples t-test. Differences in abundance among sampling techniques (YPT, SMT, SN, VA) were investigated via Independent Samples Kruskal-Wallis (KW).

Key Research Findings

Results

Cercopoidea

Bathyllus albicinctus were the only Cercopoidea recorded in this study. Thirty *B. albicinctus,* most commonly collected by sweep netting, were found across GO and NE. *B. albicinctus* abundances were not significantly different between GO and NE study sites (Independent samples t-test: $t = 0.411$, N = 1193, $p = 0.341$). *B. albicinctus* was present at both commercial farms, although VA, YPT, and SMT were more effective for collecting *B. albicinctus* at GO, and SN was the best at NE. Across sites, sweep netting caught more *B. albicinctus* than other techniques, if including empty sweep nets (Kruskal-Wallis test: $F_{3,1191} = 64.781$, $p < 0.001$). Pan traps and yellow sticky mats were minimally effective for catching *B. albicinctus* but did not differ from each other (Pairwise comparison: $t = 1.738$, $p = 0.692$, Figure 3). *B. albicinctus* were only caught from October to January (Figure 4).

These results show that the putative *X. fastidiosa* vector, *B. albicinctus*, is present in Australian commercial crop areas. Sweep nets were effective at collecting *B. albicinctus*, although only between October and January, when *B. albicinctus* was present (Figure 5). These results suggest that future efforts to research *B. albicinctus* in commercial crop should consider using a sweep net technique and avoid using sticky mats or pan traps. Furthermore, our data is consistent with findings from Selleck et al (2024) which indicated spring and summer as the ideal seasons for finding *B. albicinctus*.

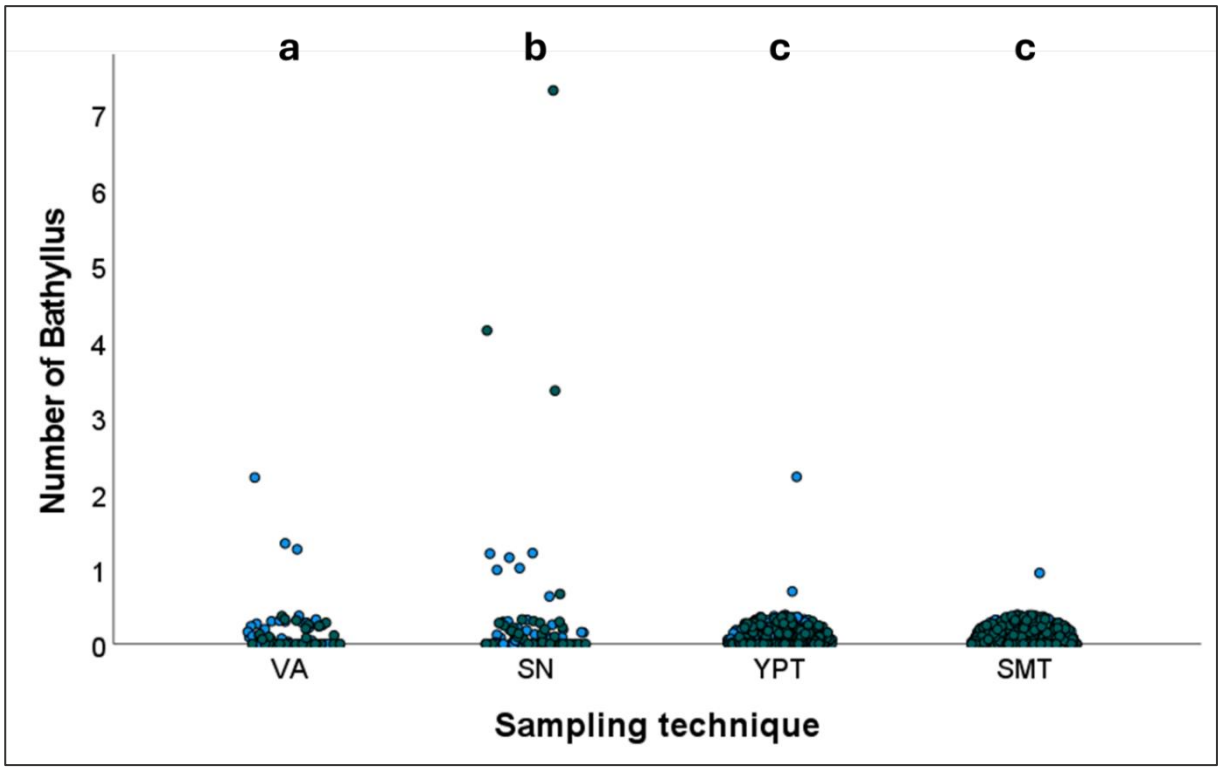


Figure 3. Abundance of *B. albicinctus* across sampling technique for collections from Grampians Olive Estate (blue dots) or Norton Estate (green dots). Lower case letters indicate statistical differences (pairwise comparison) where groups do not share a letter.

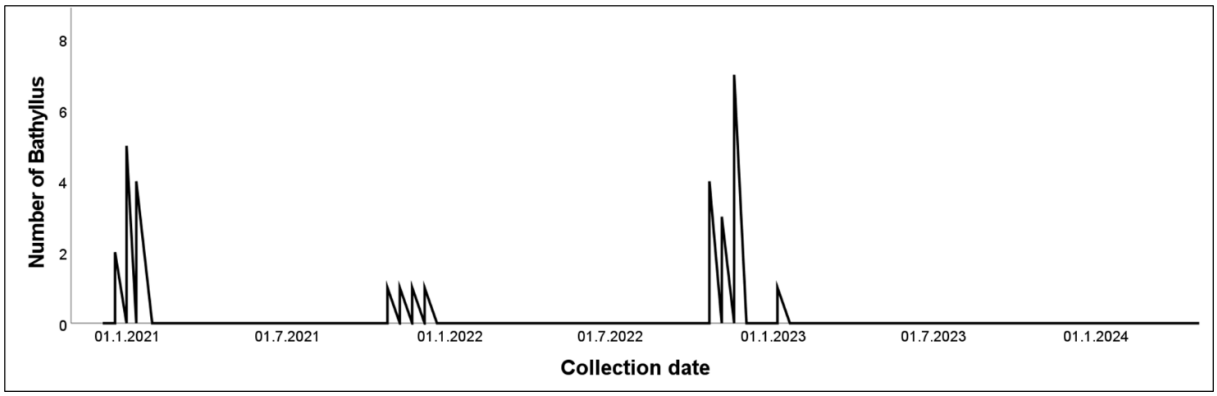


Figure 4. *Bathyllus albicinctus* abundance throughout the study period.

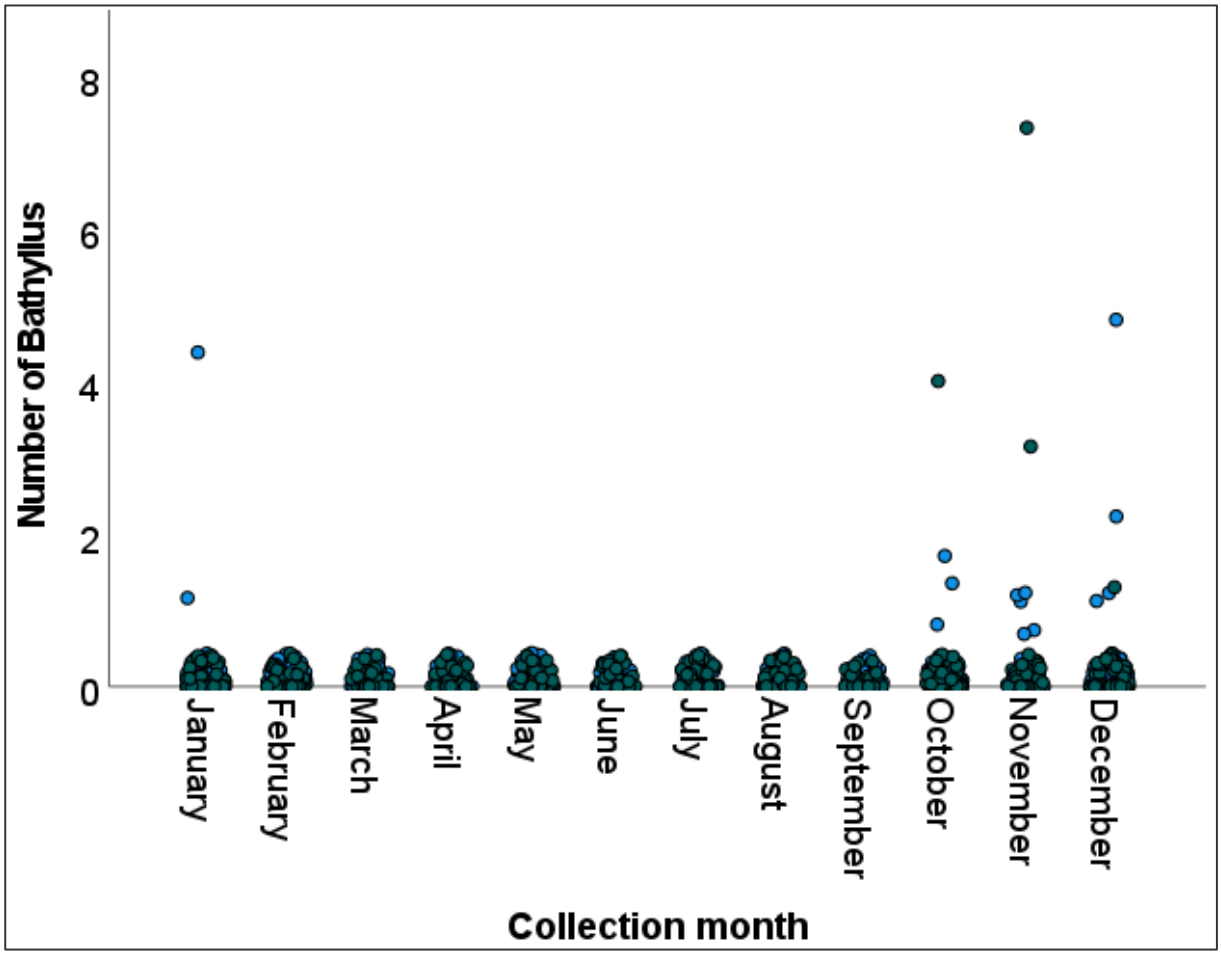


Figure 5. Abundance of *Bathyllus albicinctus* by calendar month at Grampian Olives (blue dots) and Norton Estate (green dots).

Cicadellidae

Several species of Cicadellidae were caught across both study sites by each technique except visual assessment. Cicadellidae were present at both farms and were caught more often at NE compared to GO (Independent samples t-test: $t = 2.323$, N = 822, $p = 0.010$). Differences were found in Cicadellidae abundance among all collection techniques (Kruskal-Wallis: $F_{3,820} = 40.986$, $p < 0.001$), except between SN and YPT, with SMT collecting the most Cicadellidae (Figure 6). Cicadellidae were present for most of the calendar year, with abundance reaching its minimum during winter and maximum during summer (Figure 7). Notably, no xylem-feeding species of Cicadellidae were detected during the three years of surveillance.

These results show that SMT are an effective means of surveying Cicadellidae in commercial crops, especially when deployed between December and January, as this is when Cicadellidae abundance was greatest (Figure 8). Cicadellidae were generally present at all times of the year. Here, species diversity was not assessed, and the seasonal spike in Cicadellidae may not reflect changes in the abundance of all present Cicadellidae species.

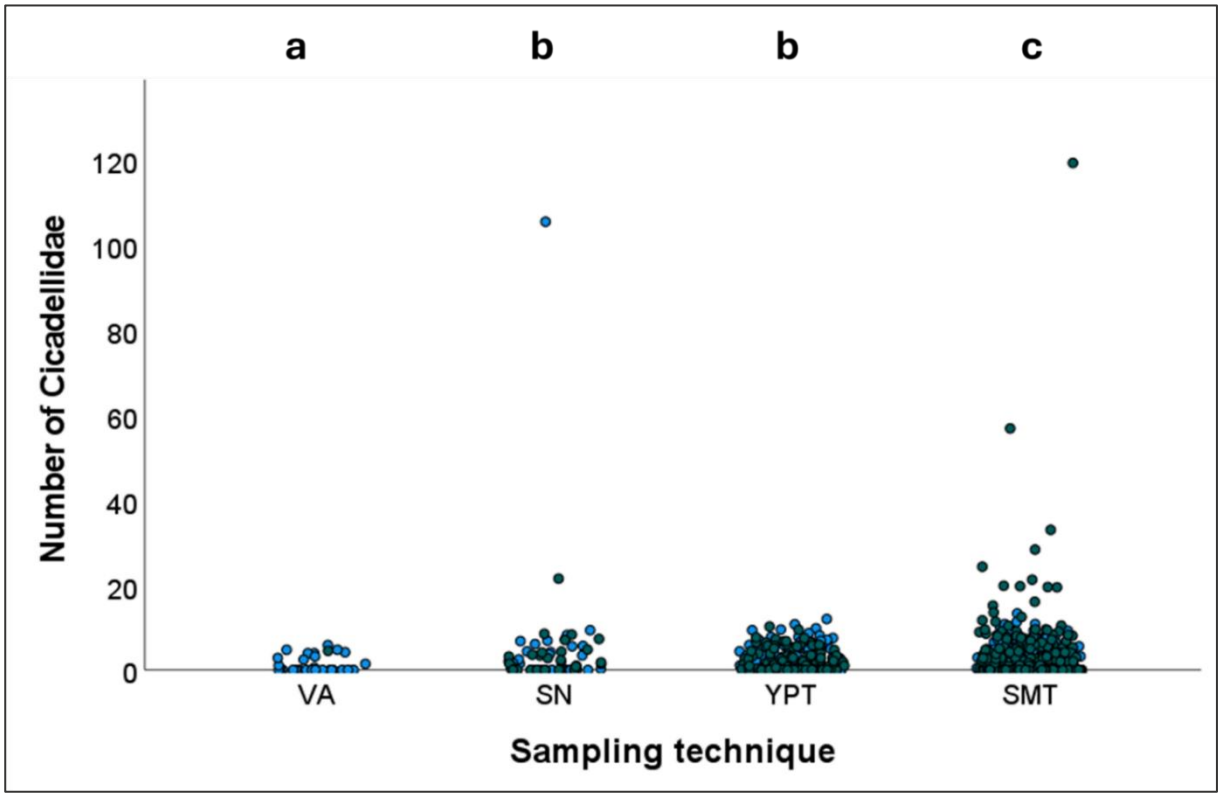


Figure 6. Abundance of Cicadellidae across sampling technique for collections from Grampians Olive Estate (blue dots) or Norton Estate (green dots). Lower case letters indicate statistical differences (pairwise comparison) where groups do not share a letter.

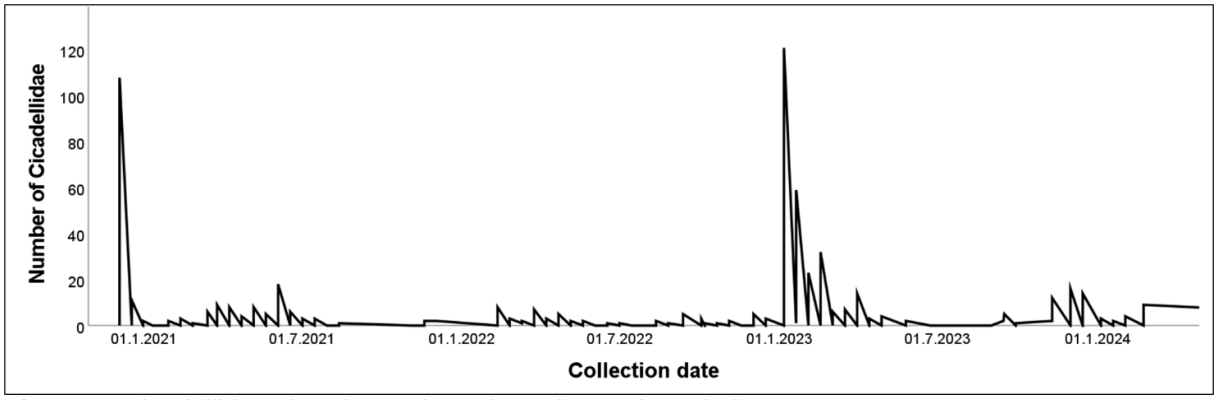


Figure 7. Cicadellidae abundance throughout the study period.

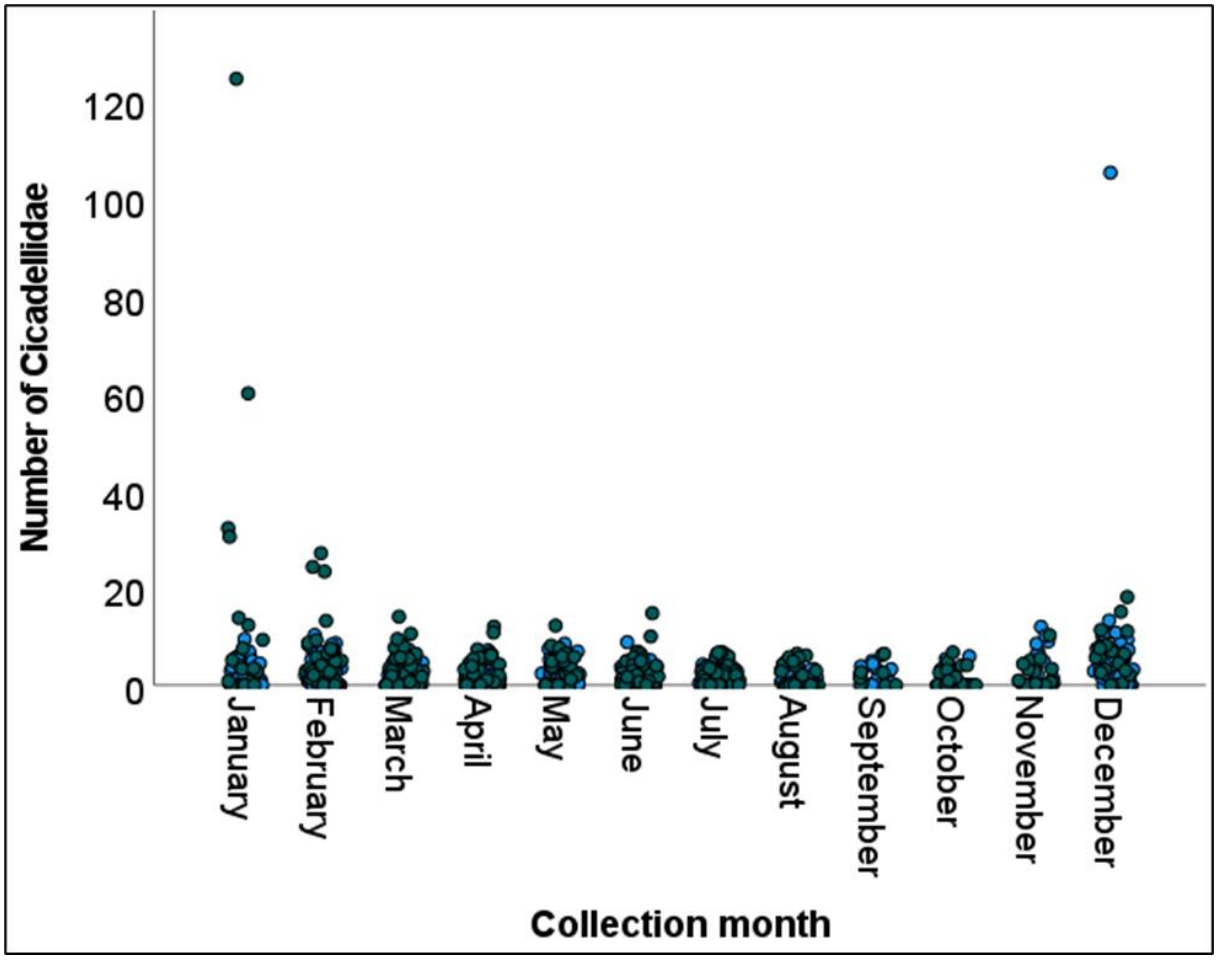


Figure 8. Abundance of Cicadellidae by calendar month at Grampian Olives (blue dots) and Norton Estate (green dots).

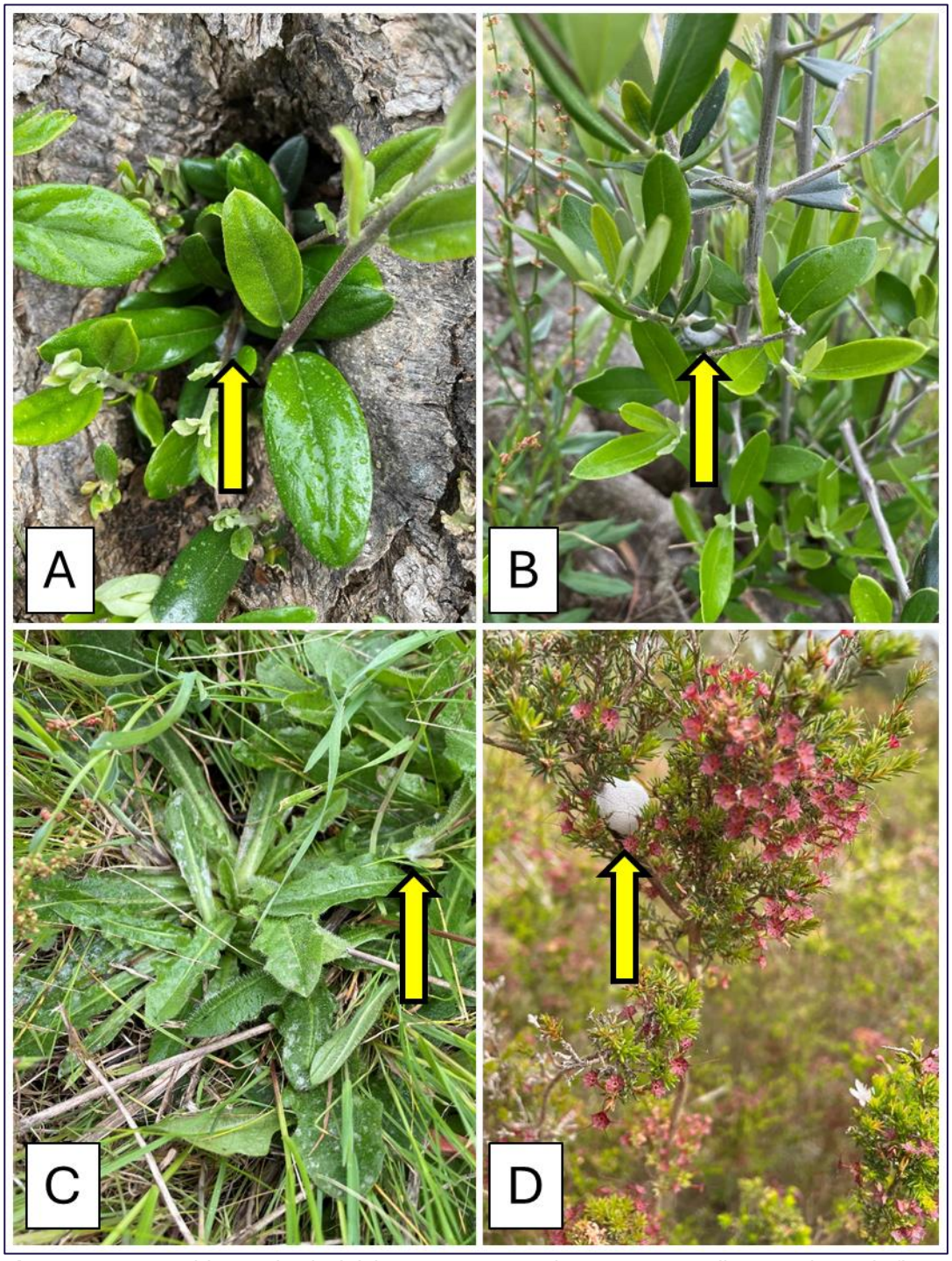


Figure 9. Cercopoid nymphs (spittle) present at GO site, Oct 2023. Olive tree (A & B), flatweed (C) and heather (*Erica* sp.) (D). Presence of Spittle is indicated by arrows.

Recommendations

Outcomes

The typically common and widespread species *B. albicinctus* was present at both commercial farms, and in far smaller abundance than Cicadellidae. Statistical analysis indicated that the effectiveness of sampling techniques was not the same between these taxa. For *B. albicinctus*, sweep netting was the most successful sampling technique. Future sampling of *B. albicinctus* should consider using sweep nets and avoid SMT or YPT.

For Cicadellidae, SMT were the most successful sampling technique, although a substantial number of SMT were mouldy, which makes it difficult to accurately identify insects to species. In contrast to *B. albicinctus*, no Cicadellidae were collected by VA at NE or GO throughout this study. Cicadellidae in this study, however, were not identified to species or genus. Therefore, there may be strong fluctuations in abundance for certain Cicadellidae genera within this group that were not resolved during statistical analyses.

For both *B. albicinctus* and Cicadellidae, abundance was highest from late spring to early summer, and hence when sampling efforts are most likely to succeed. The exception to this was December – February 2023 – 2024 summer, when no *B. albicinctus* were caught.

In NSW, another survey of commercial crops from 2020 – 2024 (Hoskins & Stevens 2024), in which the collected Cicadellidae were identified to species level. There, abundances of the *Anzygina* (Typhlocybinae: Erythroneurini) and *Orosius* (Deltocephalinae: Macrostelini) genera were orders of magnitude greater than any other Cicadellidae. Victoria's Cicadellidae communities may have similarly high variation in abundance between Cicadellidae species, although the species composition may be different between Victoria and NSW like for Cercopoidea. The NSW survey found only three Cercopoidea individuals, all *Pectinariophyes stalii* (Cercopoidea: Machaerotidae), and no *B. albicinctus*, compared to Victoria where thirty *B. albicinctus* were caught. Despite only one Cercopoidea species being caught in both crop surveys, museum collections (Selleck et al 2024) indicate that *B. albicinctus* and *P. stalii* are known to be present in NSW and VIC.

Conclusion

Future directions

This research highlighted several key questions for future research:

1. The results from surveying Cercopoidea and Cicadellidae in commercial crops revealed variation in the abundance of both insect communities. These variations were found to be associated with geographic location and taxa. Future research could support control efforts by investigating the environmental conditions and factors that lead to differences in insect abundance between the two field sites, and thereby indicate when or where high populations of Cercopoidea and Cicadellidae can be expected.

2. SN was the most effective sampling technique for *Bathyllus albicinctus*, while SMT caught the most Cicadellidae generally. Despite success with *B. albicinctus*, SN may not be ideal for other cercopoid species. Some traps were relatively better than others, for catching Cicadellidae and Cercopoidea, and generally rely on visual inspection of trap contents. The development of genetic primers specific to Cercopoidea or Cicadellidae would allow for more rapid and sensitive surveillance of putative *X. fastidiosa* vectors, rather than relying solely on visual inspection of field-vegetation or collected traps.

3.. Commercial crops in Victoria would be at risk of *X. fastidiosa* infection from Cercopoidea, namely due to the presence of *Bathyllus albicinctus* and its potential to vector *X. fastidiosa*. While Cicadellidae were also collected in large numbers, they were all assumed to be phloem-feeders, due to an absence of xylem-feeding Cicadellidae species in these commercial fruit orchards. Large fluctuations in *B. albicinctus* abundance between seasons, however, suggests that the risk of *X. fastidiosa* transmission from *B. albicinctus* would also fluctuate with season. The absence of xylem-feeders other than *B. albicinctus* from Victorian crops also elevates its importance as a potential vector, should *X. fastidiosa* arrive in Australia. As such, Southeast Australian crops may be best protected if control efforts are targeted towards *B. albicinctus*.

4.. As *B. albicinctus* the only potential vector detected in this study, a local biological control agent targeting *B. albicinctus* during its few months of high abundance could reduce the chance of *X. fastidiosa* transmission. Therefore, identifying and evaluating a biological control targeting *B. albicinctus* could provide another management tool to stymie the establishment of *X fastidiosa* in Victorian crops, as is being investigated for *Philaenus spumarius* (Cercopoidea: Aphrophoridae), Europe's primary *X. fastidiosa* vector (Molinatto et al. 2020: McCaw et al. 2024).

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Wine Australia Project WA2001

Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (*Xylella*) in Australia and New Zealand by understanding its potential vectors.

Final report - NSW DPI subcontract – April 2024

Jess Hoskins - Entomologist, Yanco Agricultural Institute
Mark Stevens – Principal Research Scientist, Yanco Agricultural Institute

Summary
We investigated the presence and distribution of potential native leafhopper and spittlebug vectors of *Xylella fastidiosa* in horticultural crops and native vegetation in the Riverina area of NSW. In three years of sampling, we found only three individuals of one spittlebug species, *Pectinariophyes stalii* in the crop sites. The low abundance of this species, and the absence of other species from groups recognised as containing xylem-feeders indicates that should *Xylella fastidiosa* enter Australia, there is a low risk of spread within the Riverina area, and any spread that does occur is likely to be slow. However, further investigation of the distribution of potential native vectors in other areas of NSW is required to better inform biosecurity response plans.

1. Introduction
Xylella fastidiosa (*Xf*) is an exotic bacterium transmitted to plants by xylem-feeding spittlebugs (Cercopoidea) and some cicadellid leafhoppers (Cicadellinae: Cicadellini). *Xf* is considered the single most significant threat to Australian crop biosecurity and plant industries. *Xf* is the cause for many plant diseases including Pierce's disease in grapes, olive quick decline syndrome and citrus variegated chlorosis. As *Xf* migrates to new areas (or invades a new country), native species of leafhopper and spittlebug are known to emerge as effective vectors of the bacterium. At present there is limited information on the native leafhopper and spittlebug fauna associated with vulnerable crops in Australia, and consequently if *Xf* enters Australia there is no information on potential native vectors that could facilitate its spread. The main aim of this project was to generate baseline data on the distribution of potential native *Xf* vectors in horticultural crops in the Riverina area of NSW from the xylem feeding groups present in Australia (Cercopoidea and Cicadellini). In addition to our main goal, our aim was also to identify all Cicadellidae encountered, since many phloem-feeding species are associated with the transmission of other plant diseases such as viruses, mycoplasmas and mycoplasma-like organisms. Additionally, as the feeding mechanisms of some species are not well known, identifying all species will be beneficial to future assessments. The data generated from this study will provide the basis for developing *Xf* biosecurity response plans which will help to mitigate the severity of damage to agricultural industries and the economy in the event of a *Xf* incursion, as well as increasing our general knowledge of native leafhopper and spittlebug diversity and distribution in Australia.

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2. Method

2.1 Site selection and establishment

Four horticultural crops across 10 different locations were selected as leafhopper sampling sites. These were grapes (4 sites), citrus (2 sites), cherries (2 sites) and olives (2 sites) (Table 1). Sampling sites were established within a 100km radius of Yanco Agricultural Institute. Site selection was based on proximity to YAI due to the need to attend to traps every other week.

Table 1: Leafhopper and spittlebug site sampling locations, crop type and establishment and removal dates.

Site	Location	GPS Co-ordinates	Crop	Establishment	Removal
YAI	Yanco	34.6149, 146.4156	Citrus (oranges)	17/11/2020	24/04/2023
GRC	Griffith	34.1919, 146.0445	Citrus (oranges)	23/11/2021	4/12/2023
GRG	Griffith	34.1915, 146.0450	Grapes	23/11/2021	4/12/2023
LIL	Leeton	34.3421, 146.2528	Grapes	13/12/2021	22/12/2023
YAR	Yenda	34.1402, 146.1056	Grapes	20/12/2021	28/12/2023
TOO	Leeton	34.3324, 146.2301	Grapes	20/01/2022	8/02/2024
RAP	Hanwood	34.2447, 146.0222	Cherries	9/03/2022	27/03/2024
GIA	Bilbul	34.1628, 146.0648	Cherries	9/03/2022	27/03/2024
MOR	Widgelli	34.2206, 146.1211	Olives	17/03/2022	27/03/2024
BRU	Ganmain	34.5324, 147.0448	Olives	27/10/2022	27/03/2024

All but two sites, YAI and BRU, were surveyed for two years. The YAI site was surveyed for three years. The initial year of sampling at YAI was undertaken as a pilot study for the remaining sites to establish an appropriate methodology. The BRU site was surveyed for 1.5 years, as identifying a willing collaborator was delayed due to the small number of commercial olive producers in the area and grower caution regarding site access. However, sampling was completed at the BRU site over two summer seasons, so we feel that the leafhopper community there was adequately sampled, due to the lower abundance and species diversity typically observed over the winter season (Figure 1).

2.2 Sampling methods

Sampling for leafhoppers and spittle bugs was conducted between November 2020 and March 2024. Three sampling methods were used at each of the 10 sites. These were yellow sticky traps, yellow tray traps and sweep netting. Four yellow sticky traps and four yellow tray traps were used at each site. The yellow sticky traps (Bugs for Bugs, Toowoomba, Qld) were positioned at a height of 1.2 to 1.8 m above the ground, attached to branches or vine trellises. The yellow tray traps were placed on the ground beneath the trees or vines. Tray traps consisted of two 2L capacity generic yellow plastic ice-cream containers. The outer tray had a hole drilled in the bottom which was used to secure the trap to ground by the addition of a small tent peg. The second container, which fitted inside the first contained propylene glycol (propane-1,2-diol) approximately 3 cm deep (~250ml). Propylene glycol was used to capture and preserve insects that flew into the trap because it didn't evaporate to any significant extent during the fortnight between each trap servicing and unlike ethylene glycol

(which has been used historically) it is non-toxic if consumed by wildlife. During the duration of the project, we experienced unseasonably wet weather. This resulted in the tray traps sometimes overflowing during the early part of the project. To counteract this, a hole about halfway up the side of the internal tray was drilled and fine mesh attached to the outside to prevent loss of insects. However, the large volume of water in the traps did mean that in some cases the samples were degraded due to dilution of the propylene glycol, which ultimately prevented the identification of some specimens. Tray traps and sticky traps were placed in rows within the crop approximately 10m apart. Sweep-netting was conducted for half an hour at each site during trap renewal. Sweep netting involved running an insect net through the plant foliage after which insects were collected using a mouth aspirator into a plastic vial. Sweeping and trap renewal occurred at all sites at two to three week intervals. Two-week intervals typically occurred between September and April, when insect prevalence was high, while when insect prevalence was low (May to August) (Figure 1) the sampling interval was 3 weeks. Sweeping was suspended for the deciduous crops (grapes and cherries) between May and September when plants were lacking well-established foliage. The yellow tray traps and yellow sticky traps were present continuously. During trap renewal, yellow sticky traps were replaced with new traps. Used yellow sticky traps were pinned open onto a cork board to prevent damage and loss of specimens during transport from the field back to the laboratory. The propylene glycol in the tray traps was poured into plastic jars (250ml) and then replaced with new propylene glycol. Sweeping samples were placed into cold storage for transport. All samples were stored in a cool room set at 4 ℃ until processing.

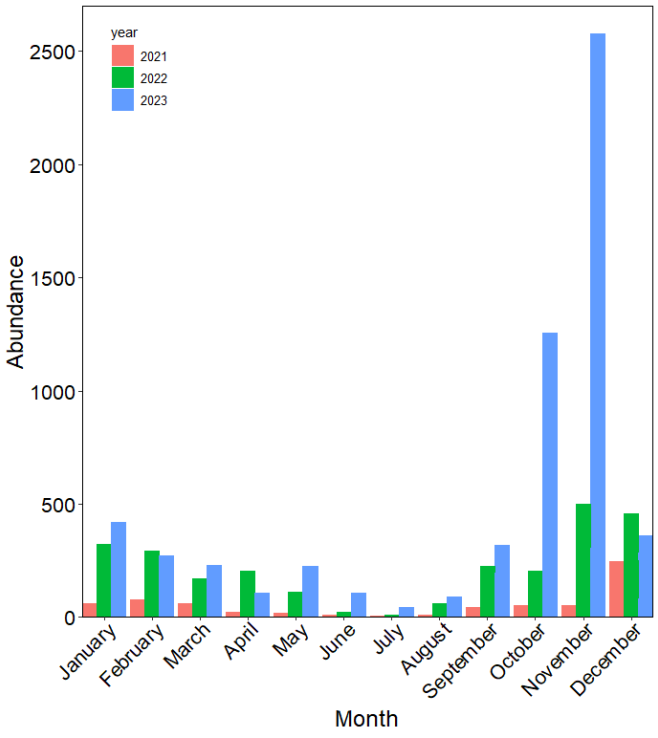


Figure 1: Abundance for each full year sampled. Large numbers in October and November of 2023 are primarily attributed to one grass feeding non-vector leafhopper species, *Anzygina* species.

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Towards the end of the collecting period, we also employed light trapping as a method of collection. We did this as we were not finding the target species and wanted to examine as many collection methods as possible to ensure that the lack of target species present in crops was a product of low abundance and distributional boundaries rather than the trapping methodology itself. Light trapping was conducted between February and April of 2024 in three locations around YAI. Two of the light trapping locations were near orange orchards and one near to an area of native vegetation. A white sheet and a light (LepiLED) were hung up slightly before sunset after which insects were either hand collected directly into vials or collected via mouth aspirator into vials for a period of one to two hours. Collections took place over several nights and no potential vectors were identified. As this method of collection was used for the sole purpose of checking our methodology, no further processing or identification was conducted for insects in these samples (although the samples were retained).

2.3 Sample processing

Specimens were transferred to 80% ethanol as soon as practicable. Insects were removed from sticky traps with Safsolvent®, a histopathology solvent that dissolves the trap adhesive. Once removed from the trap the insects were soaked in Safsolvent® overnight to remove any remaining adhesive, transferred to pure ethanol to remove traces of the Safsolvent® and then into 80% ethanol prior to identification. Insects in tray traps were sorted from the bycatch and placed directly into 80% ethanol prior to identification. Where possible the propylene glycol was recycled to reuse in tray traps. Specimens from sweeping were transferred directly into 80% ethanol. Specimens were then sorted into morphospecies (see section 2.4 for details), and the numbers of males and females recorded.

2.4 Taxonomic identification

All leafhopper and spittlebug species were identified regardless of potential vector status. Species were initially identified to morphospecies using the NSW DPI Auchenorrhyncha keys (idtools.dpi.nsw.gov.au/keys/auch) and subsequently identities were confirmed by undertaking male genitalia dissections. The last abdominal segment and pygophore (area containing male genitalia) were excised from the abdomen of each insect. This section was then placed into potassium hydroxide (KOH) overnight to clear. Clearing the specimen removes pigment and dissolves internal tissues so that the structures required for identification can clearly be seen. Morphological keys and taxonomic descriptions were then used to confirm species identifications. An identification guide was developed which included species morphological descriptions as well as photographs which allowed non-specialists to identify most specimens without dissection and facilitated much shorter sample processing times. In the case of some highly abundant species groups where multiple species were present but for which external morphological characteristics could not be used to identify insects to the species level (e.g. *Orosius* spp., *Anzygina* spp., *Horouta* spp. and *Maiestas* spp.), it was impractical to dissect all the males collected, and data was recorded collectively for these groups. Females were identified based on external morphology only and where a male was present for confirmation, except in cases where external morphology was distinctive (e.g *S. foshoi*).

2.5 Extending the search area

Sampling was extended to native vegetation after initial analysis of the crop samples led resulted in almost no potential vectors; only three individuals of one potential vector, *P. stalii,* were identified out of ~20, 000 individuals (Table 2). Sweeping was the only method used to survey the native vegetation. Sweeping was conducted approximately twice a week between March 2023 and February 2024. Areas of native vegetation were selected based on proximity to horticultural crops and vegetation type. Targeted vegetation types included *Eucalyptus* and *Melaleuca* species as well as sedges, as xylem feeding spittle bugs and leafhoppers have previously been found on these plant species. Time spent sampling was typically 30 minutes to one hour per site. Identification was completed to morphospecies and where possible species were identified based on external morphology. Due to the volume of individuals, dissections were not carried out to confirm species identities.

Towards the end of the project (February 2024) the sampling area was further extended to one area outside of the Riverina in Orange, NSW, to attempt to delimit the distribution of potential vectors after none were found during native vegetation sweeping in the Riverina area. During this sampling effort sweeping and light trapping were used. Sweeping of native vegetation was conducted at several locations in and around Orange on two separate days. Light trapping was conducted on two nights at two separate locations on the NSW Department of Primary Industries, Orange Agricultural Institute grounds. One area was in a vineyard/apple orchard, while the other was close to a nature reserve.

3. Results

3.1 Crop results

There were 458 sticky traps and tray trap collection attempts (with 4 traps deployed per attempt), of these 87.5% of sticky traps and 91.5% of tray traps were positive for the target species. There were 366 sweeping attempts of 30 minutes each, of these 51.6% were positive for the target species. Out of the positive samples 20,149 individual insects were collected and identified of which 20,146 were leafhoppers and 3 were spittlebugs. 133 dissections were performed, and 39 species were identified (Table 2).

Most leafhoppers (69.8%) were caught in yellow tray traps followed by sticky traps (26.4%) and least of all sweeping (3.8%). The most common and abundant species found were those species making up the *Anzygina* spp. complex (66%) (including those identified as *A. zealandica* and *A. honiloa*) followed by species making up the *Orosius* spp. complex (17.9%) (identified as *O. argentatus*, *O. canberrensis* and *O. orientalis*), *Batracomorphus angustatus* (Osborn) (3%), *Austroagallia torrida* Evans (3.3%), *Maiestas* spp. complex (2.14%) (identified as *M. knighti* and *M. veta*), *Xestocephalus tasmaniensis* Evans (1.7%) and *Austroasca viridigrisea* (Paoli) (1.2%). All other species were relatively rare with low abundance. In general, abundance was similar for all crop sites. RAP (cherries) had the highest abundance with 19.2% of all individuals found here, followed by GRG (grapes) with 14.1%, while LIL (grapes) and YAR (grapes) had the lowest abundance at 5.1 and 5.6%, respectively. Abundance generally declined in the cooler months (Figure 1). There were 13 species that were found in all crops (Table 2). Species diversity within crops was similar for all crops although grapes and olives were slightly more diverse with 30 species each, while

citrus and cherries had slightly less with 25 and 23 species, respectively. Species present in the crops varied with time of year, with diversity decreasing over the winter months (June to August, 18-20 spp.) and peaking during the summer months (December to February, 25 to 29 spp., although March also had high species diversity 29 spp.).

One potential native *Xf* vector was found, which was identified as the spittlebug *Pectinariophyes stalii* (Spångberg). Three individuals of *P. stalii* were found at three different site locations, RAP (cherries), GRC (citrus) and TOO (grapes) and using three different collection methods (yellow tray trap, yellow sticky trap and sweeping, respectively). No other potential native vectors, such as members of Cicadellini were found in the crops.

Table 2: Leafhopper species and abundance in crops in the Riverina area of NSW. Species highlighted in blue represent known vectors of other viruses and bacteria.

Membracoidea-Cicadellidae		**Cherry**	**Citrus**	**Grape**	**Olive**	**Total**
Deltocephalinae						
Athysanini	*Arawa pulchra* Knight	7	12	49	28	96
	Limotettix incertus Evans	7	29	44	12	92
Chiasmini	*Chiasmus varicolor* (Kirkaldy)		1			1
	Exitianus plebeius (Kirkaldy)	4	58	66	15	143
Deltocephalini	*Hourouta* spp. complex[1]	1	1	17	83	102
	Maiestas spp. complex[2]	80	123	150	79	432
Eupelicini	*Paradorydium* cf. *pseudolyricen* Kirkaldy				10	10
Goniagnathini	*Goniagnathus osmelaki* Fletcher			2		2
	Goniagnathus venenensis Fletcher				1	1
Macrostelini	*Balclutha incisa* (Matsumura)	23	63	101	2	189
	Balclutha rubrostriata (Melichar)		1			1
	Balclutha saltuella (Kirschbaum)	9	4	8		21
	Cicadulina bimaculata (Evans)			2		2
	Nesoclutha phryne (Kirkaldy)	33	70	53	54	210
	Orosius spp. complex[3]	767	400	784	1673	3624
Paralimnini	*Soractellus* nr *brunneus* Evans		1	8	2	11
Scaphoideini	*Scaphoideus foshoi* Fletcher & Semararo	1		1		2
Penthimiini	Penthimiini sp.1				1	1
Iassinae						
Batracomorphini	*Batracomorphus angustatus* (Osborn)	225	172	129	88	614
Idiocerinae						
	Idiocerinae sp.1			1		1
Macropsinae						
Macropsini	Macropsini sp.1			1		1
Megopthalminae						
Agalliini	*Austroagallia torrida* Evans	302	66	229	65	662
Typhlocybinae						
Empoascini	*Austroasca malvae* (Evans)			1		1
	Austroasca viridigrisea (Paoli)	90	67	57	30	244
Erythroneurini	*Anzygina* spp. complex[4]	4749	2530	4420	1604	13303
	Empoascanara cf. *peregrina* Dworakowska	1				1
	Zygina cf. *evansi* (Ross)				1	1
Xestocephalinae						
Xestocephalini	*Xestocephalus sidnicus* Kirkaldy				3	3
	Xestocephalus tasmaniensis Evans	226	5	96	10	337

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Membracoidea - Membracidae						
Centrotinae						
Terentiini	*Pogonotypellus australis* (Goding)				38	38
Cercopoidea - Machaerotidae						
Enderleiniinae						
Enderleiniini	*Pectinariophyes stalii* (Spångberg)	1	1	1		3
Total		**6526**	**3604**	**6220**	**3799**	**20149**

[1]*H. austrina* (Kirkaldy), *H. perparvus* (Kirkaldy), *H. jahmoi* Fletcher
[2]*M. veta* (Knight), *M. knighti* Webb & Viraktamath
[3]*O. orientalis* (Matsumura), *O. argentatus* (Evans), *O. canberrensis* (Evans), *Orosius* sp.1
[4]*A. zealandica* (Myers), *A. honiloa* (Kirkaldy)

3.2 Native vegetation results

Sweeping was conducted in native vegetation at 88 different locations, 78 within the Riverina and 10 in and around Orange, NSW. 60 morphospecies (Table 3) were identified from the Riverina native vegetation sweeping collections, but no potential vectors (i.e., members of Cercopoidea and Cicadellini) were found. Species diversity in the native vegetation was higher than that in the crops. There were 11 species that were found in both native vegetation and in crops (Table 3).

During sampling in Orange, NSW, two potential vector species were identified. These were *Pectinariophyes stalii* (Spångberg), one individual, and cf. *Anyllis liela* Kirkaldy, two individuals.

Table 3: Leafhopper species found in native vegetation in the Riverina area of NSW. Species highlighted in blue represent overlap between crops and native vegetation.

Cicadellidae	Species	Native vegetation
Austroagalloidinae		
	Austroagallodies sp.	1
	cf. *Austroagalloides grisea* Evans	7
Deltocephalinae		
Athysanini	*Arawa* sp.	1
	Limotettix incertus Evans	2
Chiasmini	*Exitianus plebeius* (Kirkaldy)	11
Deltocephalini	*Maiestas veta* (Knight)	4
Macrostelini	*Balclutha* sp.1	22
	Balclutha sp.2	89
	Balclutha incisa (Matsumura)	4
	Balclutha saltuella (Kirschbaum)	4
	Nesoclutha phryne (Kirkaldy)	2
Opsiini	*Opsius stactogalus* Fieber	6
	Orosius spp. complex	4
Penthimiini	*Ectopiocephalus australis* (Walker)	4
	Penthimiini sp.1	4
Scaphoideini	*Scaphoideus foshoi* Fletcher & Semararo	2
Eurymelinae		

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Eurymelini	cf. *Eurymeloides pulchra* (Signoret)	1
	Eurymelita terminalis Evans	1
Ipoini	Ipoini sp.1	2
	Ipoini sp.2	4
	cf. *Anacornutipo lignosa* (Walker)	3
	cf. *Bakeriana* sp.	1
	cf. *Ipoella brunneus* (Evans)	1
	cf. *Katipo* sp. (Evans)	2
	Ipoini sp.3	1
Iassinae		
Batracomorphini	*Batracomorphus angustatus* (Osborn)	16
Trocnadini	*Trocnada dorsigera* Walker	4
Idiocerinae		
	cf. *Rosopaella citrinella* (Webb)	5
	Idiocerinae sp.1	2
	Idiocerinae sp.2	36
	Idiocerinae sp.3	6
	Idiocerinae sp.4	10
Macropsinae		
	Macropsinae sp.1	4
	Macropsinae sp.2	3
	Macropsinae sp.3	1
Megopthalminae		
Agalliini	*Austroagallia torrida* Evans	3
Tartessinae		
Tartessini	cf. *Brunotartessus fulvus* (Walker)	22
	cf. *Tartessella attenuata* (Evans)	1
Typhlocybinae		
Dikraneurini	*Aneono australensis* (Kirkaldy)	7
	cf. *Kahaono viridis* Evans	74
	cf. *Kahaono yarama* (Dworakowska)	3
	Kahaono wallacei Evans	42
	Dikraneurini sp.1	84
	Dikraneurini sp.2	24
	Dikraneurini sp.3	4
	Dikraneurini sp.4	1
	cf. *Kahaono* sp.1	1
	Dikraneurini sp.5	6
Empoascini	*Austroasca viridigrisea* (Paoli)	119
Erythroneurini	*Anzygina* spp complex	171
	Erythroneurini sp.1	2
	Zygina evansi (Ross)	28
Membracidae		
Terentiini	*Sextius virescens* (Fairmaire)	5

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4. Discussion

In our three years of sampling in the Riverina area, we only found one species of potential vector, *P. stalii*, in very low numbers in the crops and none in areas of native vegetation surrounding the crops. This finding indicates that should *Xf* arrive in Australia there is probably a low risk of spread to and within the Riverina area due to a lack of species from groups generally regarded as xylem-feeders.

The most likely reason for the low numbers of known xylem-feeding species is related to distributional boundaries and abundance. In that the Riverina area is outside of, or in the case of *P. stalii* the very edge of the area in which these species naturally occur. Distributional records of *P. stalii* indicate that this species is more often found closer to coastal areas of NSW. Likewise, distributional data for other potential vector species, that were not found in this study, such as the spittlebug *Bathyllus albicuntus* (Ericson), indicate these species are probably more abundant in coastal regions of NSW. Although, further research is required to confirm this.

In this study the low numbers of known xylem-feeding species recorded are unlikely to be a consequence of the trapping methodologies we used. Even after diversifying and including native vegetation in our sampling, and including light trapping as a collection method, no additional (from what was collected in the crops) known xylem-feeding species were found in the Riverina study area. Additionally, our success in finding two different species of potential vector in Orange indicates the methods we used were effective, and further supports the idea that the low numbers of target species in the Riverina area are related to abundance and distributional boundaries.

Further work is necessary to identify and delimit distributional boundaries of potential xylem-feeding leafhoppers and spittlebugs in NSW. While the Riverina area seems relatively safe from the spread of *Xf*, there is limited knowledge of potential vectors in the horticultural areas closer to coastal regions in NSW. Knowledge of potential native vectors in these areas is required to develop a suitable biosecurity plan to limit the spread of *Xf* should it arrive in Australia.

Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (Xylella) in Australia and New Zealand by understanding its potential vectors

Technical Report: Australian Cercopoidea Reference Collection Specimens

May 2024

Cait Selleck, Duncan Jaroslow, Francesco Martoni & Mark Blacket

VICTORIA State Government | Energy, Environment and Climate Action

AGRICULTURE VICTORIA

External Project Code	WA2001 & 4-EWI2VY6
RCT No	AgVic 6162
Project Leader Name	Mark Blacket

Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.



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Executive Summary

Outside Australia, the insect group known as spittlebugs (superfamily Cercopoidea) is attributed with vectoring the lethal plant bacterium *Xylella fastidiosa* between host plants during feeding. Identifying high and low risk *X. fastidiosa* vectors in Australia is increasingly relevant to Australian agricultural security, with *X. fastidiosa* presenting a growing threat to global agriculture. Surveillance relies on effective and consistent collection methods for vector insects to screen for bacterial pathogens. If *X. fastidiosa* were to arrive in Australia, the effectiveness of surveillance and management could be restricted by a limited understanding of Australia's native cercopoid abundance, phenology, distribution, and diet.

Our study explored these key knowledge gaps. We investigated cercopoid specimens in reference collections across Australian museums and state agricultural departments. Specimens present in these collections were used to better understand the spatiotemporal patterns, common collection methods, and plants associated with Australia's cercopoid community. Specimen information including collection date, location, host plant, and insect identity (at genus level) were obtained from a total of 6350 preserved cercopoid specimens, with 5555 (87.5%) of these records deemed appropriate for further analysis. Our assessment revealed that:

a. Effective adult insect collection methods differ between cercopoid families (Table 7). For example, 'light trapping' is noted as the most common collection method overall, however for Aphrophoridae the most common collection method is 'hand collecting', followed by beating / sweeping / sieving / vacuuming (activities are that often performed together).
b. Overall Cercopoidea showed a strong pattern of seasonal abundance (Figure 5), however this varied greatly with state / territory. For example, *Anyllis* were only collected in South Australia from January to March but were collected from Queensland in all months except June.
c. The five most abundant cercopoid genera, *Anyllis*, *Bathyllus*, *Chaetophyes*, *Pectinariophyes*, and *Philagra* (Figure 7), represent more than 75% of all specimens analysed (each genus representing >10% of all specimens), are geographically distributed across multiple Australian states and are found on multiple plant families (Figures 6 & 8).
d. Notably, the genera *Bathyllus, Anyllis* and *Philagra* appear to have the greatest potential for vectoring of *X. fastidiosa* (if it was introduced to Australia) due to their broad geographic distributions, relatively wide putative host ranges, and higher abundances during spring and summer when the risk of spreading *X. fastidiosa* is likely higher. *Bathyllus* was found to have the widest host range of any recorded Australian Cercopoidea. *Bathyllus albicinctus*, the only species in the genus, was recorded on 44 plant genera spread across 27 plant families (Figure 8).
e. The plant genera *Eucalyptus* and *Acacia* may have a high risk of becoming infected with *X. fastidiosa* in the case of an incursion, in part due to the high diversity of Cercopoidea that are associated with these plants (Figure 8).

This information represents a previously unavailable resource, with approximately half of these specimen records not databased within reference collections, and a much higher percentage not available to researchers without prior connections to each reference collection. The records analysed here provide valuable insights into when and where *X. fastidiosa* is likely to establish or spread in Australia in the case of an incursion (based on Australian insect potential vectors), and thus where and how surveillance and management efforts can be most effective.

Introduction

Background

The establishment of herbivore-transmitted diseases, like *Xylella fastidiosa*, requires a capable herbivorous vector. As *X. fastidiosa* bacteria dwell inside the xylem of their host plant, they are transmitted between hosts by insects that feed on xylem vessels. In Europe, *X. fastidiosa* is vectored by cercopoid insects, the insect group known as froghoppers or spittlebugs, particularly by the European meadow spittlebug (*Phileanus spumarius*) (Cavalieri et al. 2019).

The introduction of *X. fastidiosa* to Australia would be disastrous for Australian agriculture and natural vegetation. While known exotic *Xylella* vectors have a small potential to enter Australia unnoticed (e.g. meadow spittlebug, glassy-winged sharpshooter), the far likelier scenario is that *Xylella* would be vectored within Australia by local xylem feeding insects. Understanding the community of potential *Xylella* vectors in Australia is of great utility for preparative management efforts.

Cercopoidea, and other xylem feeders, are expected to be able to transmit *Xylella* across Australia if exposed to the bacterium. However, in part due to being relatively harmless without *Xylella*, the cercopoid community of Australia is currently poorly understood. Understanding and resolving key knowledge gaps would greatly improve the readiness of management efforts to respond to an incursion by *X. fastidiosa*. Some key information includes: seasonal abundance, host preferences, geographic distribution, and relative abundance of cercopoid genera.

Despite the knowledge gaps for Australian Cercopoidea, many preserved specimens with observational collection records are available in reference collections throughout Australia. These records are a largely un-databased, unexplored resource for revealing crucial information about the cercopoid community in Australia to better prepare for a *X. fastidiosa* incursion.

In this study, we utilised reference specimen records of Cercopoidea collected in Australia. Most of these records and specimens include geographic, temporal, associated plant, sex, and/or developmental stage details. Here, we use this information to map the seasonal abundance of the cercopoid genera represented in Australian reference collections. We highlight the most abundant genera collected and preserved, as these may be most likely to spread *X. fastidiosa* within Australia. We also highlight the plant groups on which Cercopoidea have commonly been found on, and by association, the plants *X. fastidiosa* might first infect during an Australian incursion.

Project Objectives

A major aim of this project was to improve surveillance methods of potential Australian vectors of *X. fastidiosa*. Including, better understanding of the taxonomic diversity of potential vector insects and range of host plants potential vectors they can utilise.

Study Aims

The two main aims were to inform Xylella vector surveillance activities by:

1) Compiling cercopoid specimen records from Australian invertebrate reference collections.
2) Analysing temporal and spatial trends of specimen records, determine common collection methods and examine Australian Cercopoidea plant associations.

Methodology

Reference Specimen Records

We contacted representatives of museums and state agricultural government departments and universities across Australia. This included Orange Agricultural Institute (OAI), South Australian Museum (SAM), Queensland Department of Agriculture and Fisheries (QDAF), Museums Victoria (MusVic), Tasmanian Museum and Art Gallery (TMAG), Queensland Museum (QLD Mus), Western Australian Department of Primary Industries and Regional Development (WA DPIRD), Western Australian Museum (WAM), Cairns Northern Australian Quarantine Strategy (Cairns NAQS), University of Adelaide (AU), and the South Australian branch of the Department of Agriculture, Fisheries and Forestry (SA AFF). Additionally, Dr Melinda Moir provided her personal cercopoid collection (hereafter referred to as Moir Coll.), and we accessed our own reference collection, the Victorian Agricultural Insect Collection (VAIC), for use in the project.

In total, 14 collections at 12 locations contributed to the final dataset (Figure 1, Table 1); note the QLD Museum permanently houses the University of QLD (UQ) invertebrate collection, and Dr Moir's collection was viewed while visiting WA DPIRD. Twelve collections were personally visited by AVR staff, and the remaining two collections (SA AFF and TMAG) were contacted via email after which collection data was sent in a digital format (Table 1). Collection dates range from October 1883 (*Bathyllus albicinctus* from SAM) to March 2024 (*Pectinariophyes* from VAIC). More than 99% of specimens recorded were pinned or card mounted (i.e. dry preserved).

OAI contained 1942 records, 35% of the total record count (Figure 1). All other collections consisted of fewer than 800 cercopoid records each, with SA AFF being the smallest cercopoid collection with only one specimen. The size of each collection was broadly indicative of the genus and species diversity, with a few exceptions (Figure 2); e.g., while the Moir Collection ranked 8th in size, it ranked 4th in species diversity;

likewise, the Cairns NAQS collection was the 3rd smallest collection with 76 cercopoid specimens, but outperformed both WA DPIRD and WAM in species diversity.

Approximately 51.6% of specimens were databased (i.e. label information was digitised; Figure 3). As part of this project, all OAI and VAIC specimens were databased. The single specimen from SA AFF was previously databased, and the TMAG, and WAM had over 85% of their cercopoid specimens databased. The remaining collections had fewer than 15% of their Cercopoidea databased, with Cairns NAQS, the Moir Collection, UQ, and AU contained no databased specimens. As such, copies of each collection's own digitised specimen data will be provided back to the curator of that collection, to assist with future data sharing.

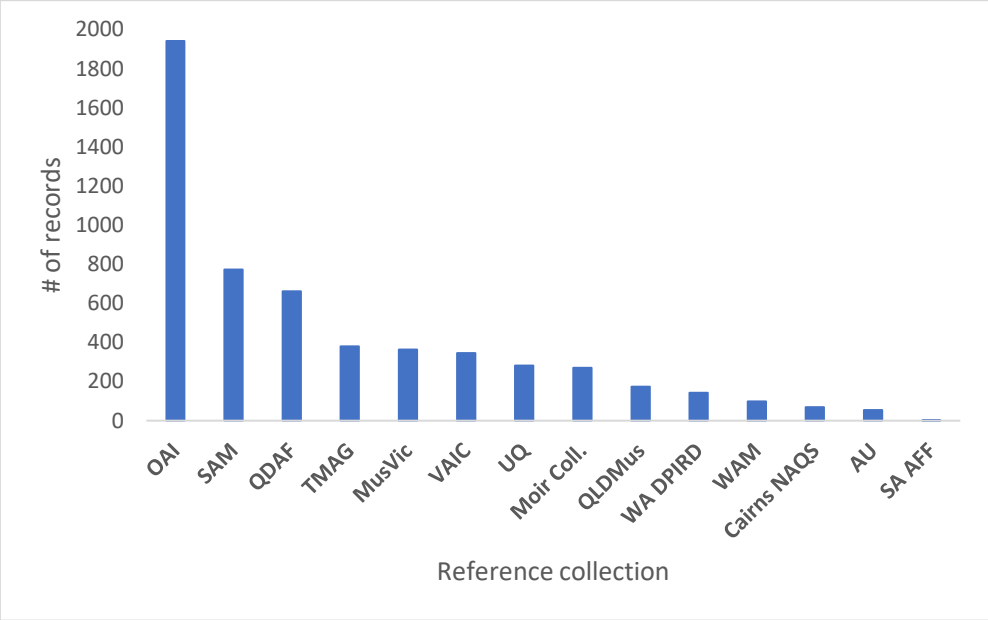


Figure 1. Cercopoid record count from each contributing reference collection.

Table 1. Australian reference collections visited throughout the project.

Institute visited	Location	Date of visit	AVR staff member/s involved	Comments
OAI	Orange, NSW	25 - 28th Apr 2023	C. Selleck	
MusVic	Melbourne, Vic	11 Jul 2023	C. Selleck, M. Blacket	
WA DPIRD	Perth, WA	26, 30th Jun 2023	C. Selleck	
Moir Coll.	Perth, WA	26, 30th Jun 2023	C. Selleck	
QLD Mus	Brisbane, QLD	7-8th Aug 2023	C. Selleck	
UQ	Brisbane, QLD	7-8th Aug 2023	C. Selleck	
QDAF	Brisbane, QLD	9-11th Aug 2023	C. Selleck	
Cairns NAQS	Cairns, QLD	28th Aug 2023	F. Martoni	
SA AFF	Adelaide, SA	28th Jul 2023	-	Data sent digitally by Kate Sparks
AU	Adelaide, SA	24 Nov 2023	C. Selleck	
SAM	Adelaide, SA	20 - 23 Nov 2023	C. Selleck	
WAM	Perth, WA	9th Nov 2023	M. Blacket	
TMAG	Hobart, Tas	8th Sep 2023	-	Data sent digitally by Simon Grove
VAIC	Melbourne, Vic	Ongoing access	C. Selleck	Final specimens databased 11th Apr 2024

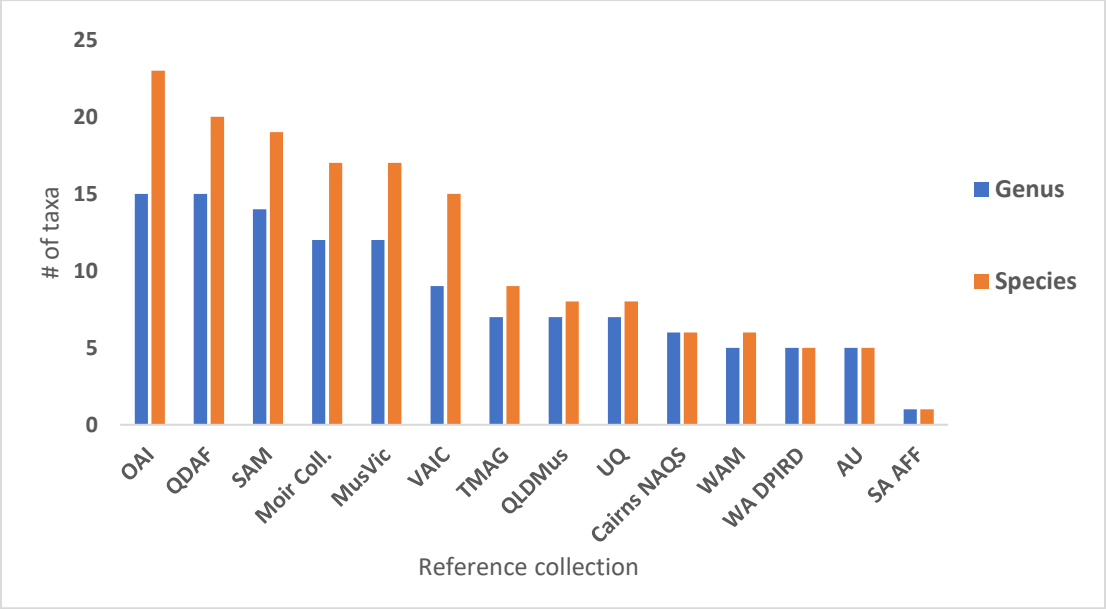


Figure 2. Cercopoid genus and species count in each reference collection.

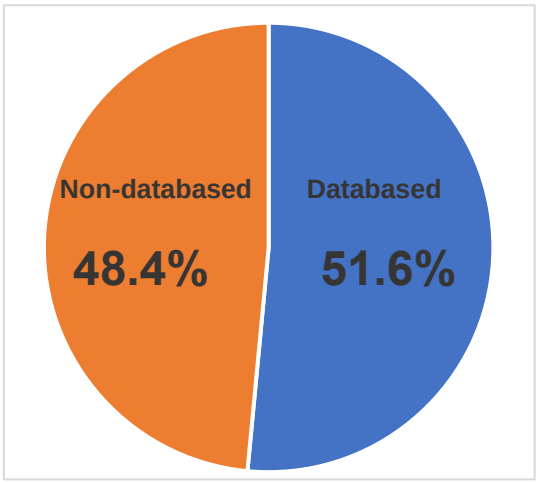


Figure 3. The ratio of databased to non-databased cercopoid specimens from included reference collections.

Complete data for two collections (QLD Museum and University of QLD) were not recorded. Due to the unexpectedly large number of cercopoid specimens present in both collections. The Aphrophoridae specimens were prioritised for data recording. As a result, the Cercopidae and Machaerotidae are under-represented from these collections in our dataset.

Upon arrival at each collection, C. Selleck assessed the specimens currently categorised under the superfamily Cercopoidea. If non-cercopoid specimens were detected, they were removed before data recording began. Additionally, specimens in the Membracoidea and Fulgoroidea drawers were checked for any mis-placed Cercopoidea, of which there were usually a small number. Any undetermined Cercopoidea were identified to genus where possible and placed in the correct unit tray.

After all specimens were sorted as accurately as possible, data labels from each specimen were transcribed,

along with number of specimens per pin, life-stage, and sex for *Bathyllus albicinctus.* Examples of reference collection specimens are shown in Figure 4.

Post collection visits, the datasets were collated in Excel and enhanced in various ways: 1) GPS coordinates were added for locations which were specific enough to allow this; 2) cercopoid family and genus names were updated (e.g., Aphrophoridae is here treated as a family, not a subfamily), and 3) plant scientific names were added if the common name was species/genus specific, or updated in other cases (e.g. *Acacia* is now in family Fabaceae, not Mimosaceae). Some data was also removed. This included specimens which had tentative or questionable identifications attributed to them, the questionable identification level was removed (e.g., a specimen labelled "*Chaetophyes admittens*" was conservatively reduced to *Chaetophyes*, as the species identification was uncertain).

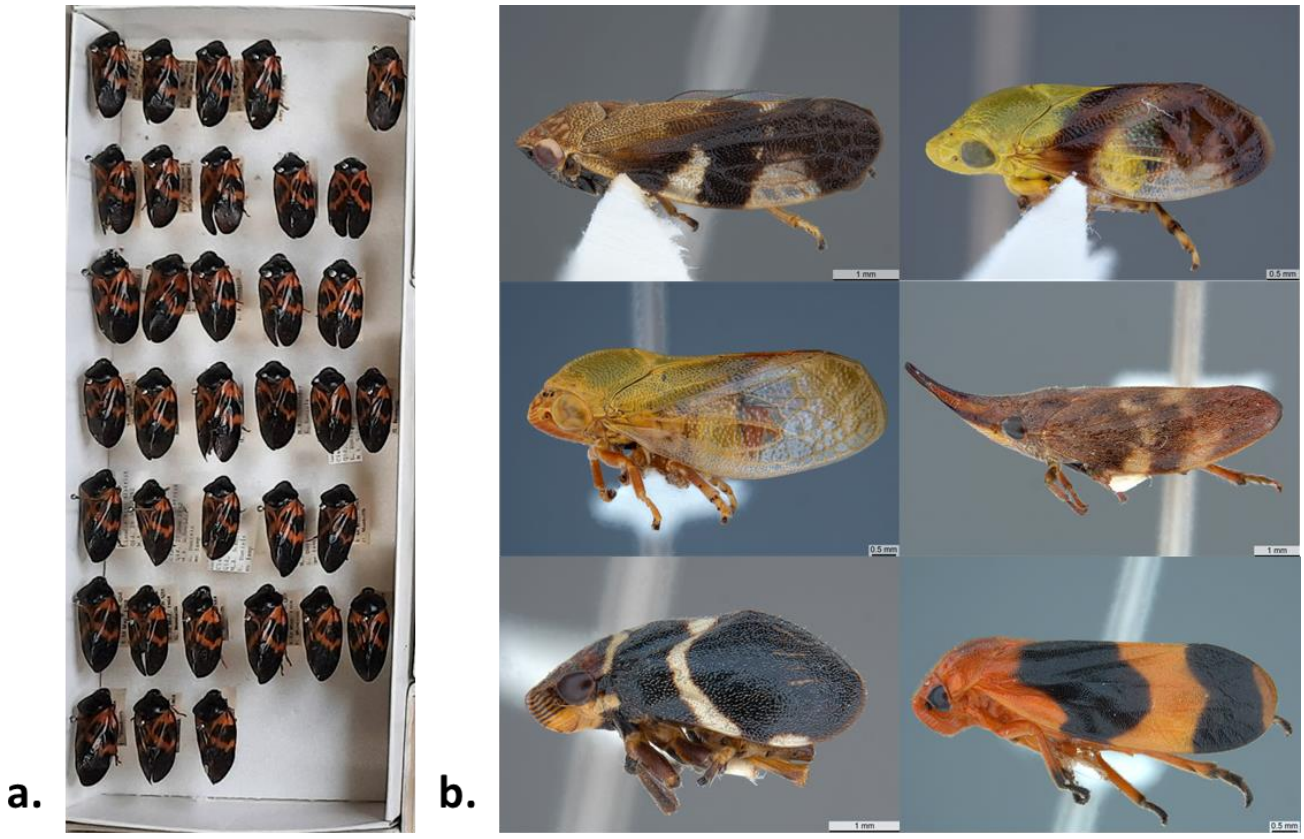


Figure 4. Examples of Cercopoidea from reference collections. **a.** A unit tray of *Megastethodon* specimens housed in the QLD Mus collection. **b.** The six most abundant genera recorded from the specimen reference collections visited (Genus images from left to right, and top to bottom: *Anyllis*, *Chaetophyes*, *Pectinariophyes*, *Philagra*, *Bathyllus*, *Euryaulax*).

Additional Insect Collection

In addition to the reference collection preserved insect records, we also collected new Cercopoidea on several field visits in Victoria (Vic), New South Wales (NSW), Queensland (QLD), and Western Australia (WA). These specimens were lodged in the VAIC, with accompanying DNA barcodes and high-resolution images also generated (see Martoni et al. 2024).

Data Analysis

To focus on the Australian Cercopoidea, records of specimens in Australian collections collected overseas were omitted from the analyses. This includes records from offshore Australian territories, such as Christmas Island, which is geographically closer to Indonesia than Australia.

Many specimens did not have all the desired details available, yet the records were retained where the information relevant for a given analysis was present. Any Australian specimen with the following data was retained: collecting location (state/territory or more specific), collecting month, and specimen identification to family or a lower taxonomic rank.

A small subset of records contained information on the plant associated with the insect during the collection event. Where a common name was given for the associated plant, we added the relevant family, genus, and species names if the common name was taxonomically unambiguous (e.g. “fortnight lily” applies specifically to *Dietes iridioides*; while “bush” does not have any taxonomic relevance).

For records including a collecting method, terminology was standardised, and methods grouped for easier analysis (Appendix 2); for instance, all light-based trapping methods were grouped, and “at light” was considered the same as “light”, “light trap(ping)”, and other typographical variations.

Sankey diagrams were generated for each cercopoid family using R software v4.2.2. (The R Core Team 2024), with a combination of the packages *networkD3* v0.4 (Gandrud et al. 2017) and *flipPlots* v1.3.6. Sankey diagrams considered only the insect records that were identified at least to the genus level and had a corresponding host plant identified at least to the genus level.

Key Research Findings

Summary of Results

- The compiled specimen record dataset represented 19 genera and 31 species across the three cercopoid families (Component 1).
- We revealed that the Australian cercopoid families were most commonly collected using different insect collection methods (Component 2).
- Our results also supported the initial expectation that some Australian Cercopoidea have very wide geographic ranges (Component 3), and are associated with many different plant types (Component 4).

As with many other insects, the expectation that Cercopoidea would be more abundant in summer months was supported across Australia, although seasonal fluctuations in insect abundance were less apparent in Queensland and the Northern Territory than other states. Several insect genera were identified as particularly relevant for potential vectoring or establishing *X. fastidiosa* in Australia:

- *Anyllis* was the most abundant genus, although it was absent from NT and ACT.
- *Chaetophyes* and *Pectinariophyes* were the most widely distributed and were present in all Australian states and mainland territories.
- *Bathyllus* may be perhaps the most serious potential vector due to its extremely wide range of associated plants (44 plant genera across 27 families), although it was absent from NT and ACT.

Component 1: Composition of Insect Collections

The most abundantly recorded cercopoid genus was *Anyllis*, with 989 individual records across six Australian states (Table 2), representing 19.5% of Cercopoidea collected in Australia. *Hindoloides* was the least collected cercopoid genus on the Australian mainland, with three individuals collected from NSW and five from QLD, accounting for 0.2% of Australian records. Three *Basilioterpa* specimens were collected on Lord Howe Island. Two Machaerotidae, *Chaetophyes* and *Pectinariophyes*, were the most widespread genera within Australia and were collected from all states, NT, and ACT (Table 3).

Most collections were recorded in and around Australia's most populated areas (Figure 5), although some differences appeared apparent between families. Aphrophoridae and Cercopidae were generally more abundant around Australian population centres, particularly across Australia's south-east (Figure 6 and 7). In contrast, Machaerotidae were relatively evenly distributed across Australia, and with a few exceptions, were the only subfamily to have a presence in Central Australia (Figure 8).

The five most abundant genera (*Anyllis*, *Pectinariophyes*, *Bathyllus, Chaetophyes* and *Philagra*) each represented more than 10% of recorded specimens and collectively accounted for 77.75% of Australian cercopoid records. Except for *Euryaulax*, which represented 7.83% of records, all other genera accounted for less than 3.5% of the Cercopoidea record (Table 2).

Table 2. Summary of cercopoid records by genus. Absolute counts and percentage of total records are included. Only Cercopoidea collected within Australian states and territories are included.

Family	Genus	# of records	% of total
Aphrophoridae	*Anyllis*	989	19.5
Machaerotidae	*Pectinariophyes*	950	18.8
Aphrophoridae	*Bathyllus*	853	16.9
Machaerotidae	*Chaetophyes*	607	12.0
Aphrophoridae	*Philagra*	555	11.0
Cercopidae	*Euryaulax*	398	7.9
Cercopidae	*Petyllis*	167	3.3
Cercopidae	*Aufidus*	144	2.8
Cercopidae	*Tonnoiria*	132	2.6
Machaerotidae	*Polychaetophyes*	73	1.4
Aphrophoridae	*Amarusa*	73	1.4
Aphrophoridae	*Liorhina*	34	0.7
Cercopidae	*Megastethodon*	20	0.4
Cercopidae	*Aufiterna*	19	0.4
Aphrophoridae	*Interocrea*	17	0.3
Machaerotidae	*Machaerota*	14	0.3
Machaerotidae	*Hindoloides*	8	0.2
Aphrophoridae	*Clovia*	6	0.1
Aphrophoridae	*Basilioterpa*	3	0.1
	Subtotal	5062	100

Table 3. Cercopoidea species presence in each state and territory of Australia, in alphabetical order and grouped by cercopoid family. Records with no species designation were pooled under the genus determination. Records with only family designation (493 records) were omitted.

		Present in:									
Genus	**Species**	**Mainland**	**ACT**	**NSW**	**NT**	**QLD**	**SA**	**Tas**	**Vic**	**WA**	**Offshore**
APHROPHORIDAE											
Amarusa		**Y**		**Y**		**Y**					
Amarusa	*australis*	Y		Y		Y					
Anyllis		**Y**		**Y**		**Y**	**Y**	**Y**	**Y**	**Y**	
Anyllis	*leiala*	Y		Y		Y	Y	Y	Y		
Anyllis	*spinostylus*	Y						Y			
Anyllis	*tiegsi*	Y		Y					Y		
Basilioterpa		**N**									**Y**
Basilioterpa	*pallida*	N									Y
Bathyllus		**Y**		**Y**		**Y**	**Y**	**Y**	**Y**	**Y**	
Bathyllus	*albicinctus*	Y		Y		Y	Y	Y	Y	Y	
Clovia		**Y**				**Y**					
Clovia	*eximia*	N									
Interocrea		**Y**				**Y**					
Interocrea	*immaculata*	Y				Y					
Interocrea	*nigrofasciata*	Y				Y					
Liorhina		**Y**		**Y**		**Y**					
Liorhina	*loxosema*	Y		Y		Y					
Philagra		**Y**	**Y**	**Y**		**Y**	**Y**	**Y**	**Y**	**Y**	
Philagra	*concolor*	Y		Y		Y					
Philagra	*fulvida*	Y		Y		Y					
Philagra	*parva*	Y	Y	Y		Y	Y	Y	Y		
Philagra	*recurva*	Y		Y		Y					
CERCOPIDAE											
Aufidus		**Y**				**Y**					
Aufidus	*trifasciatus*	Y				Y					
Aufiterna		**Y**				**Y**					
Aufiterna	*kirkaldyi*	Y				Y					
Euryaulax		**Y**			**Y**	**Y**		**Y**		**Y**	
Euryaulax	*carnifex*	Y			Y	Y		Y		Y	
Euryaulax	*kirkaldyi*	Y			Y	Y				Y	
Euryaulax	*vacuola*	Y			Y						
Megastethodon		**Y**				**Y**					
Megastehodon	*urvillei*	Y				Y					
Petyllis		**Y**		**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	
Petyllis	*deprivata*	Y		Y		Y	Y	Y	Y	Y	
Tonnoiria		**Y**	**Y**	**Y**		**Y**		**Y**	**Y**	**Y**	

Tonnoiria	*chinai*	Y				Y					
Tonnoiria	*tasmaniae*	Y	Y	Y		Y		Y	Y		

MACHAEROTIDAE

Chaetophyes		**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	
Chaetophyes	*admittens*	Y		Y		Y		Y	Y		
Chaetophyes	*compacta*	Y	Y	Y		Y	Y	Y	Y		
Chaetophyes	*pulcherrima*	Y		Y		Y					
Chaetophyes	*vicina*	Y		Y		Y	Y	Y	Y	Y	
Hindoloides		**Y**		**Y**		**Y**					
Hindoloides	*appendiculata*	Y		Y		Y					
Machaerota		**Y**			**Y**	**Y**				**Y**	
Machaerota	*finitima*	Y				Y					
Machaerota	*pugionata*	Y			Y	Y				Y	
Pectinariophyes		**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	
Pectinariophyes	*reticulata*	Y							Y	Y	
Pectinariophyes	*stalii*	Y		Y		Y	Y	Y	Y	Y	
Polychaetophyes		**Y**	**Y**	**Y**	**Y**	**Y**				**Y**	
Polychaetophyes	*serpulidia*	Y		Y	Y	Y				Y	

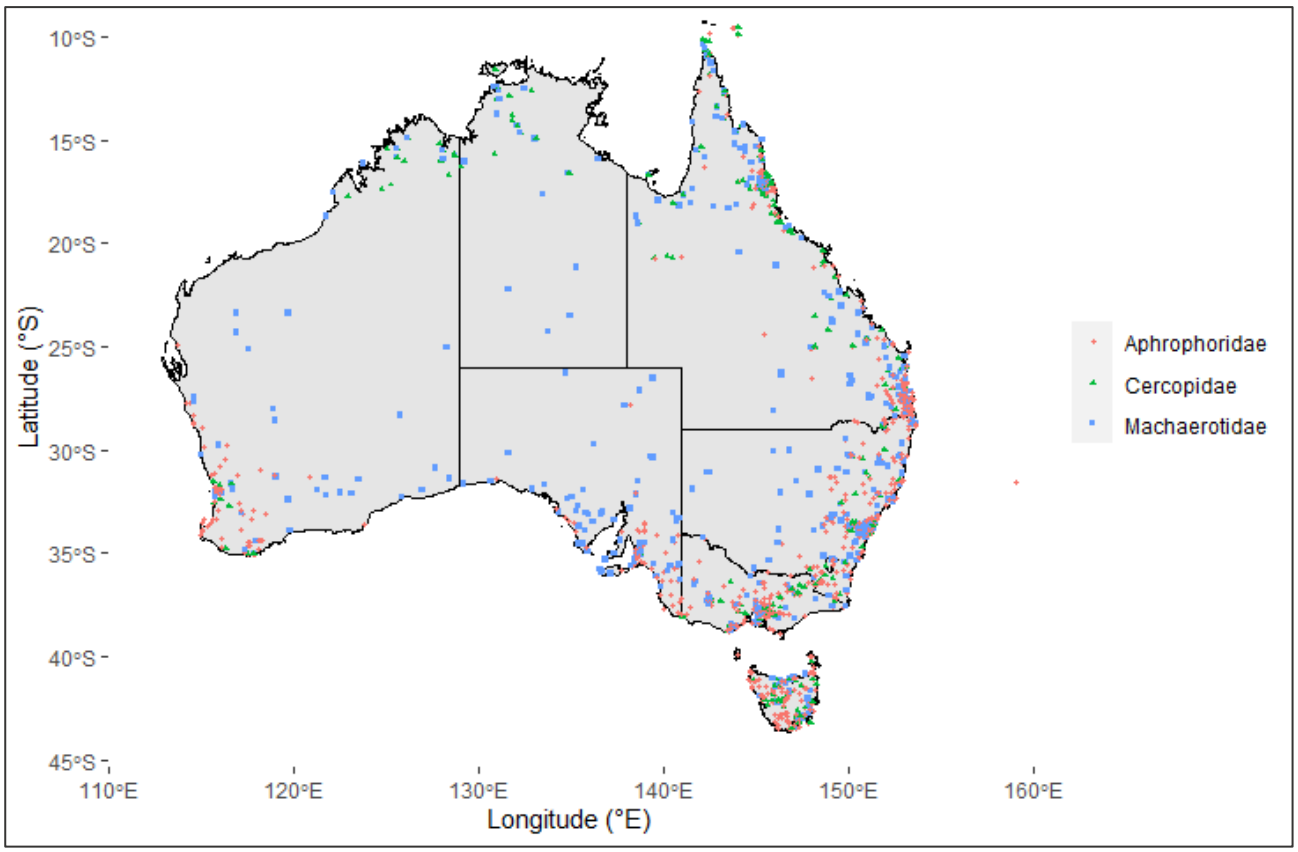


Figure 5. Collection locations of Aphrophoridae (red crosses), Cercopidae (green triangles) and Machaerotidae (blue squares) recorded across mainland Australia and the surrounding islands.

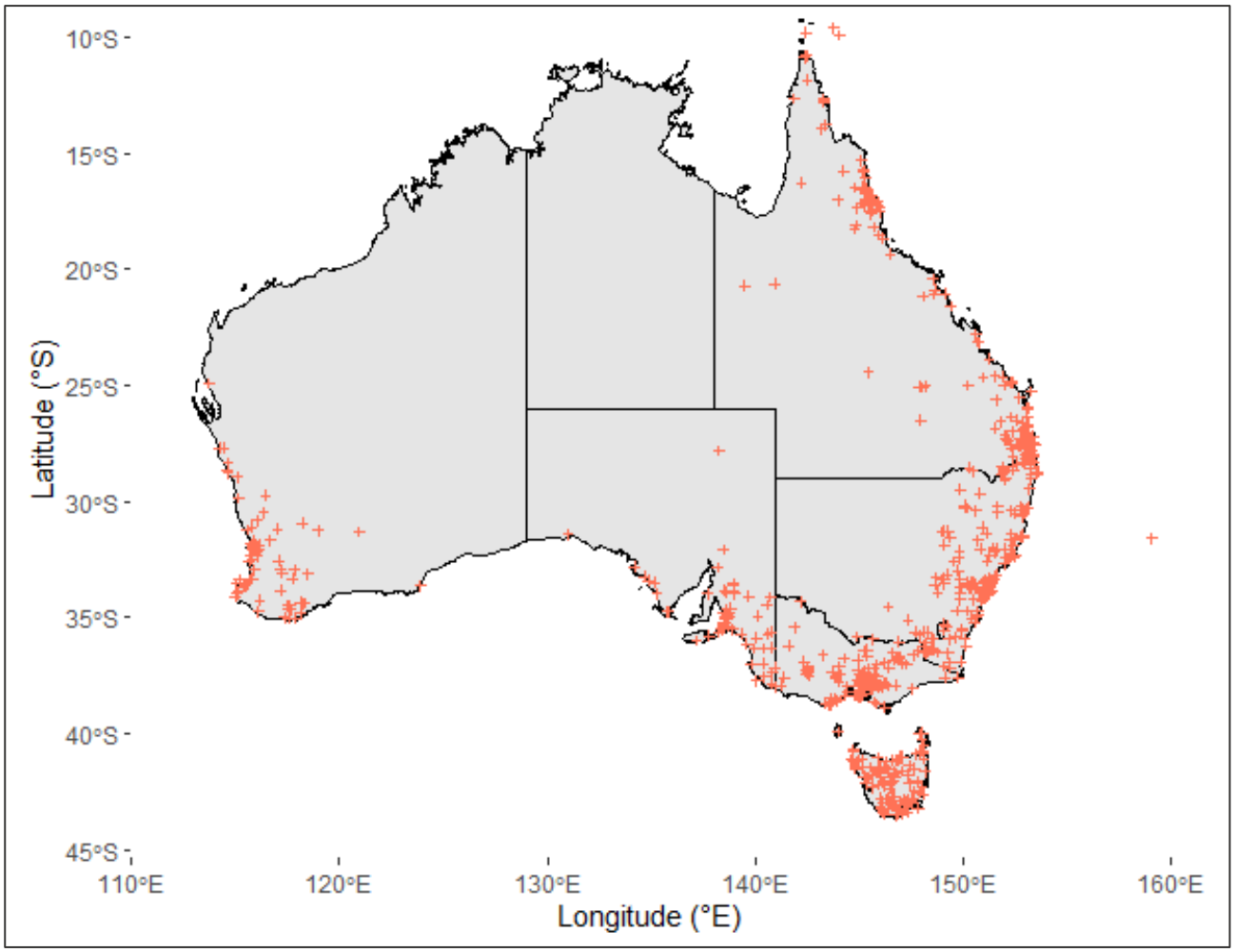


Figure 6. Collection locations of Aphrophoridae (red crosses) recorded across mainland Australia and the surrounding islands.

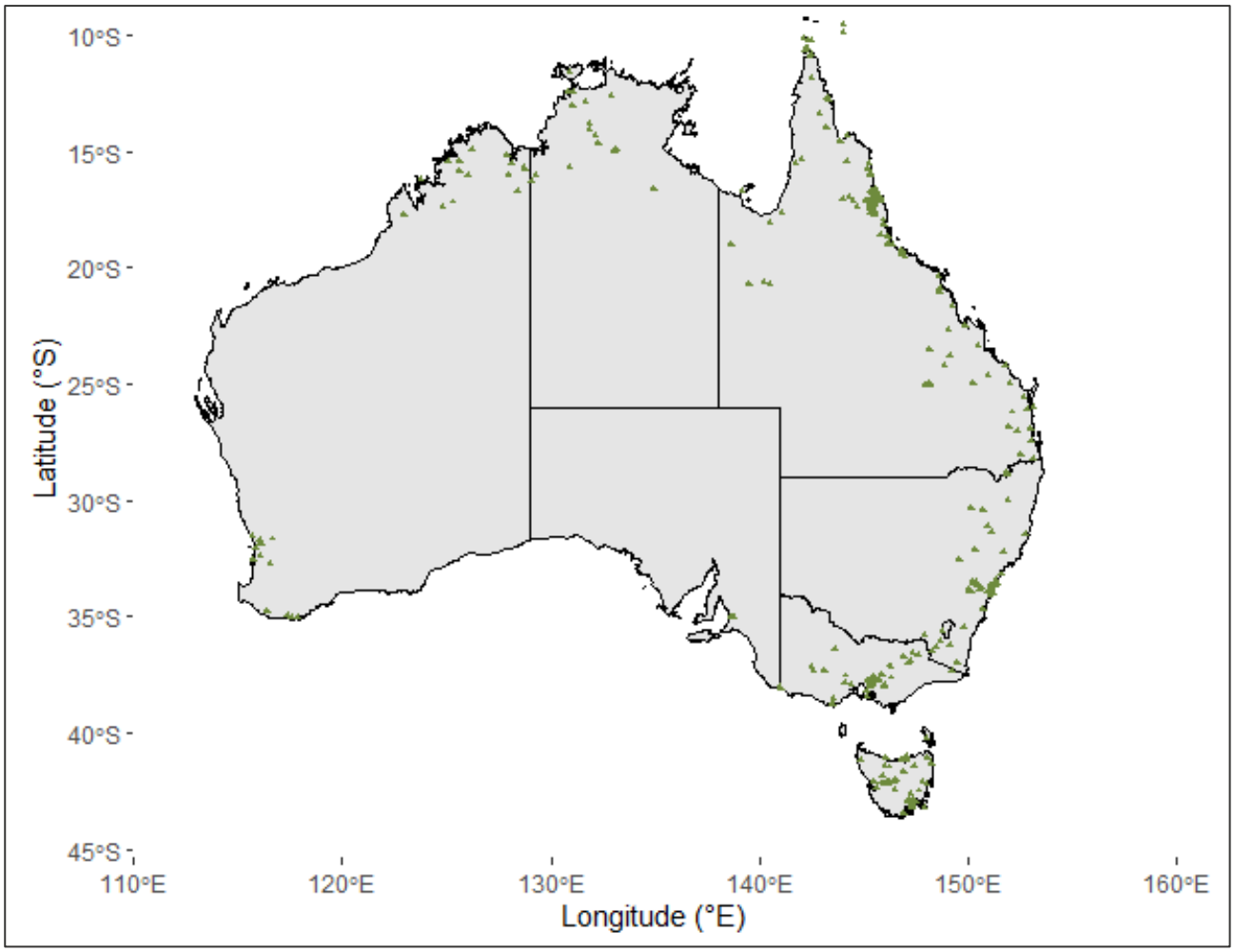


Figure 7. Collection locations of Cercopidae (green triangles) recorded across mainland Australia and the surrounding islands.

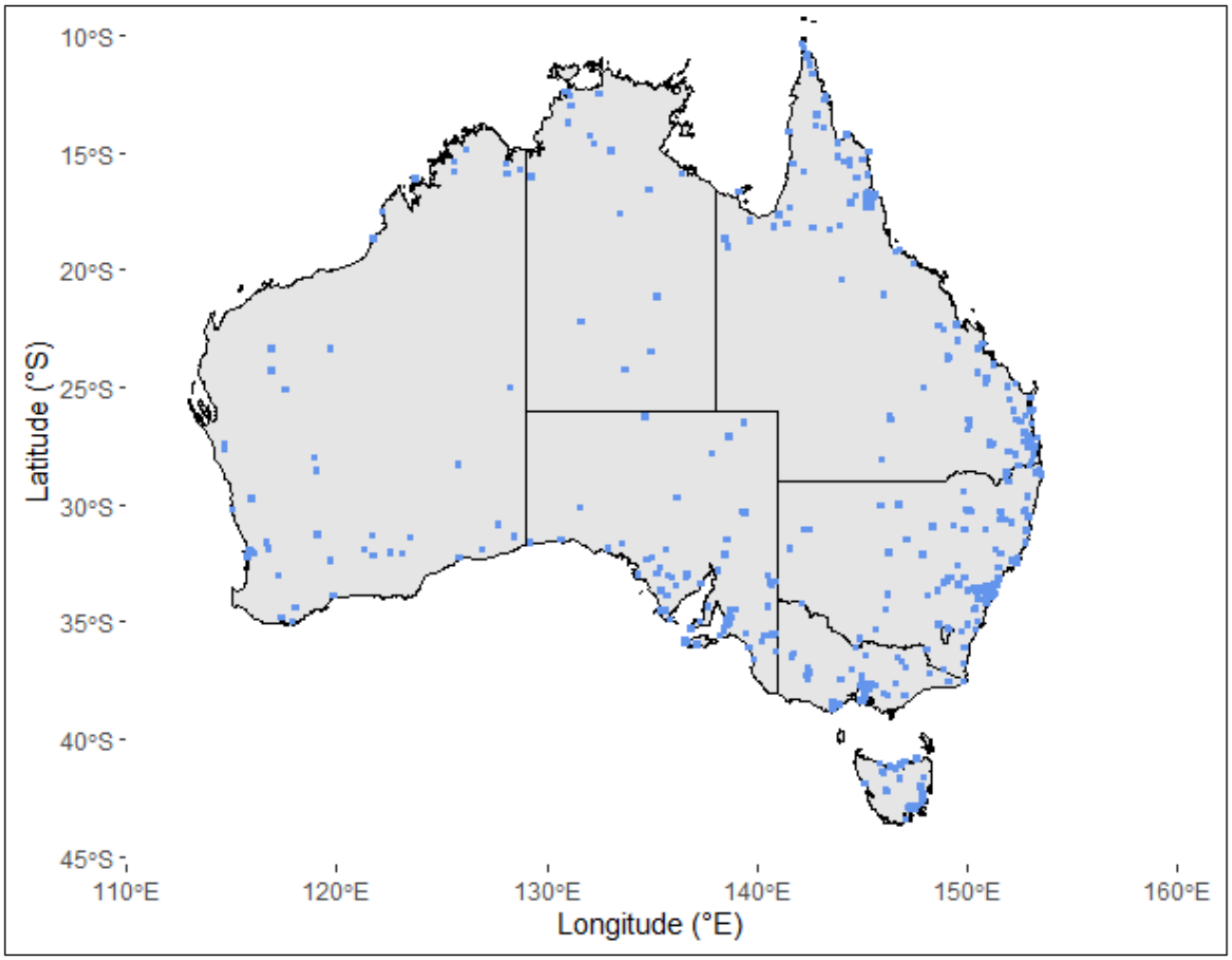


Figure 8. Collection locations of Machaerotidae (blue squares) recorded across mainland Australia and the surrounding islands.

Component 2: Methods of Insect Collection

Collection methods were available for 3208 of the 5555 analysed records (57.7%). Forty-eight different collection methods were recorded in the data, however typographical variation accounted for many of the differences. For example, there are 13 variations in wording on a collection method involving light, including “light”, “MV light”, “UV light”, “Blacklight”, “Campsite light” etc. As such, the 45 collection methods were grouped into 8 primary types of collection method (Table 4), for easier analysis. All 45 collection methods are shown in Appendix 2. Overall,

- Light trapping represented the most records in the collection data, with 93% of the Machaerotidae with collection method data collected using a light.
- Aphrophoridae were collected most often (36%) using ‘hand collecting’, while combined beating, sweeping, sieving, vacuum method, and light trapping accounted for another 26% of aphrophorid records.
- Cercopidae contained approximately half the number of records of the Aphrophoridae and Machaerotidae, with light trapping accounting for 65% of cercopid records.

As many records did not have the full complement of data accompanying the specimen, it is possible that light trapping was more often noted than other types of collecting; for instance, if an insect is hand collected on a plant, the plant information is generally recognised as more important, and the actual collection method may be omitted in favour of other details on the label. Thus, light trapping may be over-represented as a collection method. Notably, other types of insect traps account for <10% of cercopoid records (Table 4).

Table 4. Collection method for cercopoid records, separated by cercopoid family.

	Cercopoid family			
Collection method	**Aphrophoridae**	**Cercopidae**	**Machaerotidae**	**Grand Total**
Light	267	350	1228	1845
Hand	451	73	86	610
Beat/Sweep/Sieve/Vac	325	89	22	436
Flight trap	150	38	26	214
Ground trap	63	12	5	80
Pyrethrum	5	0	6	11
Reared	2	5	1	8
Trap	3	1	0	4
Total	1266	568	1374	3208

Component 3: Spatial and Temporal Insect Distribution

Overall, when considering all insects caught in Australia, Cercopoidea were collected most often during December (summer) and least often during July (winter), with Cercopoidea being broadly more abundant during spring and summer months, and less abundant during autumn and winter months (Figure 9). The five most abundant genera, *Anyllis*, *Bathyllus*, *Chaetophyes*, *Pectinariophyes*, and *Philagra* (Table 2), follow a broad trend of high numbers collected in spring and summer months, and low numbers over the winter months (Figure 9, Appendix 1).

Spatially, Cercopoidea were most abundantly collected in QLD (1710 records, 30.7% of total), and least often in the ACT (46 records, 0.8% of total) (Figure 10). However, considering the data by cercopoid family, Cercopidae was the only family with most collections in QLD, while both Aphrophoridae and Machaerotidae were most often collected in NSW, perhaps as a result of the NSW OAI collection containing the most cercopoid specimens.

Records in each state and territory display different temporal trends, which match the dominant climatic region in the area (Figure 11). Vic, Tas, SA and ACT each contain fewer than 10 cercopoid records for the winter months (June, July, August), suggesting it is too cold for adult spittlebugs at these times. In contrast, QLD, NSW and WA retain higher records numbers in the winter months, with QLD records not dipping below 50 at any point in the year. These two distinct patterns match temperate and tropical climatic trends, respectively. NT, a tropical region, has fewer than 10 records in the winter months; however, NT records are low in general and only rise above 20 records in September and October.

However, some genera were collected in higher abundance during Autumn and Winter than in spring and summer. *Megastethodon*, for example, was only recorded in April, May and July (Figure 12). This inverse seasonal abundance could potentially be due to the relatively low number of insects (< 50 individuals) recorded for most genera (Table 2), which may not represent true population dynamics. Indeed, multiple genera have at least one major peak in autumn or winter (*Amarusa*, *Aufidus*, *Aufiterna*, *Basilioterpa*, *Clovia, Euryaulax*, *Interocrea*, *Machaerota*, *Megastethodon*, *Polychaetophyes*) and are almost exclusively found in the northern parts of Australia (Appendix 1). Three specimen record exceptions to this pattern include, one record for *Polychaetophyes* from the temperate ACT is possibly a misidentification; and the two *Euryaulax* records from temperate Tasmania, which may be intercepted specimens as there are no more records of *Euryaulax* in Tasmania after these two 1927 specimens. A wet/dry season explanation is more fitting for these seemingly subtropical/tropical genera.

Variation in seasonal abundance was observed between some state-based populations of the same genus. This is particularly evident for the five most collected genera: *Anyllis*, *Bathyllus*, *Chaetophyes*, *Pectinariophyes*, and *Philagra* (Appendix 1). For example, *Philagra* records showing a strong summer peak in Vic and Tas, both temperate states, while multiple peaks occur in summer, autumn and winter/spring for the more tropical states of QLD and NSW. Overall, *Philagra* record numbers never dipped below 10 per month and remained above 35 records per month in all seasons outside winter (Figure 13).

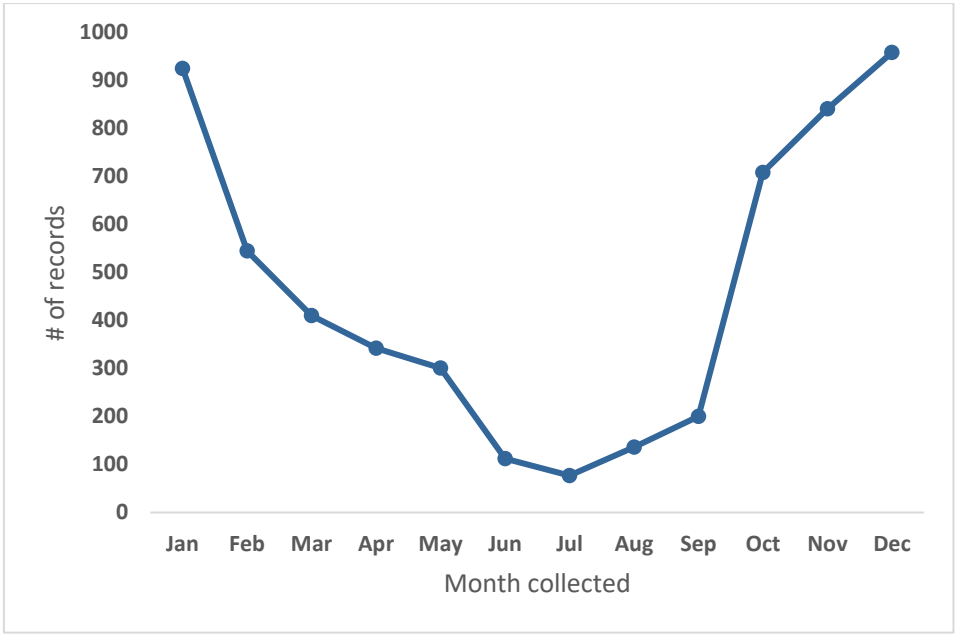


Figure 9. Count of adult cercopoid records for each month. All records included in the analysis contained information on month of collection.

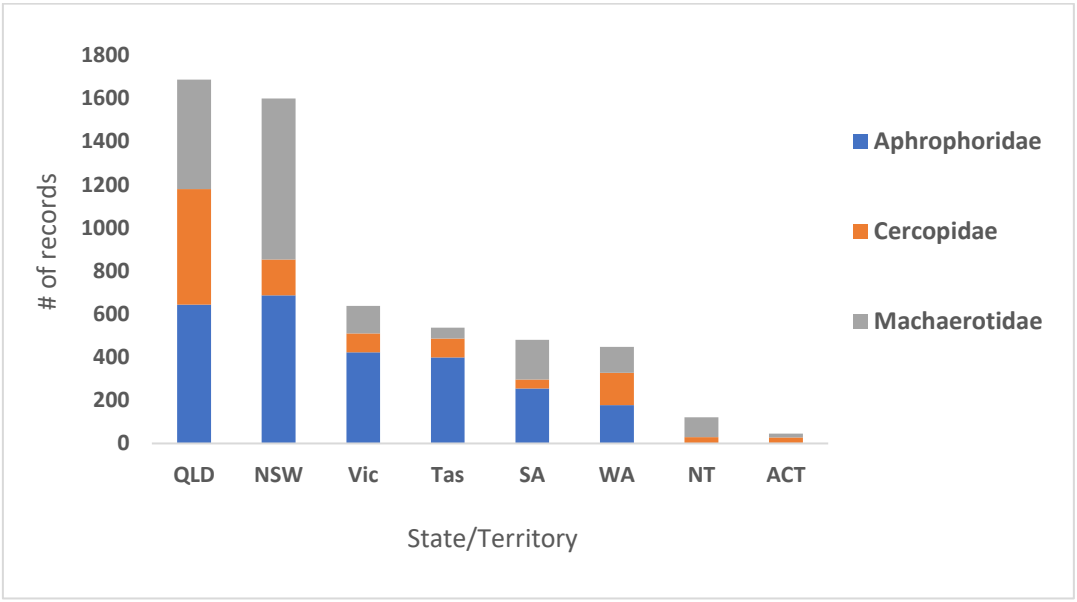


Figure 10. Count of adult records from each Australian state/territory, colour-coded by cercopoid family.



Figure 11. Total count of cercopoid records per month in each Australian state/territory.

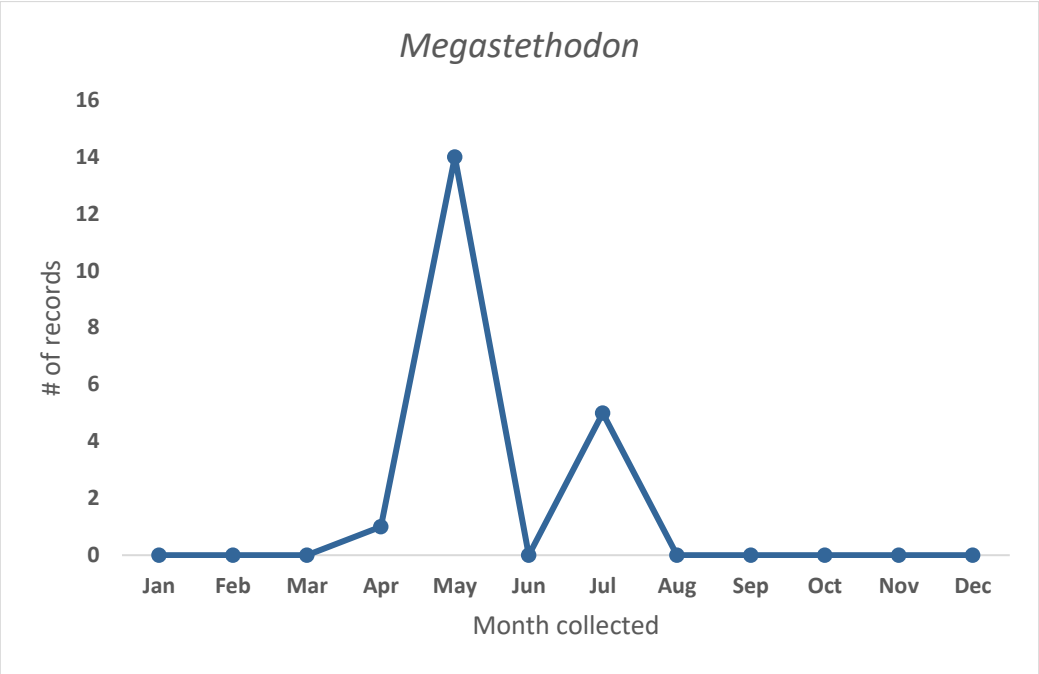


Figure 12. Count of *Megastethodon* records per month across Australia.

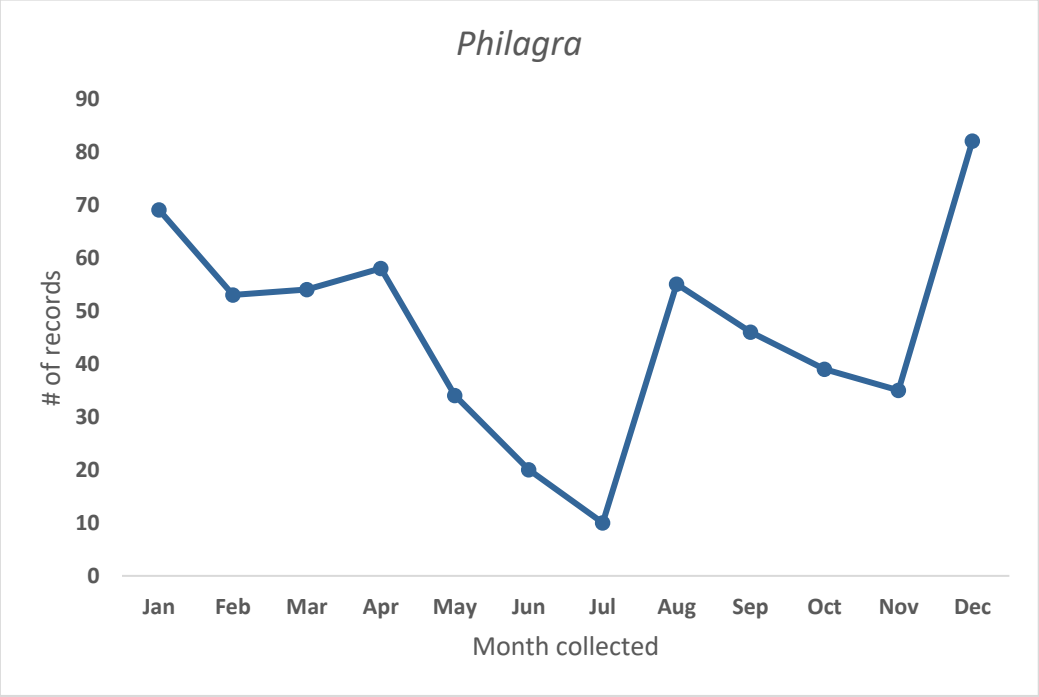


Figure 13. Count of *Philagra* records from each month across Australia.

Component 4: Associated Plants Among Insect Genera

An associated plant was recorded for 13% of the 5555 cercopoid records, from 12 of the 19 genera across the three cercopoid families. These 719 insect records with associated plant data coveried 47 plant families (Appendix 3), 603 records included a plant genus and insect genus, thus were able to be visualised using a Sankey diagram (Table 5; Figures 14, 15, 16). There were 472, 49 and 82 records each for Aphrophoridae, Cercopidae and Machaerotidae, respectively. All three cercopoid families contained genera which were associated with more than one plant family. Only one insect genus was recorded to be monophagous on a single plant genus: *Interocrea* (Aphrophoridae), recorded only on *Eucalyptus* (Myrtaceae).

The reference collection records examined here were from adult cercopoids (>99%), and provide an indication of which plants these adults might be utilising. As such, it is important to note that the plant an insect was collected on it not necessarily a host plant for that insect. Particularly for Cercopoidea, which jump in seemingly random directions when disturbed, adult specimens recorded on a plant may simply be resting, hiding or in transit to a true host plant. True host status can be confirmed by rearing an insect from egg to adult on one plant. Feeding behaviour for Cercopoidea can be confirmed by witnessing spittle on a suspected host plant, or by the presence of calcareous tubes in the case of Australian Machaerotidae.

The plant families with which most records were associated are: Myrtaceae, Fabaceae, and Poaceae (Table 5), representing 35%, 24% and 6%, respectively of the total 603 records. Myrtaceae is dominated by *Eucalyptus*-associated records, and Fabaceae by *Acacia*-associated records. These genera might be over-represented in the data because they are easily recognisable to amateur collectors, or collectors with little knowledge of botany.

Further information on the plants associated with cercopoid records can be found in Appendix 3.

Table 5. Plant families associated with cercopoid records. Only records which contained plant and insect genus information are included.

Plant family	# of records
Myrtaceae	210
Fabaceae	147
Poaceae	38
Casuarinaceae	25
Proteaceae	24
Solanaceae	24
Asteraceae	20
Ericaceae	11
Oleaceae	11
Malvaceae	9
Cyperaceae	8
Hypericaceae	6
Combretaceae	5
Pinaceae	5
Rutaceae	5
Asphodelaceae	4
Dioscoreaceae	4
Rosaceae	4
Lamiaceae	3
Moraceae	3
Verbenaceae	3
Vitaceae	3
Anacardiaceae	2

Plant family	# of records
Asparagaceae	2
Boraginaceae	2
Fagaceae	2
Gleicheniaceae	2
Iridaceae	2
Phyllanthaceae	2
Santalaceae	2
Alstroemeriaceae	1
Atherospermataceae	1
Convolvulaceae	1
Cunoniaceae	1
Cupressaceae	1
Euphorbiaceae	1
Juncaceae	1
Loganiaceae	1
Meliaceae	1
Nothofagaceae	1
Orchidaceae	1
Plantaginaceae	1
Polygonaceae	1
Sapindaceae	1
Thymelaeaceae	1
Grand Total	603

Aphrophoridae

Aphrophoridae seems to have the most polyphagous Australian Cercopoidea, with five insect genera recorded in association with 73 plant genera across 38 plant families (Figure 14). Within Aphrophoridae, *Bathyllus* was the most polyphagous genus and was recorded on 44 plant genera across 27 families.

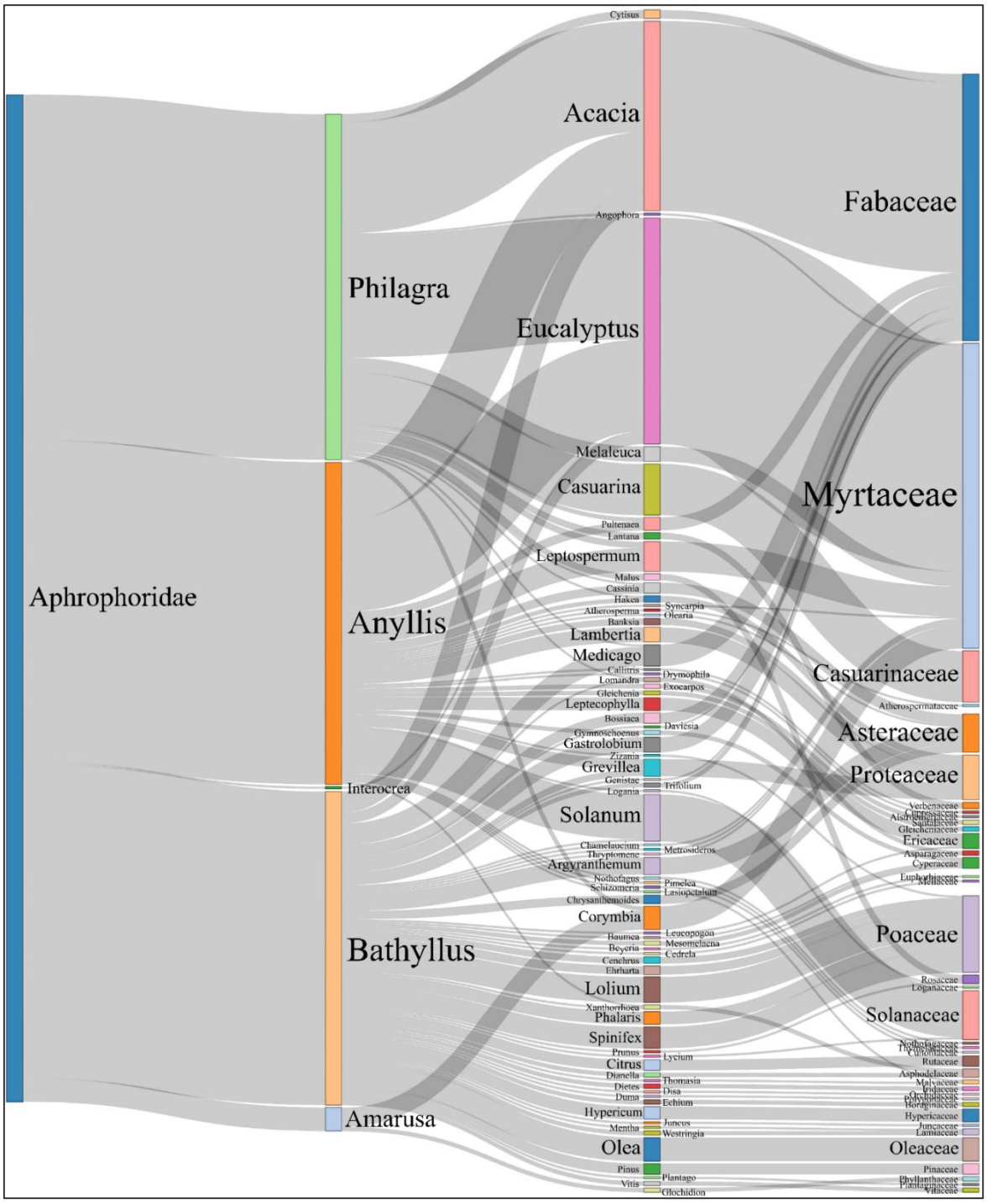


Figure 14. Plant genera associated with genera of the Aphrophoridae from 472 insect records. The thickness of grey lines is relative to the proportion of records flowing from (left to right): insect family to insect genus, insect genus to plant genus, and plant genus to plant family.

Cercopidae

Cercopidae had four insect genera recorded to feed on 20 plant genera across 12 plant families (Figure 15). Multiple plant genera, however, were only recorded in association with one cercopid genus. For example, the plant genera *Cochliasanthus* (Fabaceae), *Dioscorea* (Dioscoreaceae), *Ficus* (Moraceae), and several others were only recorded as host plants for *Euryaulax*.

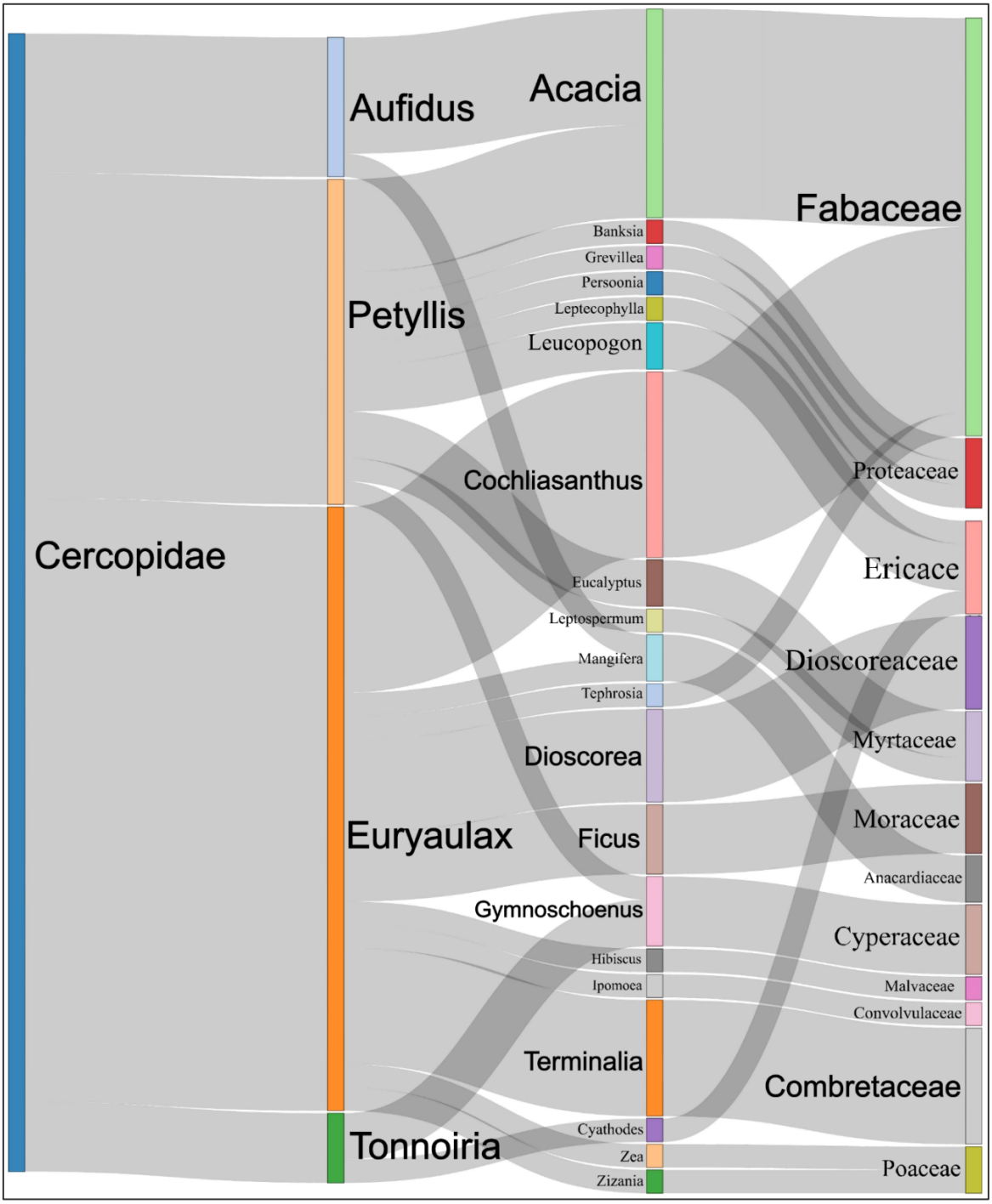


Figure 15. Plant genera associated with genera of the Cercopidae from 49 records. Connecting bar width is relative to the proportion of records flowing from (left to right): insect family to insect genus, insect genus to plant genus, and plant genus to plant family.

Machaerotidae

Machaerotidae was the least polyphagous subfamily, with three insect genera recorded to feed on 12 plant genera across nine plant families (Figure 16). *Chaetophyes* and *Pectinariophyes* were most often recorded in association with *Eucalyptus* (Myrtaceae), but were also recorded from distantly related plant families Casuarinaceae, Vitaceae, and Fabaceae. *Machaerota* has one record on *Sapindus* (Sapindaceae), while seven records are associated with *Sida* (Malvaceae).

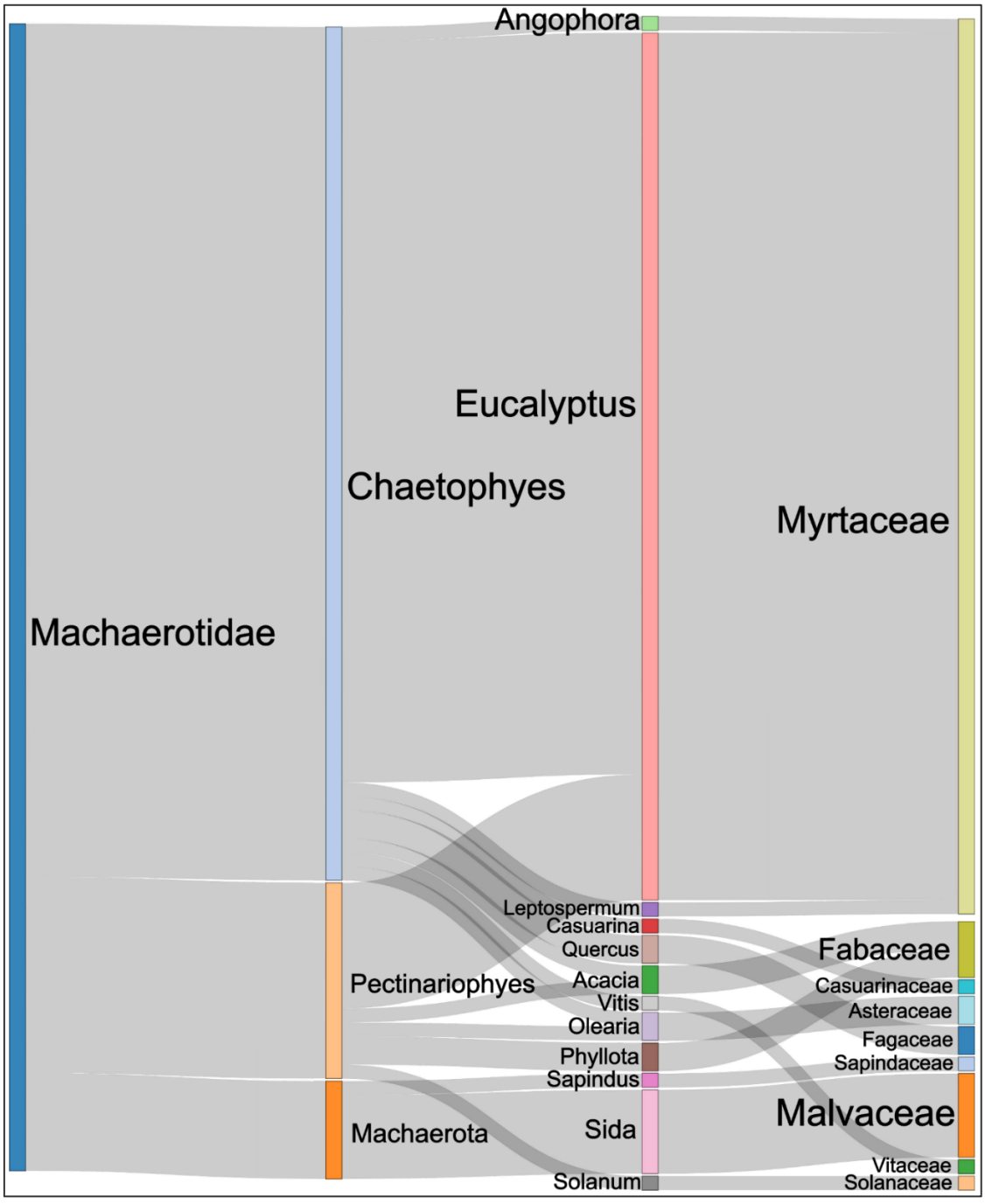


Figure 16. Plant genera associated with genera of the Machaerotidae from 82 records. Connecting bar width is relative to the proportion of records flowing from (left to right): insect family to insect genus, insect genus to plant genus, and plant genus to plant family.

Recommendations

Outcomes

1. The majority of cercopoid genera each accounted for less than 3% of the total number of Cercopoidea recorded. The five most abundant genera, in contrast, each represented over 10% of the cercopoid records. The five most represented genera of Cercopoidea were: *Anyllis*, *Pectinariophyes*, *Bathyllus*, *Chaetophyes* and *Philagra*. *Chaetophyes* and *Pectinariophyes*, both in family Machaerotidae, were also the most widespread genera, being found in all eight of the main Australian states and territories. As the five most widespread and most often caught Cercopoidea, found on the ubiquitous *Eucalyptus*, *Acacia*, and even *Solanum* plants, these genera should be considered for future *Xylella* vector studies.

2. We ascertained that the effectiveness of common collection methods varied between cercopoid taxa and should be considered when targeting surveillance efforts toward specific genera. The importance of using an effective collection/surveillance method is also demonstrated in the surveillance trapping Technical Report (cite). Aphrophoridae were most often recorded with active search methods such as beating, sweeping, sieving, vacuuming, and hand collecting. However, Aphrophoridae were also collected with light trapping at a high rate, and light trapping was by-far the most commonly recorded collection method for both Cercopidae and Machaerotidae. This information should prove especially useful for biosecurity officers when planning field collecting for potential *Xylella* vectors.

3. Cercopoid genera varied greatly in distribution throughout Australia and in seasonal presence. For each state, the season of first collection and peak collection month is relatively similar between genera (Appendix 1). Each cercopoid family was recorded in all eight main Australian states and territories, with the exception of Aphrophoridae in the Northern Territory. The five most represented genera were each collected in at least six states/territories. Cercopids, *Petyllis* and *Tonnoiria,* are notable in that although the number of records is relatively low, these genera were collected in seven and six of the Australian states/territories, respectively.

4. Eleven cercopoid genera were associated with more than one plant family (Appendix 3). The aphrophorid genera were found on the widest variety of plants, with *Bathyllus* found on 44 plant genera across 27 plant families. However, *Interocrea*, also in Aphrophoridae, was found on only one plant genus. The machaerotid genera were recorded on the fewest plant families (nine plant families). This suggests that Aphrophoridae have the highest potential to transmit *Xylella fastidiosa* to a wide range of plant families, including agriculturally relevant plants (grasses, olives, pine trees, citrus, grapes etc., see Appendix 3).

Conclusion

Future Directions

This research highlighted several key questions for future research:

1. Which environmental factors influence the distribution and development of more and less commonly collected Cercopoidea?

Based on the historical collection data, some genera appear to have wide environmental tolerances while others are more restricted. When teasing the data apart further into species-level trends, it becomes clear that certain genus-level distribution trends are driven by one common species which masks the trends of other congeneric species; for example, *Philagra parva* is distributed over six states, while the other three described species have only been collected in NSW and QLD. Reassuringly, however, genus-level trends did not significantly change as more data was added; the trends present when analysing ~3,500 records (i.e., earlier analyses, not shown) were solidified, not altered, when analysing the complete ~5,600 records.

Additionally, state/territory of collection was a good predictor of peak collection period, suggesting cercopoid abundance varies with geographic region and, by extension, climate conditions. Future work could focus on key climatic measurements that can predict areas in which Australian Cercopoidea are most likely to develop and thrive. This will greatly assist the management of *X. fastidiosa* if it arrives, notably predicting vectors, locations, and seasons suitable for transmitting *X. fastidiosa*.

2. Do cercopoid genera have preferred host plants among recorded associated plants? Are all associated plants viable hosts?

As the genus with most associated plant families recorded, *Bathyllus* is a good candidate to test questions regarding viable host plants. The genus *Bathyllus* is monotypic, with *Bathyllus albicinctus* the only described species. This species has been collected on 27 different plant families, and has been seen to feed on most of these plant families (including the gymnosperm *Pinus radiata*). However, it is unknown whether nymphs will feed and produce spittle on plants that are not viable food sources in the long-term. No-choice and choice feeding experiments could be conducted to confirm host plant use of this seemingly polyphagous insect.

3. Does the seasonal phenology or development of Cercopoidea differ with host plant?

Many insects have different developmental times depending on their food source. As xylem-feeders with nutritional requirements, it is likely that cercopoid nymphs will develop more quickly on optimal plants versus sub-optimal plants. The ways in which a plant is “optimal” or “sub-optimal”, however, are not yet known for Australian Cercopoidea, and may be different for different taxa. Physical toughness of plant stems, nutritional composition of xylem fluid, daily and seasonal xylem pressure changes, and plant defences are just a small number of variables that may affect nymphal development, which could be further explored.

4. Can Australian spittlebugs transmit *Xylella fastidiosa* in practice?

While *Xylella fastidiosa* transmission by native cercopoid vector insects in case of a *Xylella* incursion seems probable considering the polyphagous nature of many Australian spittlebugs and the apparent lack of host plant specificity of *X. fastidiosa*, this should be tested to confirm the hypothesis, and assess if transmission is similar to that of European spittlebugs which are known vectors. The most feasible option is to develop colony-maintenance protocols for Australian spittlebugs and send live insects overseas (e.g., Europe) for *Xylella*-transmission tests.

Mechanically, feeding in at least one Australian spittlebug species appears to be very similar to feeding in known European vectors. Preliminary feeding experiments involving the Electrical Penetration Graph (EPG) technique have discovered that the egestion feeding period, in which *Xylella fastidiosa* is transmitted by European spittlebugs, lasts longer in *B. albicinctus* adults than in European spittlebugs. This experiment is described and discussed in the *B. albicinctus* feeding behaviour Technical Report (Jaroslow et al 2024). *B. albicinctus* have high vectoring potential based on life history traits (e.g., wide geographic distribution, broad host range, high abundance in urban and natural areas alike), and this insect could therefore act as a ‘super-spreader’ of *X. fastidiosa* due to feeding behaviour. Considering these factors combined, there is a very high likelihood that *B. albicinctus* is a comparable, if not superior, vector to *P. spumarius*. As such, investigating the population dynamics and actual vectoring potential of *B. albicinctus* would greatly improve Australia’s preparedness for a *X. fastidiosa* incursion.

New Zealand is an optimal place to compare known *Xylella* vectors with potential vectors. Both the Australian *Bathyllus albicinctus*, and the European *Philaenus spumarius*, are currently present in New Zealand. As the most effective European vector of *Xylella fastidiosa*, *P. spumarius* is a good yardstick against which to measure *B. albicinctus* as a potential vector. This represents a unique opportunity to develop collaborative relationships with our New Zealand neighbours, to explore this further.

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Jaroslow, D., Rako, L., Nancarrow, N., Trebicki, P., Selleck, C., Martoni, F., & Blacket, M. (2024). Host plant preferences and survival of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa*. In *Tools and knowledge to mitigate the potential spread of* Xylella fastidiosa *(Xylella) in Australia and New Zealand by understanding its potential vectors*. Project Technical Report, Agriculture Victoria.

Martoni, Francecso, Lea Rako, Duncan Jaroslow, Cait Selleck, Pragya Kant, Jess Hoskins, & Mark Blacket (2024). Biodiversity and COI barcoding of the Australian spittlebugs (Hemiptera: Cercopoidea. In *Tools and knowledge to mitigate the potential spread of* Xylella fastidiosa *(Xylella) in Australia and New Zealand by understanding its potential vectors*. Project Technical Report, Agriculture Victoria.

R Core Team (2024) R: A language and environment for statistical computing. R Foundation for 6504 Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org/.

Appendices

Appendix 1: Phenological trends for each genus

Aphrophoridae:

Amarusa

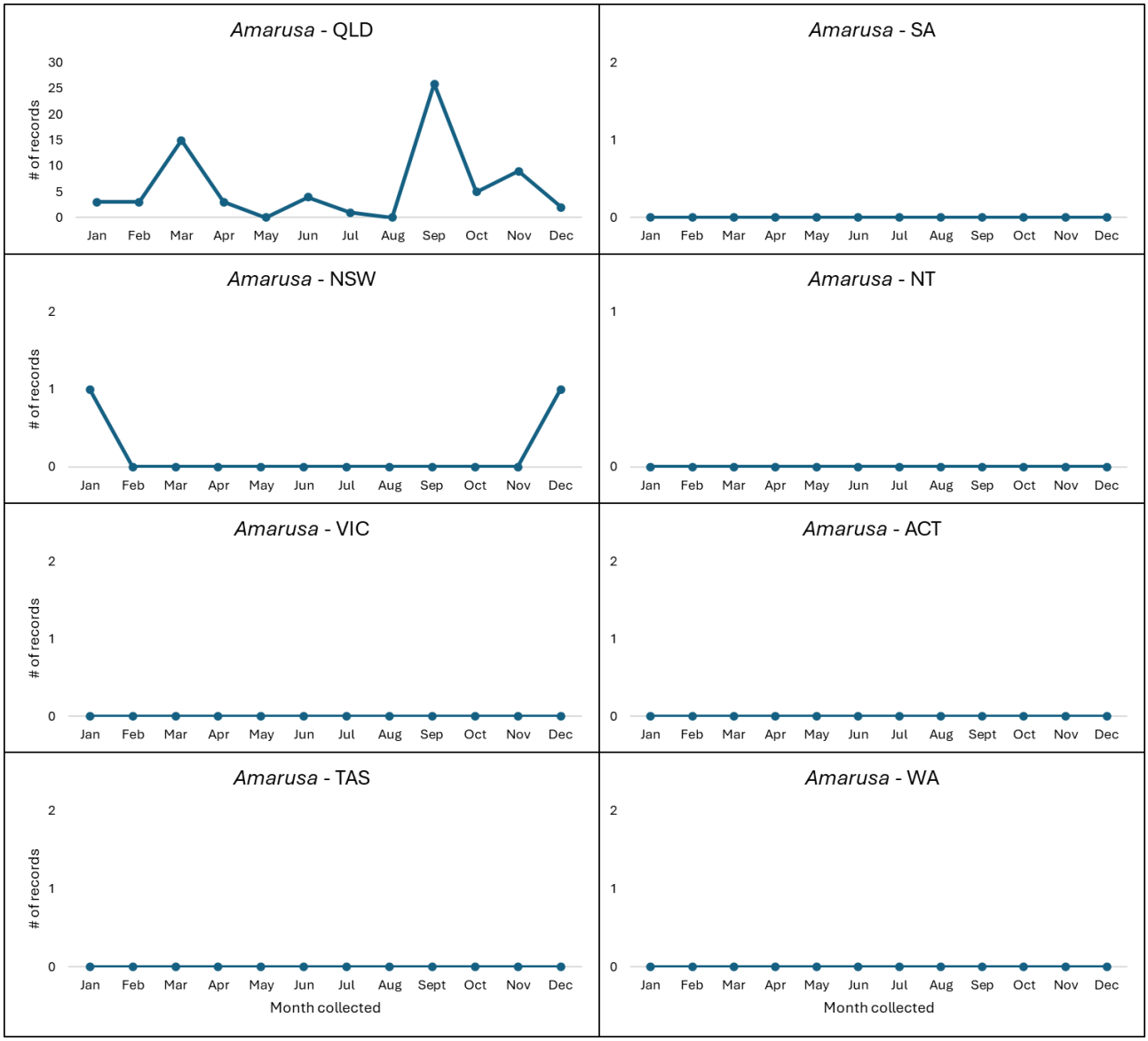


Figure A1.1. Count of *Amarusa* records per calendar month for each Australian state and mainland territory.

Anyllis

Anyllis (Figure A1.2) in QLD were collected in abundance from August until the end of December, while *Anyllis* in NSW were only abundant between December and April, and *Anyllis* in VIC were most abundant in November, January, and February. In TAS, *Anyllis* were generally found during January and February, and their occurrence in SA was virtually restricted to February and March. No *Anyllis* were collected from the NT or ACT, and only a single *Anyllis* was collected in April from WA.

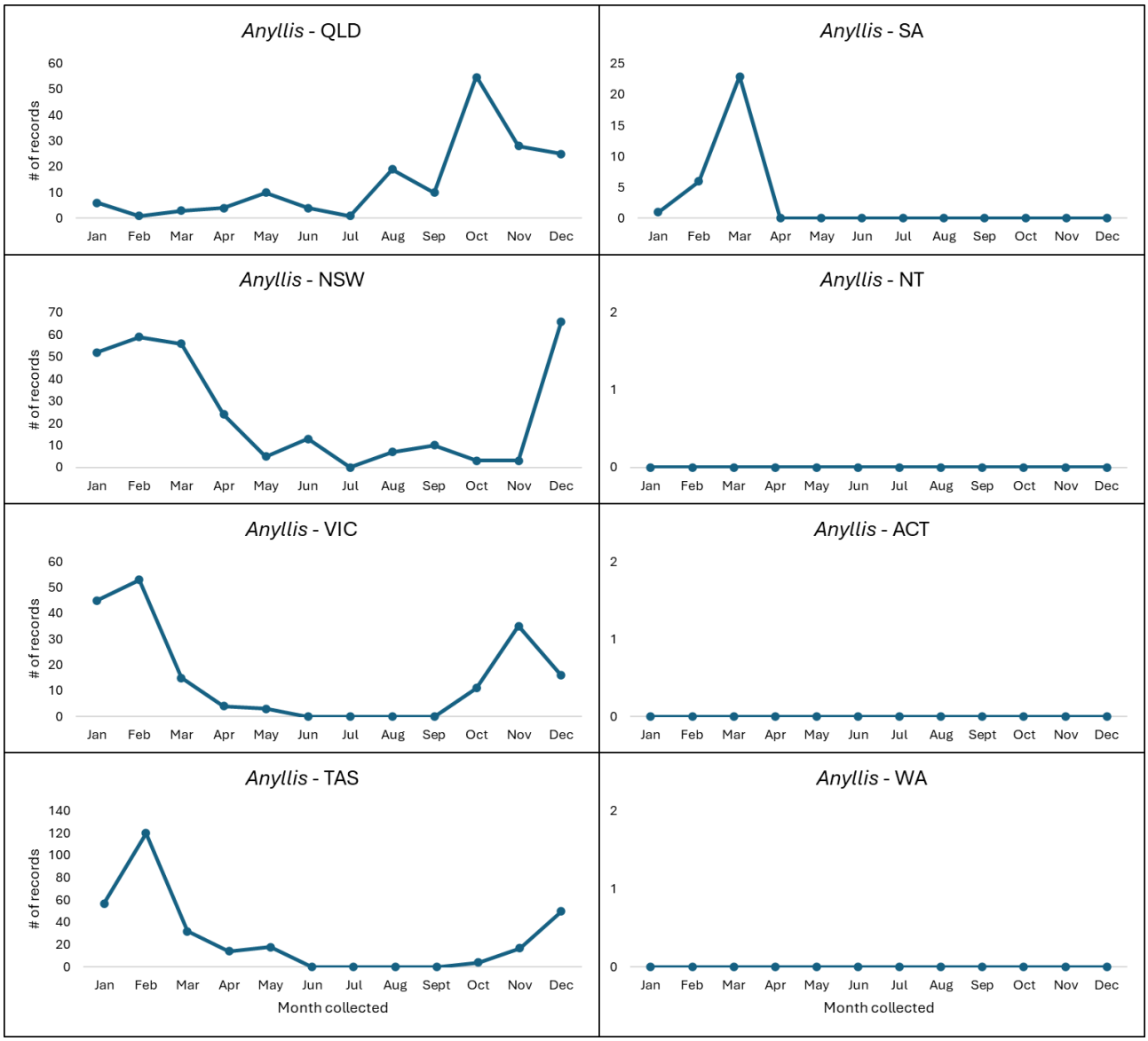


Figure A1.2. Count of *Anyllis* records per calendar month for each Australian state and mainland territory.

Basilioterpa

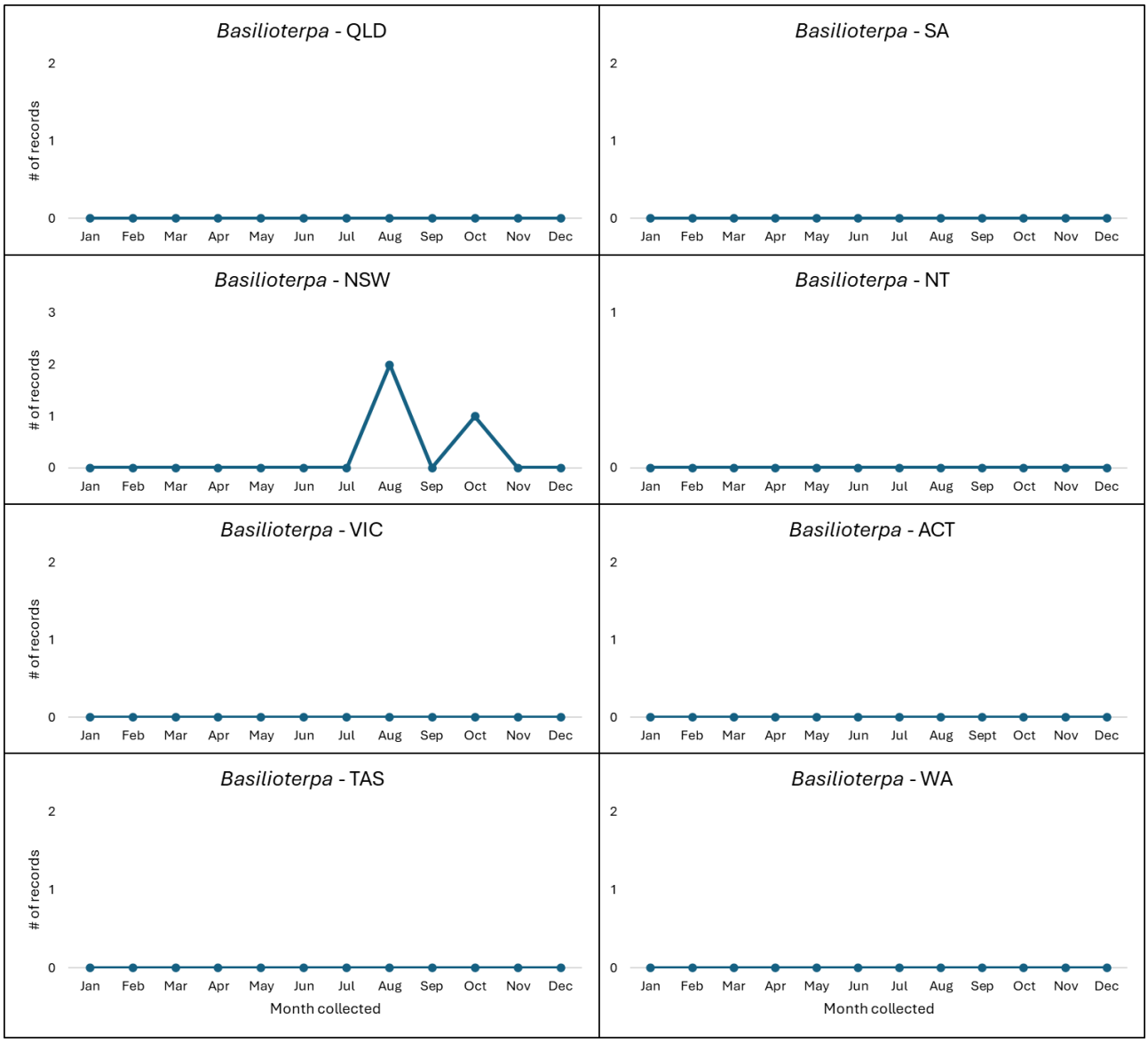


Figure A1.3. Count of *Basilioterpa* records per calendar month for each Australian state and mainland territory.

Bathyllus

Where it was present, *Bathyllus* abundance was generally highest in the spring and summer months (Figure A1.4). In QLD, *Bathyllus* was only collected in February, October, and November. For NSW and VIC, *Bathyllus* was abundant from October to February. For WA and SA, *Bathyllus* was more common in November and October. In TAS, other than one *Bathyllus* collected in November, they were only collected in January and February. *Bathyllus* were not found in the NT or ACT.

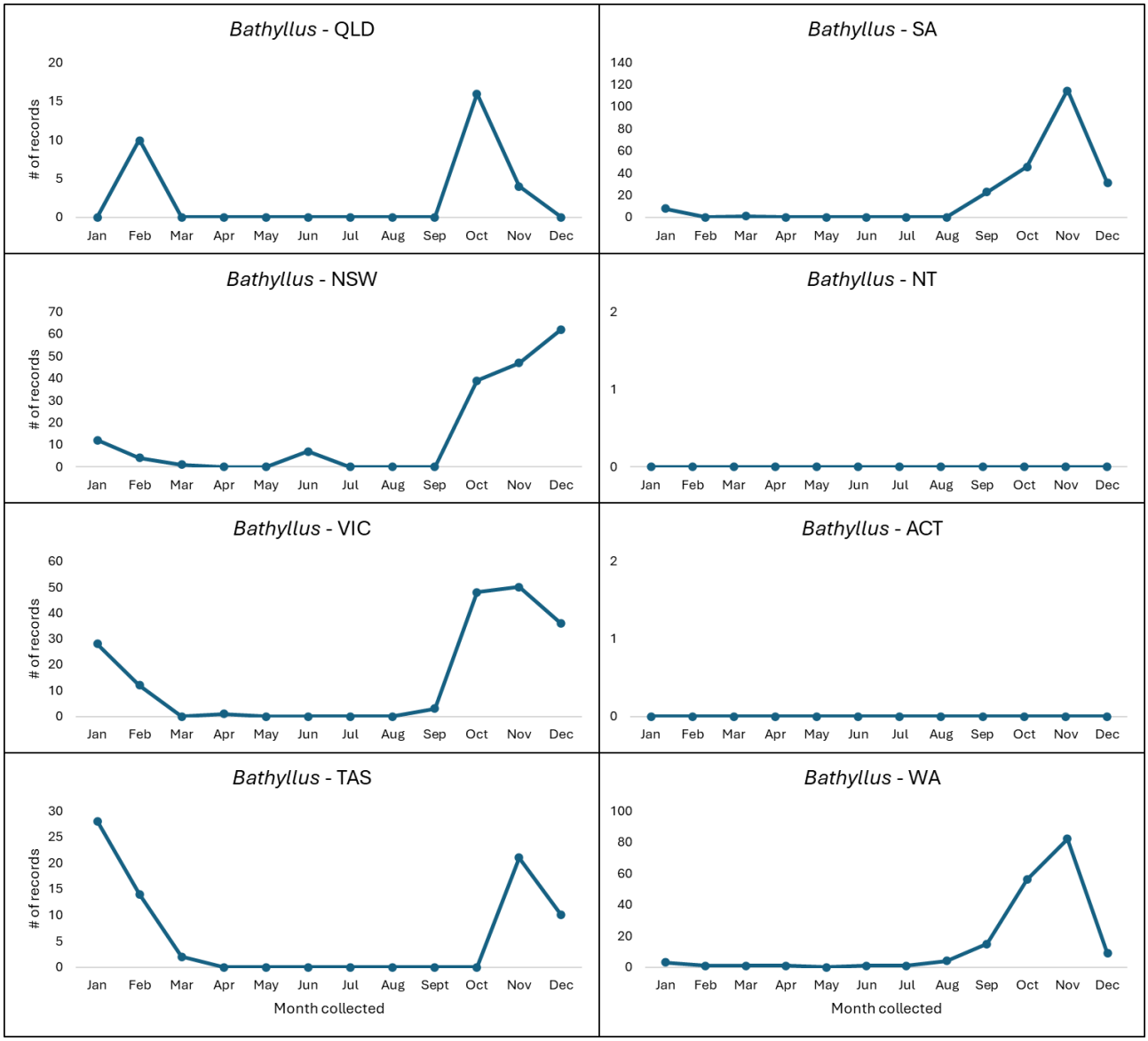


Figure A1.4. Count of *Bathyllus* records per calendar month for each Australian state and mainland territory.

Clovia

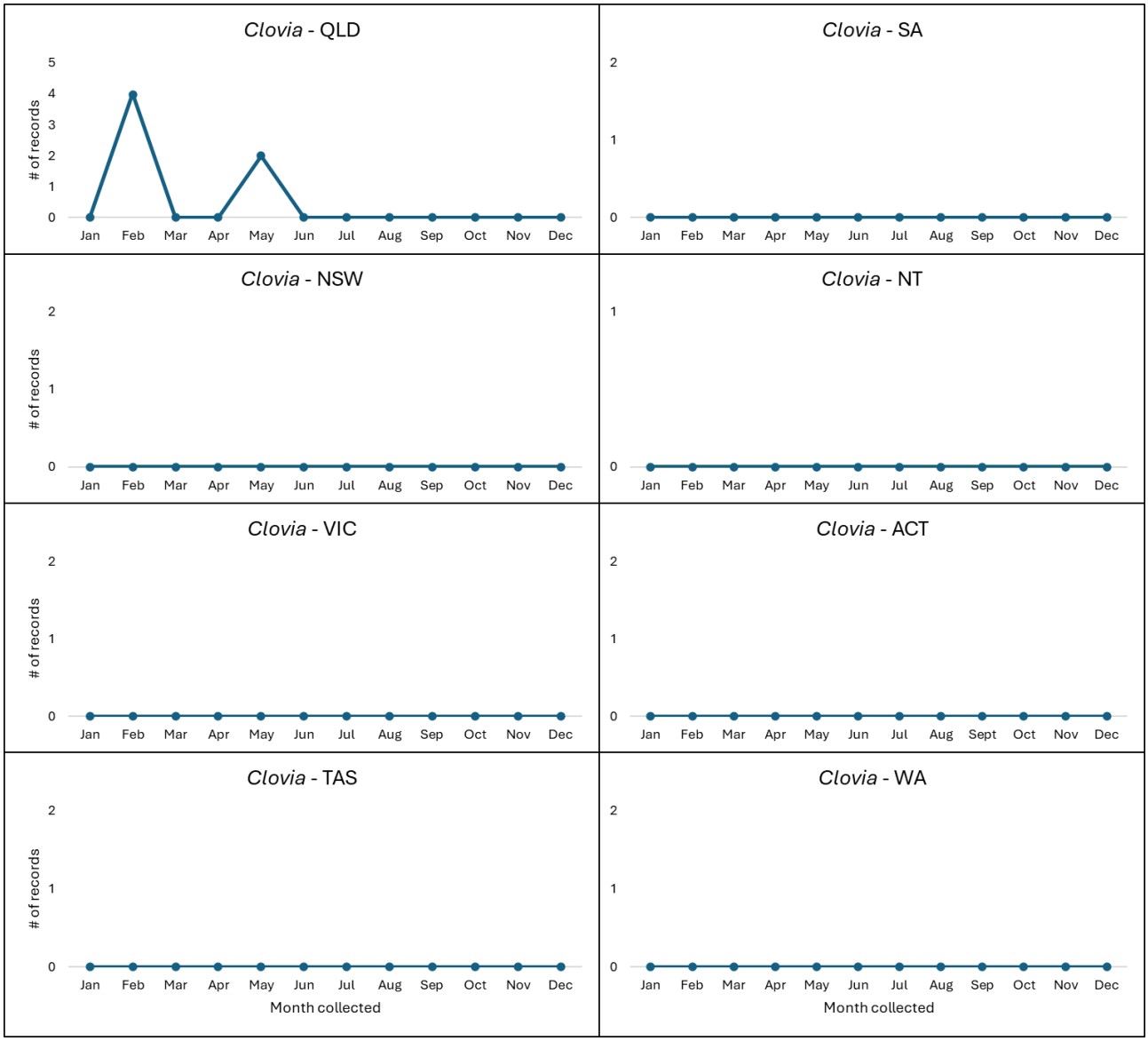


Figure A1.5. Count of *Clovia* records per calendar month for each Australian state and mainland territory.

Interocrea

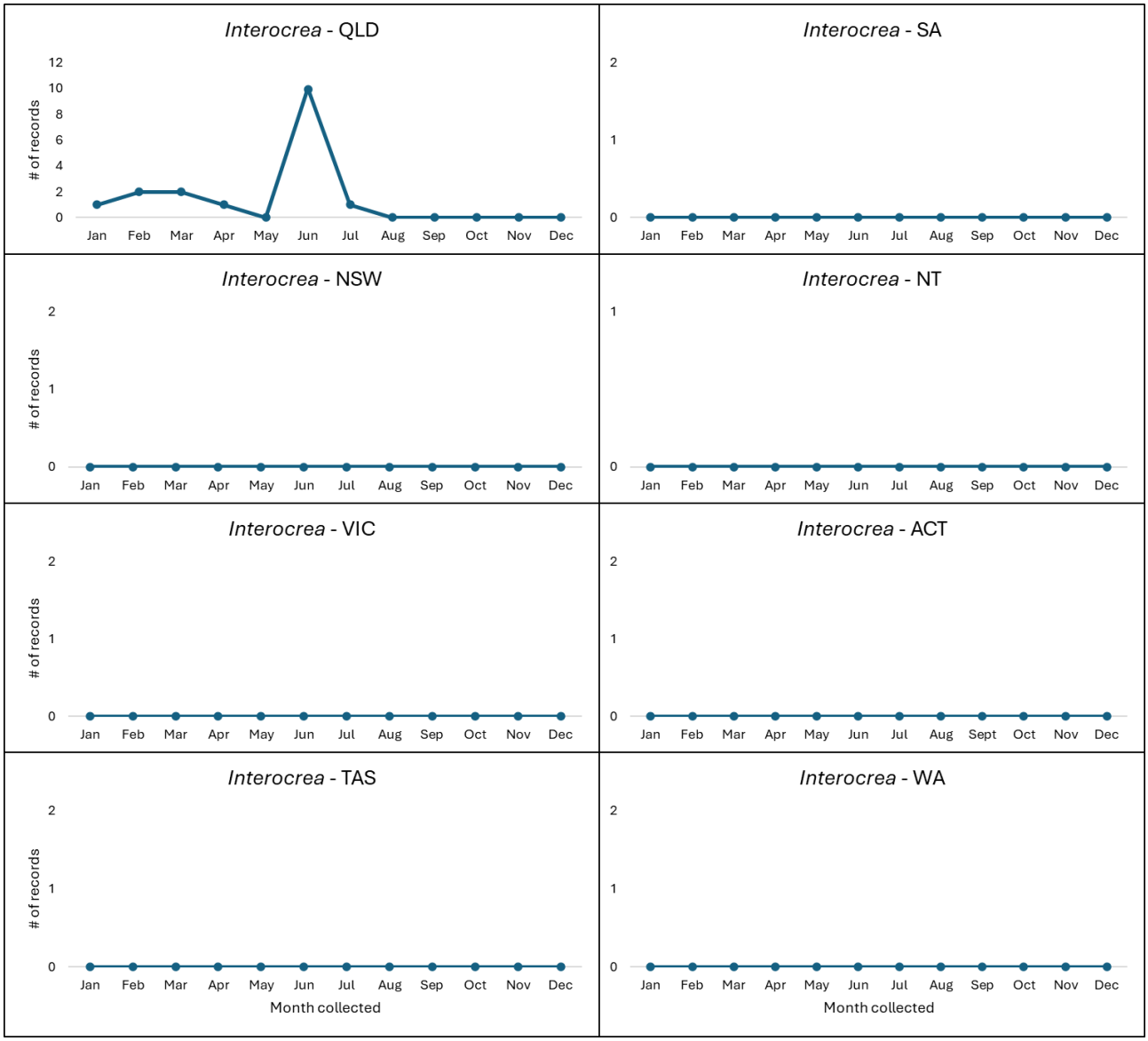


Figure A1.6. Count of *Interocrea* records per calendar month for each Australian state and mainland territory.

Philagra

Philagra abundance varied greatly between states (Figure A1.7). *Philagra* were collected during all calendar months in QLD and NSW, were completely absent from NT and WA records, and only one was caught in SA during March. *Philagra* were only present in ACT during February and March. This genus was absent from TAS and VIC collections between March and October, and June and October, respectively.

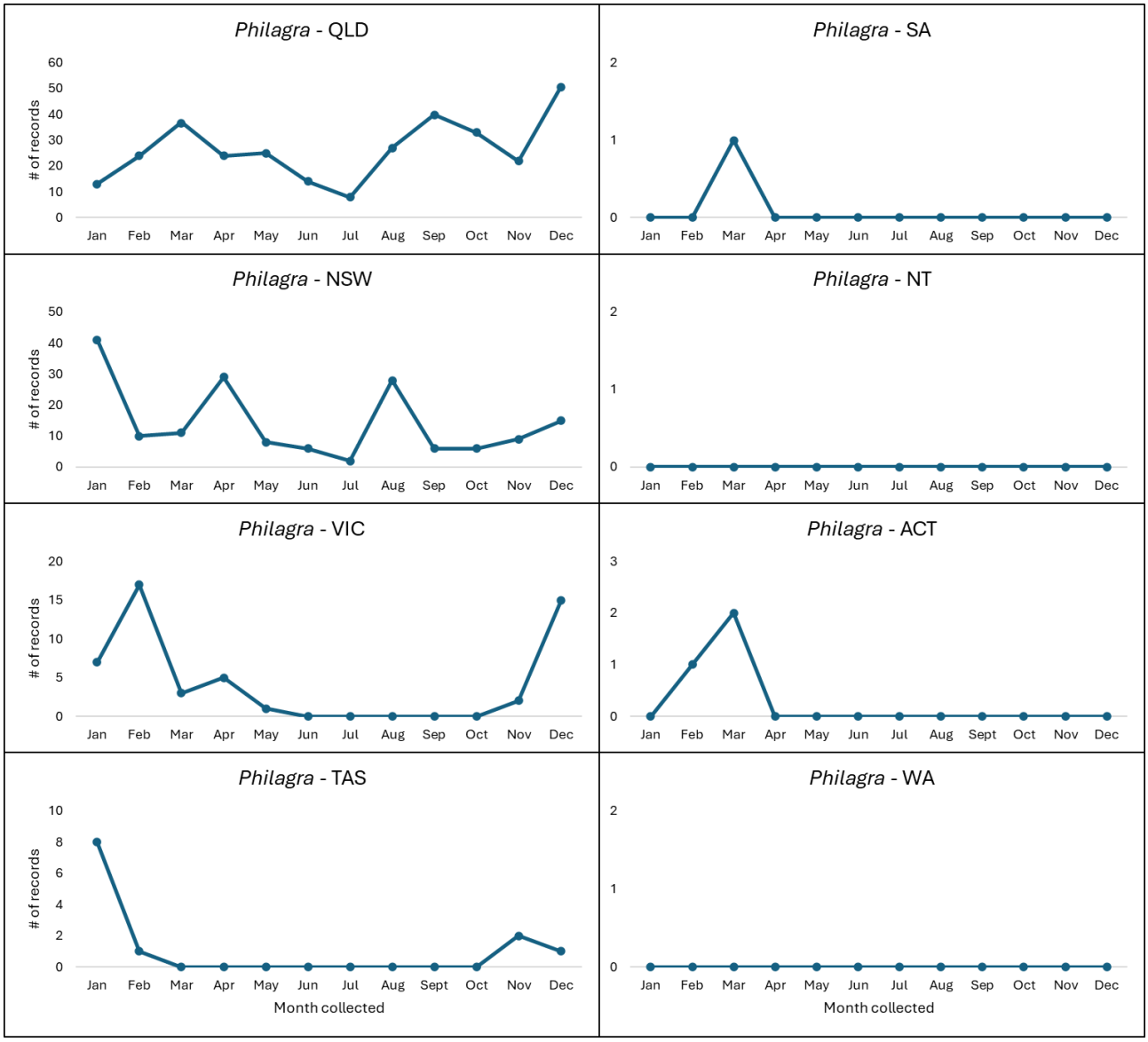


Figure A1.7 Count of *Philagra* records per calendar month for each Australian state and mainland territory.

Cercopidae

Aufidus

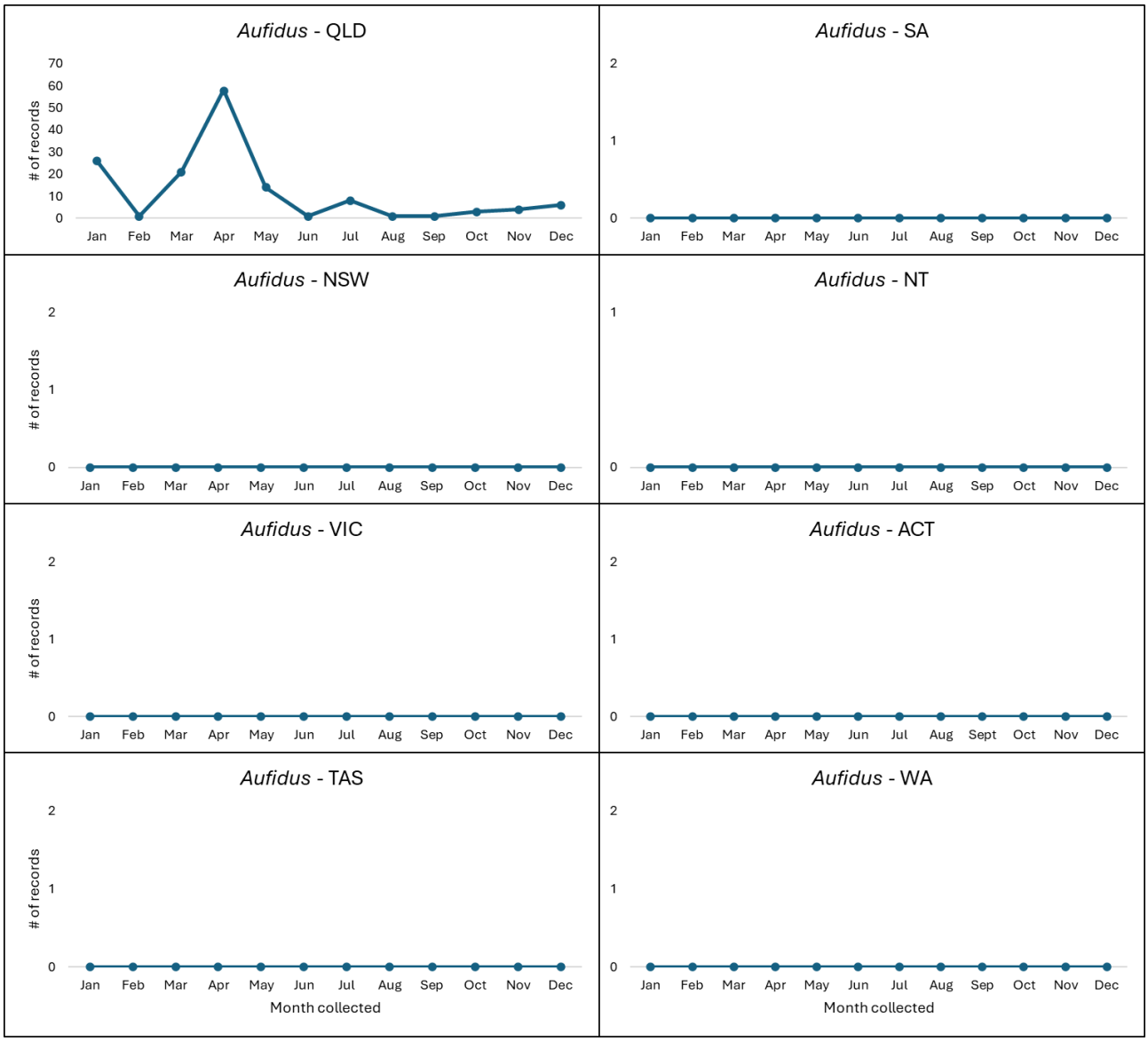


Figure A1.8. Count of *Aufidus* records per calendar month for each Australian state and mainland territory.

Aufiterna

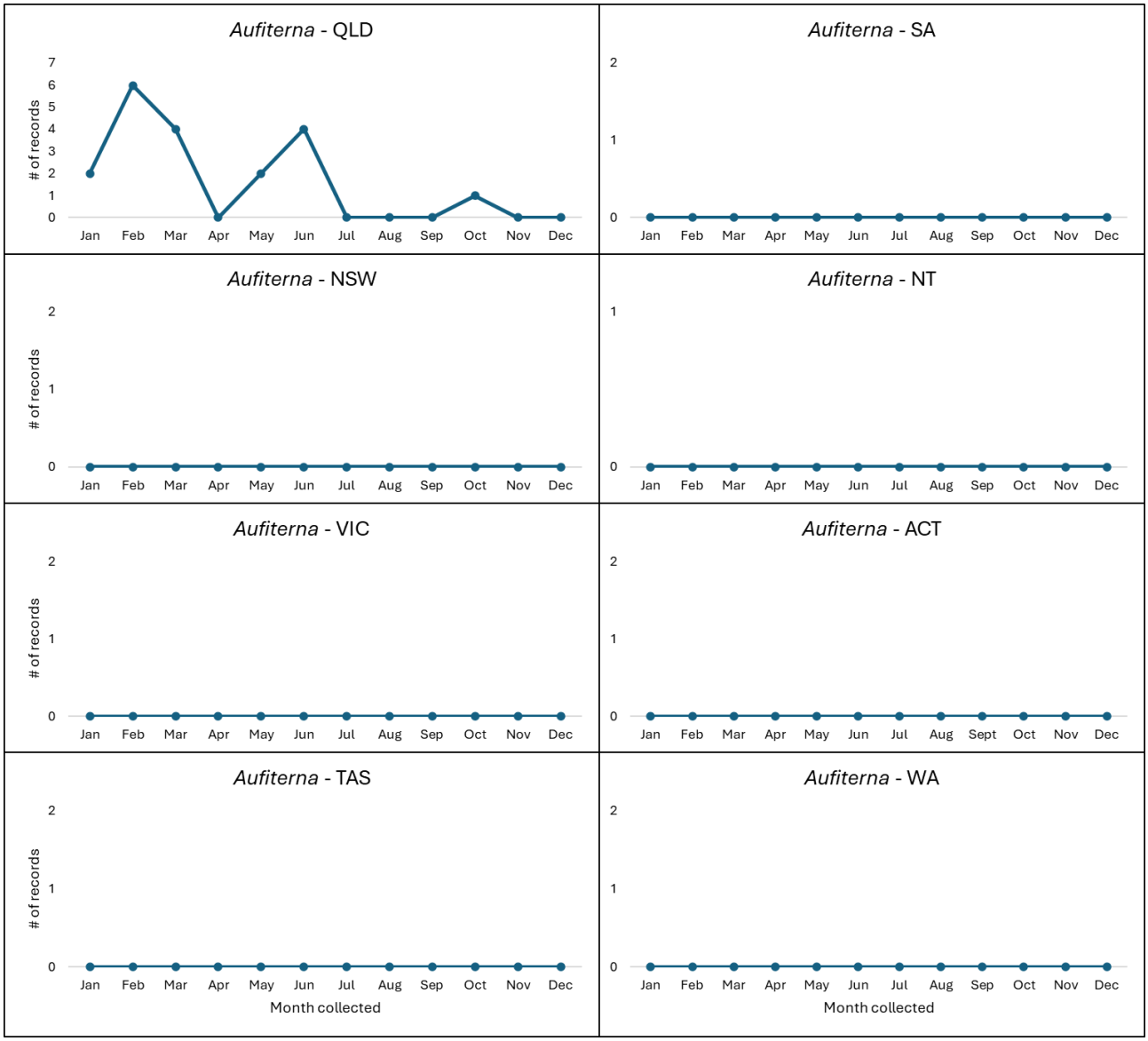


Figure A1.9. Count of *Aufiterna* records per calendar month for each Australian state and mainland territory.

Euryaulax

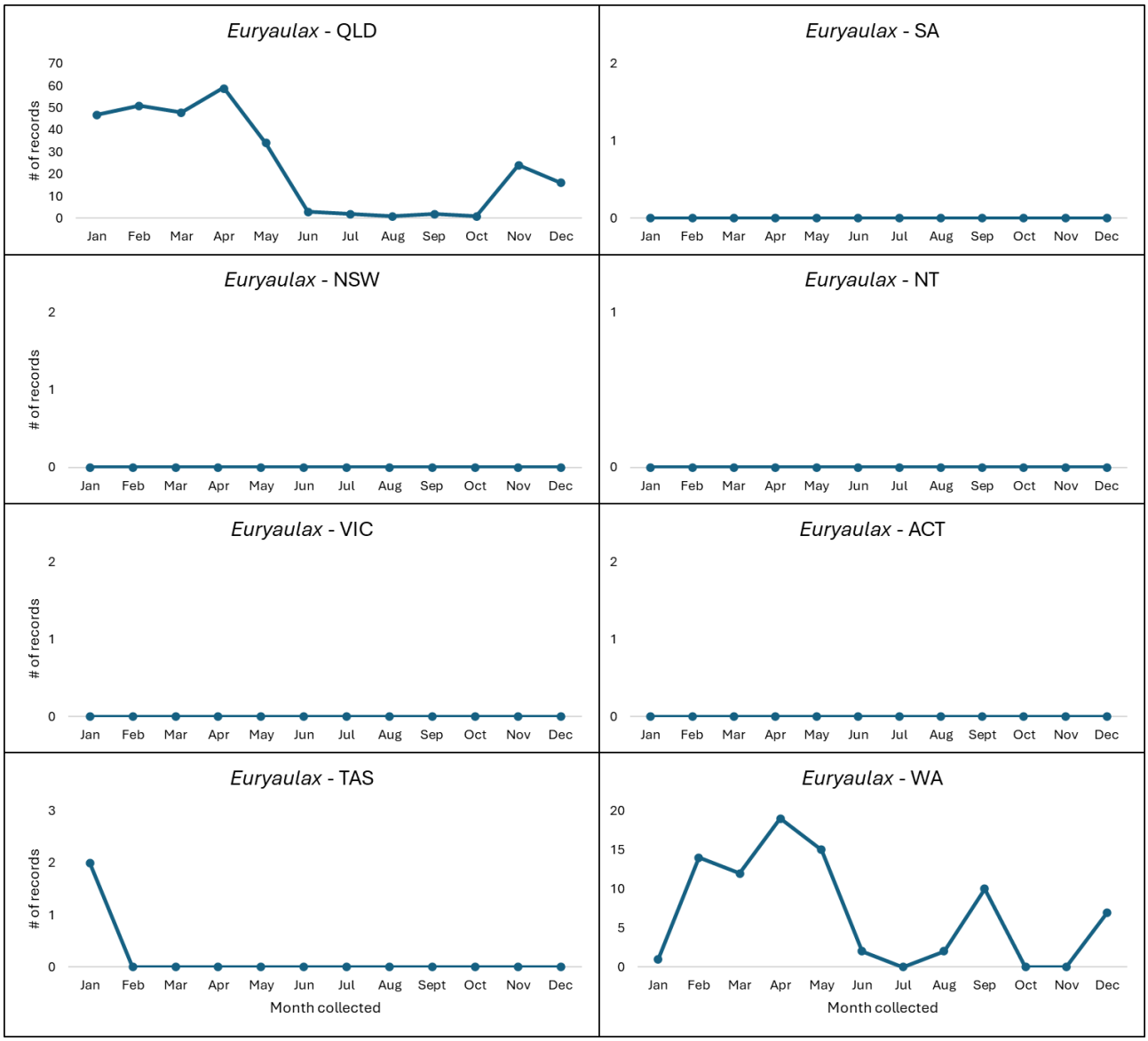


Figure A1.10. Count of *Euryaulax* records per calendar month for each Australian state and mainland territory.

Megastethodon

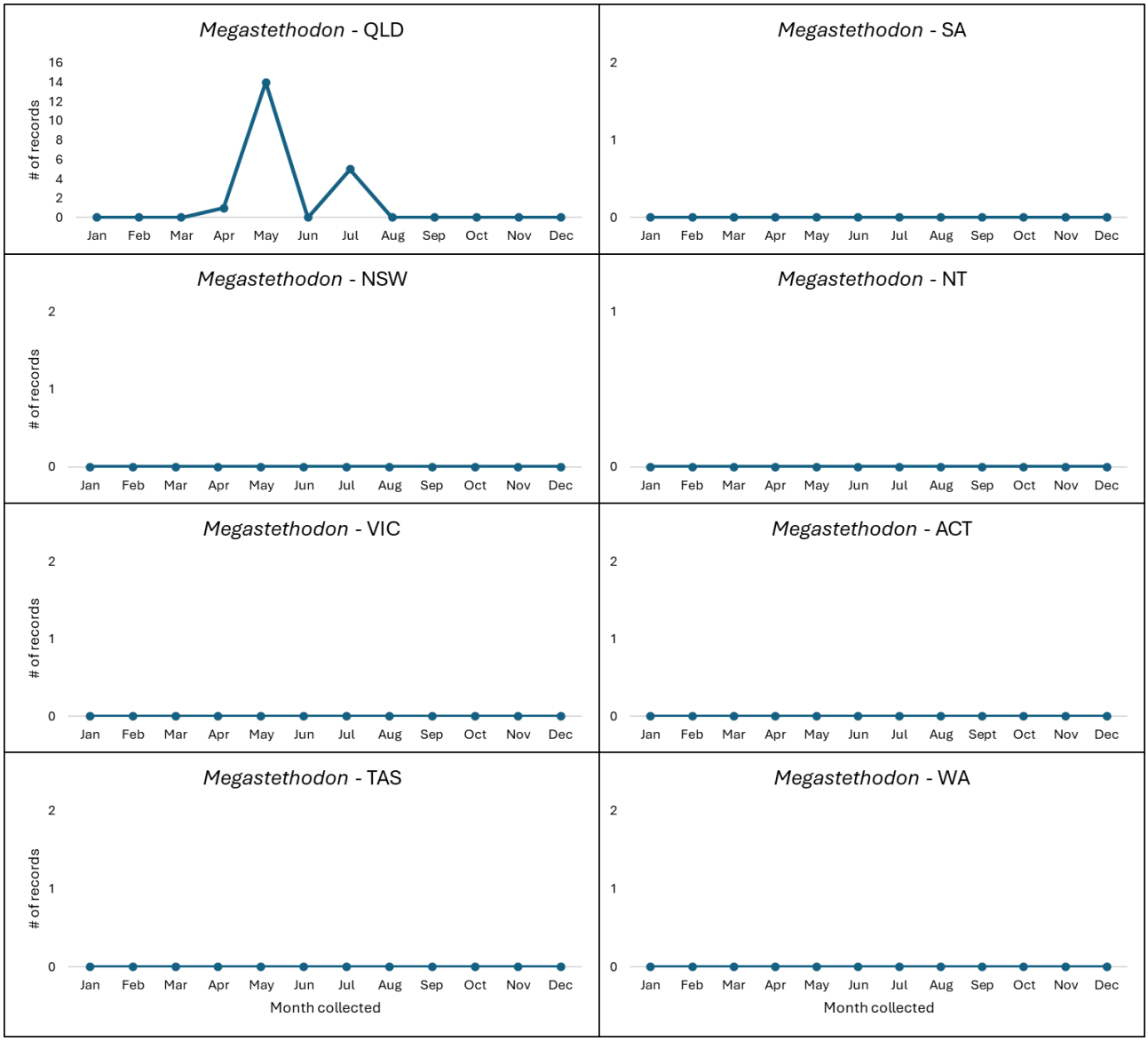


Figure A1.11. Count of *Megastethodon* records per calendar month for each Australian state and mainland territory.

Petyllis

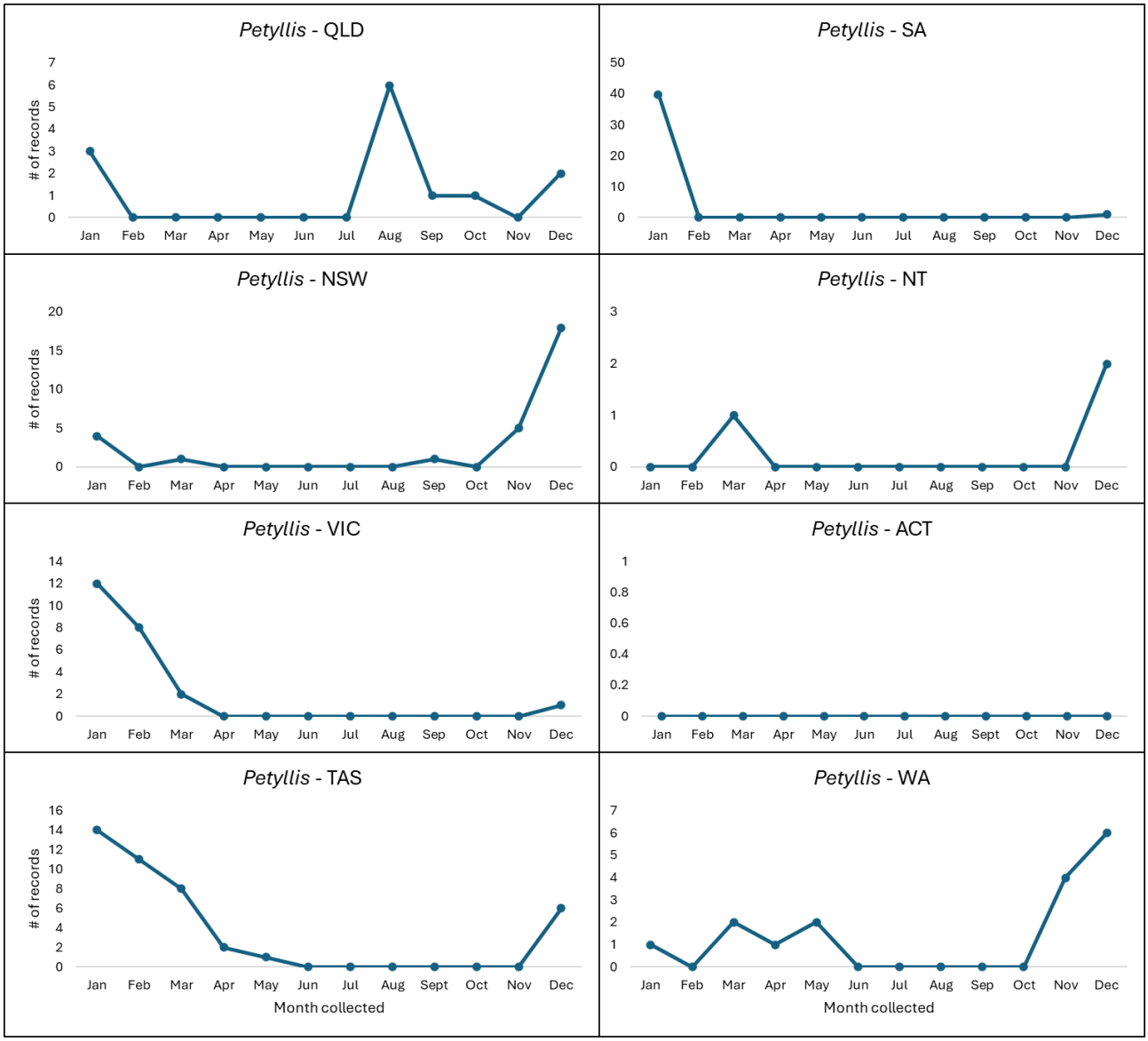


Figure A1.12. Count of *Petyllis* records per calendar month for each Australian state and mainland territory.

Tonnoiria

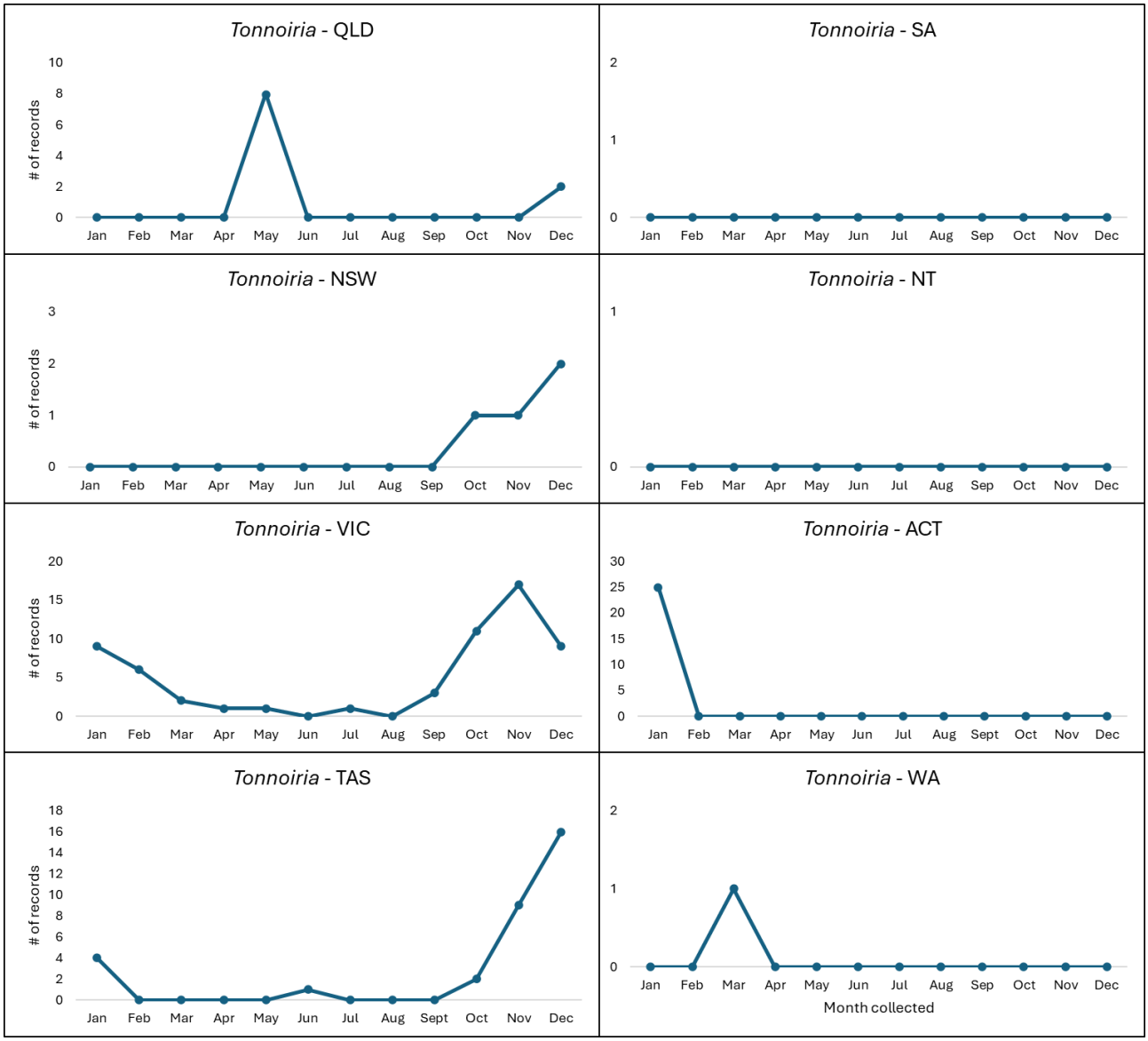


Figure A1.13. Count of *Tonnoiria* records per calendar month for each Australian state and mainland territory.

Machaerotidae

Chaetophyes

The seasonal presence of *Chaetophyes* across Australia was relatively uniform (Figure A1.14). These insects were generally only caught in NSW, VIC, SA, NT and ACT from October to February, with a few exceptions. *Chaetophyes* were caught in TAS during this period, but some were also caught in April. *Chaetophyes* were most abundant in QLD during October and November, however they were present across all calendar months except June and August. NSW collections provided the most *Chaetophyes*, although none were collected between April and September.

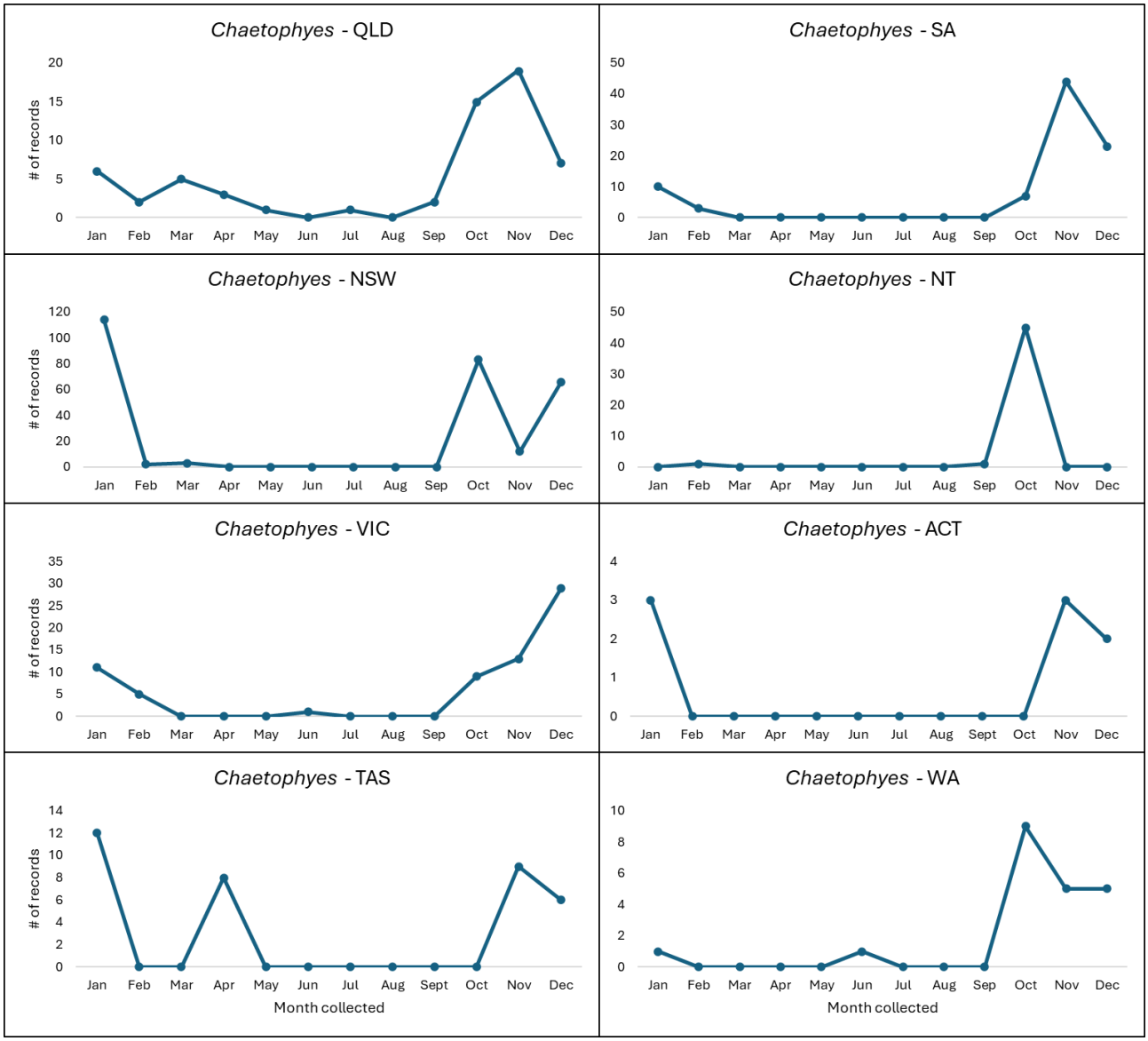


Figure A1.14. Count of *Chaetophyes* records per calendar month for each Australian state and mainland territory.

Hindoloides

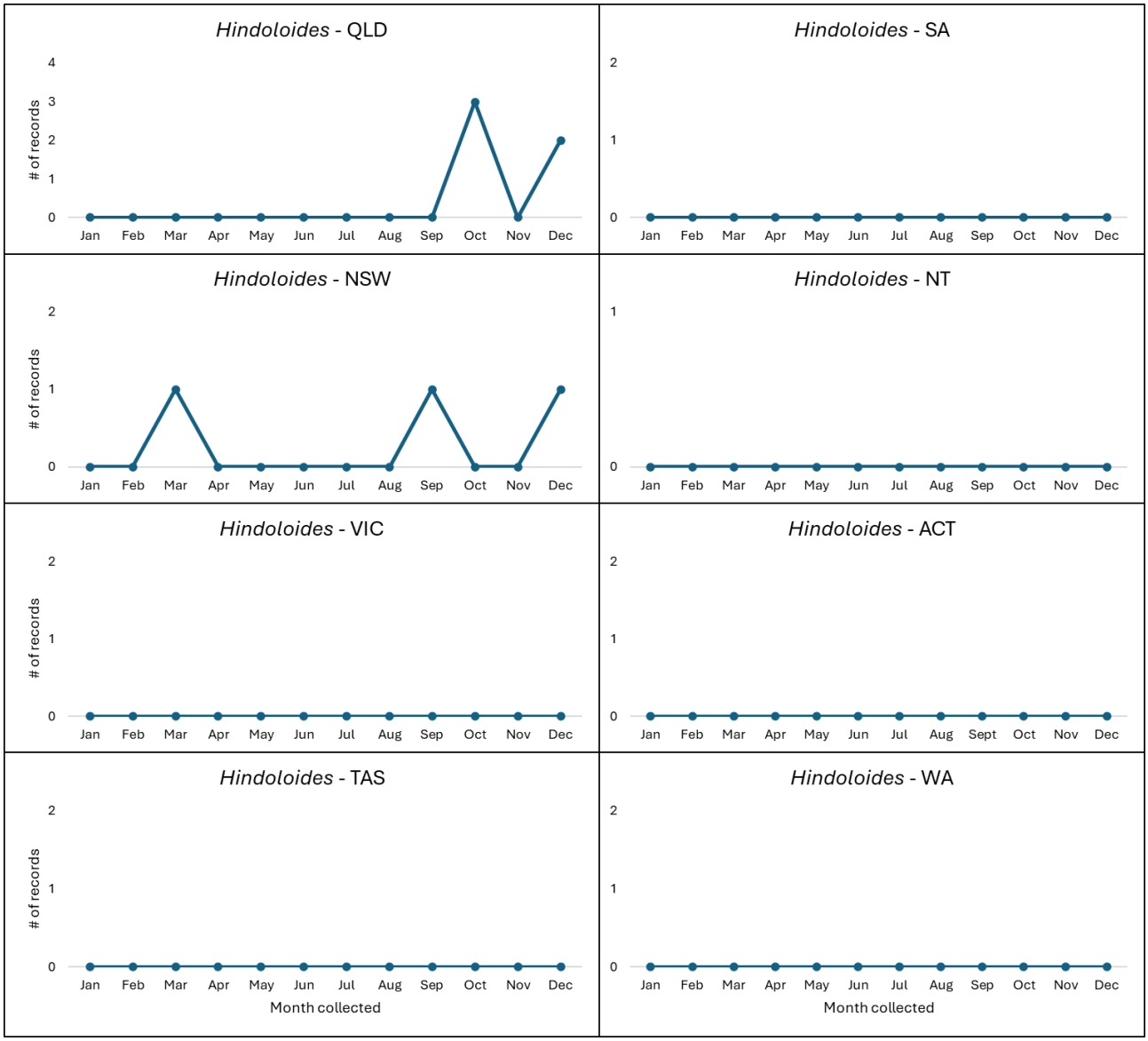


Figure A1.15. Count of *Hindoloides* records per calendar month for each Australian state and mainland territory.

Machaerota

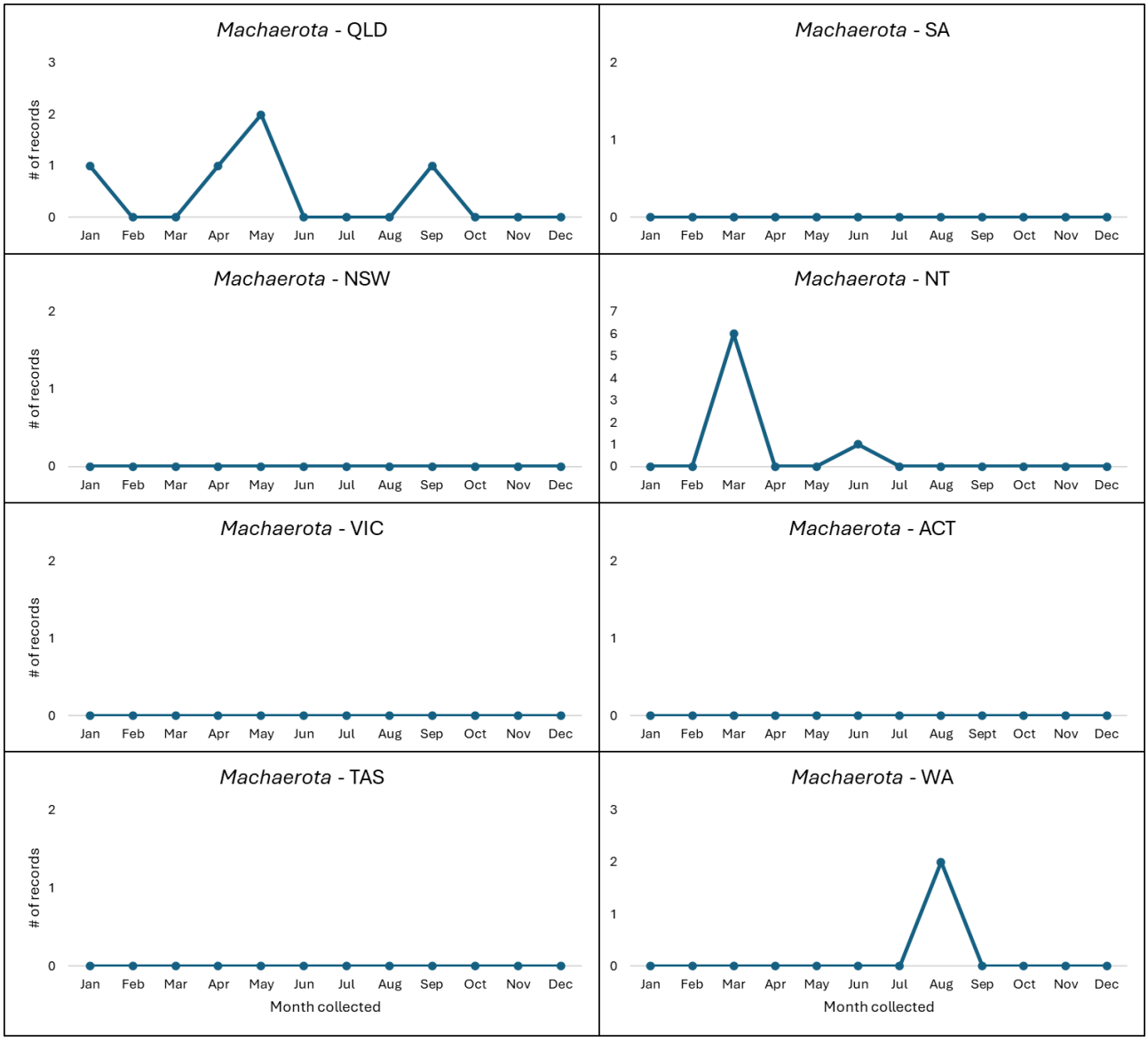


Figure A1.16. Count of *Machaerota* records per calendar month for each Australian state and mainland territory.

Pectinariophyes

Seasonal presence of *Pectinariophyes* was generally lower in winter across states and territories, although specific timing of insect presence was dynamic (Figure A1.17). For example, in QLD *Pectinariophyes* were present in all months, whereas they were absent from May to September in VIC, TAS, ACT, and, with one exception, SA. This spatial temporal variation is further illustrated during autumn and spring; *Pectinariophyes* was collected during May in QLD, NSW, and WA, but was absent from VIC, TAS, SA, NT, and ACT collections at this time. Australian states and territories also differed in the month when the most *Pectinariophyes* were collected. These insects were most abundant during December, November, October, or January depending on the state/territory of collection.



Figure A1.17. Count of *Pectinariophyes* records per calendar month for each Australian state and mainland territory.

Polychaetophyes

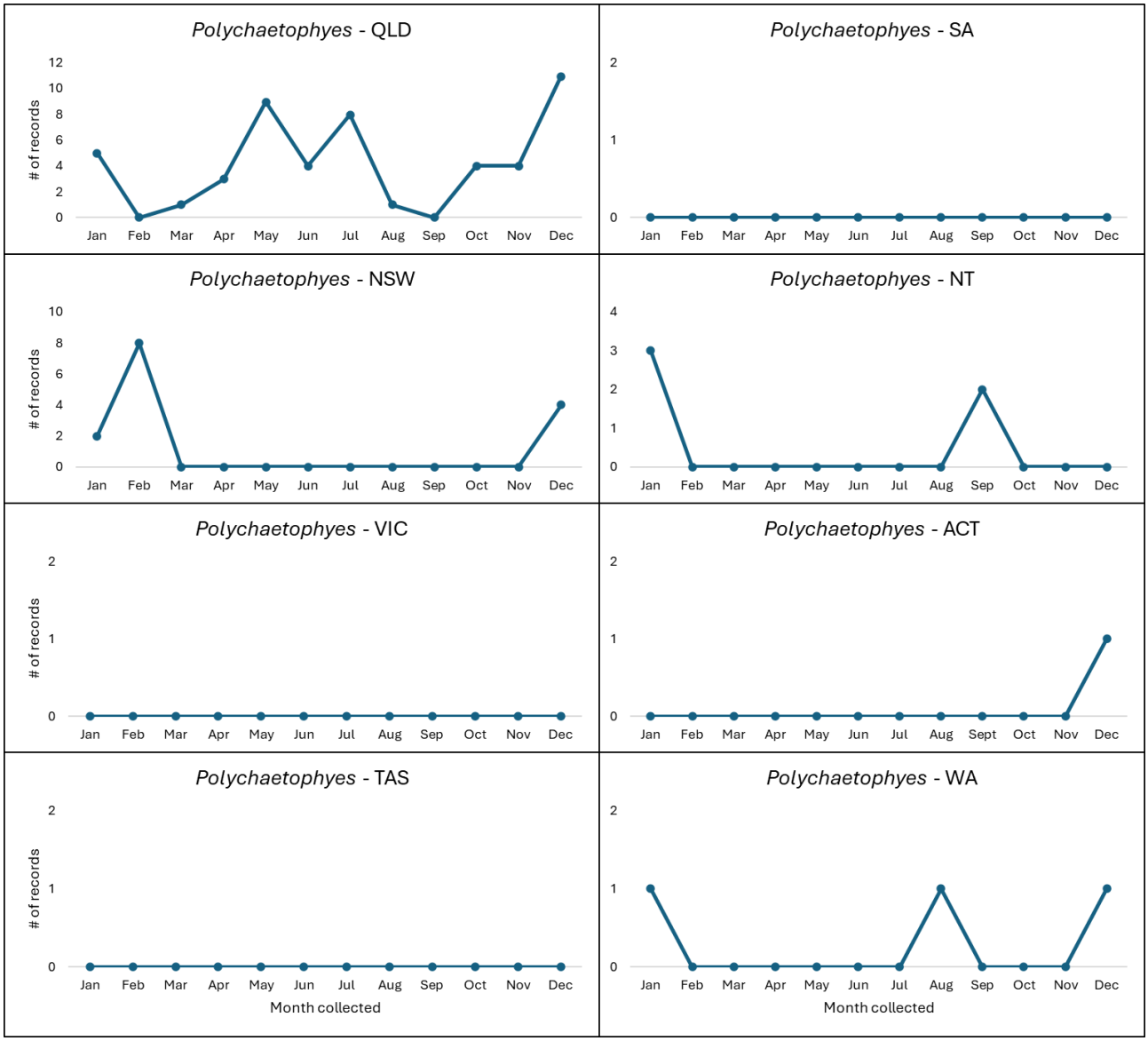


Figure A1.18. Count of *Polychaetophyes* records per calendar month for each Australian state and mainland territory.

Appendix 2: Recorded collection methods for cercopoid genera and families.

Table A2.1. Recorded collection methods grouped by cercopoid family. Colours indicate into which method category each collection method is grouped (green = light, orange = hand, blue = beat/sweep/sieve/vac, yellow = flight trap, grey = ground trap, purple = pyrethrum, pink = reared, brown = generic or mixed trap).

	Cercopoid family			
Collection method	**Aphrophoridae**	**Cercopidae**	**Machaerotidae**	**Total**
Light	90	206	611	907
MV light	155	70	565	790
Sweeping	215	78	17	310
By hand	438	73	86	597
Malaise trap	135	32	4	171
Beating	78	5	4	87
UV light (sheet)	0	42	15	57
Pitfall trap	45	4	3	52
Blacklight	3	24	3	30
At light	0	0	30	30
Vacuum	21	3	1	25
Sticky trap	6	1	10	17
Pan trap (yellow)	10	2	0	12
By hand – day	11	0	0	11
Pyrethrum	5	0	6	11
UV light	5	2	3	10
Reared	2	5	1	8
Vehicle net	0	0	8	8
Beating/sweeping	6	0	0	6
Bucket light-trap	6	0	0	6
D-vac	3	3	0	6
Pan trap (blue)	2	4	0	6
campsite light	0	4	0	4
MV light, beating	4	0	0	4
Fruition Fruitfly trap	3	0	0	3
Pan trap (white)	1	2	0	3
Panel trap	0	2	1	3
Pipe trap	3	0	0	3
Pressure lamp	2	1	0	3
Trap	2	1	0	3
By hand – night	2	0	0	2
car mounted intercept trap	0	0	2	2
Culicoidea trap	0	2	0	2
Intercept trap	2	0	0	2

Panel trap, α-pinene+EtOH lure	2	0	0	2
Sieved litter	2	0	0	2
Spotlighting at night	2	0	0	2
EHB Lure	1	0	0	1
EL light	0	0	1	1
Flight Intercept Trap	0	0	1	1
Light trap / Vacuum sampler	0	1	0	1
Log emergence trap	1	0	0	1
macro pitfalls	0	0	1	1
Malaise trap (SLAM)	1	0	0	1
Pan trap	1	0	0	1
Pitfall and Malaise traps	1	0	0	1
Pitfall trap (glycol)	0	0	1	1
QFF trap	0	1	0	1
Total	1266	568	1374	3208

Table A2.2. Collection methods recorded for Aphrophoridae, grouped by genus.

Aphrophorid genus	Collection method	# of records
Amarusa	By hand	2
	Malaise trap	1
	MV light	1
	Light	1
	Total	5
Anyllis	MV light	123
	Malaise trap	113
	By hand	112
	Light	70
	Beating	45
	Sweeping	33
	Vacuum	20
	Sticky trap	6
	Beating/sweeping	5
	Pitfall trap	4
	Pyrethrum	4
	Pan trap (yellow)	3
	Blacklight	3
	Bucket light-trap	3
	D-vac	3
	Pipe trap	3
	Intercept trap	2
	UV light	2
	Reared	2
	Sieved litter	2
	MV light, beating	2
	Pan trap (blue)	2
	Log emergence trap	1
	Pan trap (white)	1
	Pressure lamp	1
	Total	565
Basilioterpa	UV light	1
	Sweeping	1
	Total	2
Bathyllus	By hand	185
	Sweeping	137
	Pitfall trap	41
	Beating	20
	Light	13
	Malaise trap	8
	Pan trap (yellow)	6
	Bucket light-trap	3

	Spotlighting at night	2
	Pressure lamp	1
	Pyrethrum	1
	EHB Lure	1
	Beating/sweeping	1
	Vacuum	1
	Pitfall and Malaise traps	1
	Malaise trap (SLAM)	1
	Total	422
Interocrea	Light	1
	By hand	1
	Total	2
Liorhina	MV light	1
	Total	1
Philagra	By hand	126
	Sweeping	43
	MV light	16
	Malaise trap	13
	Beating	13
	By hand – day	11
	Light	5
	Fruition Fruitfly trap	3
	Panel trap, α-pinene+EtOH lure	2
	Trap	2
	UV light	2
	By hand – night	2
	MV light, beating	2
	Pan trap (yellow)	1
	Pan trap	1
	Total	242
Undet aphrophorid	MV light	14
	By hand	12
	Sweeping	1
	Total	27
	Grand Total	1266

Table A2.3. Collection methods recorded for Cercopidae, grouped by genus.

Cercopidae genus	Collection method	# of records
Aufidus	Light	62
	Malaise trap	12
	By hand	9
	MV light	1
	Total	84
Aufiterna	Malaise trap	7
	Total	7
Euryaulax	Light	114
	Sweeping	48
	Blacklight	24
	MV light	16
	By hand	16
	UV light (sheet)	7
	campsite light	4
	D-vac	3
	Culicoidea trap	2
	UV light	1
	Trap	1
	QFF trap	1
	Vacuum	1
	Light trap / Vacuum sampler	1
	Malaise trap	1
	Total	240
Megastethodon	Light	9
	Total	9
Petyllis	By hand	21
	Sweeping	17
	Malaise trap	12
	MV light	8
	Light	6
	Beating	5
	Panel trap	2
	Vacuum	1
	Sticky trap	1
	Total	73
Tonnoiria	Sweeping	11
	By hand	10
	Light	9
	Pitfall trap	4
	Pan trap (blue)	4
	Pan trap (yellow)	2
	Pan trap (white)	2

	MV light	1
	Total	43
Undet cercopid	MV light	44
	UV light (sheet)	35
	By hand	17
	Light	6
	Reared	5
	Sweeping	2
	Pressure lamp	1
	Vacuum	1
	UV light	1
	Total	112
	Grand Total	568

Table A3.4. Collection methods recorded for Cercopidae, grouped by genus.

Machaerotid genus	Collection method	# of records
Chaetophyes	Light	201
	MV light	166
	By hand	46
	Sweeping	11
	Sticky trap	10
	UV light (sheet)	5
	Vehicle net	5
	Pyrethrum	5
	car mounted intercept trap	2
	Malaise trap	2
	UV light	2
	Pitfall trap	2
	Blacklight	1
	Panel trap	1
	Reared	1
	Beating	1
	Pitfall trap (glycol)	1
	Total	462
Hindoloides	Flight Intercept Trap	1
	Beating	1
	Total	2
Machaerota	By hand	7
	Vacuum	1
	Light	1
	Total	9
Pectinariophyes	Light	368
	MV light	295
	At light	30
	By hand	17
	UV light (sheet)	9
	Sweeping	6
	Vehicle net	3
	Beating	2
	Malaise trap	2
	UV light	1
	Blacklight	1
	EL light	1
	Pyrethrum	1
	Pitfall trap	1
	macro pitfalls	1
	Total	738
Polychaetophyes	Light	30

	MV light	6
	UV light (sheet)	1
	Blacklight	1
	Total	38
Undet machaerotid	MV light	98
	By hand	16
	Light	11
	Total	125
	Grand Total	1374

Appendix 3: Plants associated with cercopoid records

Table A3.1. Plant species associated with cercopoid records.

Plant family	Plant identity	# of records
Alstroemeriaceae	Drymophila	1
	Total	**1**
Anacardiaceae	Mangifera indica	2
	Total	**2**
Asparagaceae	Lomandra	2
	Total	**2**
Asphodelaceae	Bulbine bulbosa	1
	Dianella	2
	Xanthorrhoea	3
	Total	**6**
Asteraceae	Argyranthemum fructescens	8
	Cassinia	4
	Cassinia laevis	1
	Chrysanthemoides monilifera	4
	Olearia lirata	2
	Olearia viscosa	1
	Senecio amygdalifolius	1
	Senecio lautus	1
	Senecio quadridentatus	2
	Total	**24**
Atherospermataceae	Atherosperma moschatum	1
	Total	**1**
Boraginaceae	Cynoglossum australe	2
	Echium plantagineum	2
	Total	**4**
Casuarinaceae	Casuarina	12
	Casuarina cunninghamiana	3
	Casuarina glauca	11
	Total	**26**
Combretaceae	Terminalia muelleri	5
	Total	**5**
Convolvulaceae	Ipomoea batatas	1
	Total	**1**
Cunoniaceae	Schizomeria	1
Myrtaceae	Angophora	2
	Chamelaucium uncinatum	1
	Corymbia maculata	2
	Corymbia tessellaris	9
	Eucalyptus	118
	Eucalyptus amygdalina	5
	Eucalyptus camaldulensis	12
	Eucalyptus coccifera	1
	Eucalyptus crebra	2
	Eucalyptus deanei	1
	Eucalyptus delegatensis	1
	Eucalyptus dunnii	3
	Eucalyptus globulus	3
	Eucalyptus gomphocephala	3
	Eucalyptus grandis	2
	Eucalyptus haemastoma	12
	Eucalyptus leucoxylon	2
	Eucalyptus moluccana	3
	Eucalyptus nitens	1
	Eucalyptus ovata	2
	Eucalyptus pulchella	1
	Eucalyptus regnans	1
	Eucalyptus robusta	1
	Eucalyptus sieberi	1
	Eucalyptus tricarpa	1
	Eucalyptus viminalis	6
	Leptospermum	4
	Leptospermum flavescens	1
	Leptospermum laevigatum	1
	Leptospermum lanigerum	10
	Melaleuca	1
	Melaleuca linarifolia	2
	Melaleuca quinquenervia	4
	Metrosideros armillaris	1

	Total	**1**
Cupressaceae	Callitris	1
	Total	**1**
Cyperaceae	Baumea articulata	1
	Gymnoschoenus sphaerocephalus	5
	Mesomelaena tetragona	2
	(blank)	13
	Total	**21**
Dioscoreaceae	Dioscorea alata	4
	Total	**4**
Ericaceae	Cyathodes glauca	1
	Leptecophylla juniperiana	7
	Leucopogon	2
	Leucopogon australis	1
	Total	**11**
Euphorbiaceae	Beyeria	1
	Total	**1**
Fabaceae	Acacia	53
	Acacia aneura var. intermedia	1
	Acacia binervata	2
	Acacia dealbata	33
	Acacia decurrens	4
	Acacia longifolia	1
	Acacia mearnsii	1
	Acacia melanoxylon	4
	Acacia myrtifolia	2
	Acacia saligna	1
	Acacia verniciflua	3
	Acacia verticillata	2
	Bossiaea aquifolium	5
	Cochliasanthus caracalla	8
	Cytisus scoparius	4
	Daviesia brevifolia	1
	Gastrolobium calycinum	7
	Genistae	1
	Medicago lupulina	3
	Medicago sativa	7
	Phyllota phylicoides	2
	Pultenaea glabra	4
	Pultenaea villosa	2

	Syncarpia	1
	Thryptomene calycina	1
	Eucalyptus acaciiformis	1
	Eucalyptus acaciiformis	1
	Total	**226**
Nothofagaceae	Nothofagus	1
	Total	**1**
Oleaceae	Olea europaea	11
	Total	**11**
Orchidaceae	Disa bracteata	1
	Total	**1**
Paeoniaceae	Paeonia	1
	Total	**1**
Phyllanthaceae	Glochidion ferdinandi	2
	Total	**2**
Pinaceae	Pinus elliottii	1
	Pinus radiata	4
	Total	**5**
Plantaginaceae	Plantago	1
	Plantago laceolata	1
	Total	**2**
Poaceae	Cenchrus clandestinus	3
	Ehrharta	4
	Lolium	12
	Phalaris	6
	Saccharum	1
	Spinifex	10
	Zea mays	1
	Zizania	2
	(blank)	42
	Total	**81**
Polygonaceae	Duma (florulenta?)	1
	Total	**1**
Proteaceae	Banksia spinulosa	4
	Grevillea	2
	Grevillea buxifolia	1
	Grevillea caleyi	5
	Grevillea teretifolia	1
	Hakea gibbosa	1

	Tephrosia	1
	Trifolium fragiferum	2
	(blank)	2
	Total	**156**
Fagaceae	Quercus suber	2
	Total	**2**
Gleicheniaceae	Gleichenia dicarpa	2
	Total	**2**
Hypericaceae	Hypericum perforatum	19
	Total	**19**
Iridaceae	Dietes iridioides	2
	Total	**2**
Juncaceae	Juncus	1
	Total	**1**
Lamiaceae	Mentha	1
	Westringia	3
	(blank)	2
	Total	**6**
Loganiaceae	Logania	1
	Total	**1**
Malvaceae	Hibiscus cannabinus	1
	Lasiopetalum ferrugineum	1
	Sida cordifolia	6
	Thomasia	1
	Waltheria virgata	5
	Total	**14**
Meliaceae	Cedrela australis	1
	Total	**1**
Moraceae	Ficus coronate	3
	Total	**3**

	Hakea microcarpa	1
	Hakea sericea	1
	Lambertia formosa	7
	Persoonia levis	1
	Total	**24**
Rosaceae	Malus domestica	3
	Prunus persica	1
	Total	**4**
Rutaceae	Citrus	1
	Citrus sp. x tangelo	4
	(blank)	3
	Total	**8**
Santalaceae	Exocarpos	2
	Total	**2**
Sapindaceae	Sapindus	1
	Total	**1**
Solanaceae	Lycium ferocissimum	1
	Solanum lycopersicum	22
	Solanum tuberosum	1
	Total	**24**
Tamariaceae	(blank)	1
	Total	**1**
Thymelaeaceae	Pimelea linifolia	1
	Total	**1**
Verbenaceae	Lantana camara	3
	Total	**3**
Vitaceae	Vitis	1
	Vitis vinifera	2
	Total	**3**

Table A3.2. Plants associated with Aphrophoridae, grouped by insect genus. Records with no associated plant were omitted.

Aphrophorid genus	Plant scientific name	# of records
Amarusa	*Corymbia tessellaris*	9
	Glochidion ferdinandi	2
	Total	**11**
Anyllis	*Eucalyptus*	35
	Leptospermum lanigerum	10
	Eucalyptus amygdalina	3
	Myrtaceae	3
	Eucalyptus camaldulensis	2
	Syncarpia	1
	Leptospermum	1
	Eucalyptus regnans	1
	Eucalyptus sieberi	1
	Eucalyptus coccifera	1
	Acacia dealbata	18
	Acacia	6
	Pultenaea glabra	4
	Acacia verticillata	2
	Acacia myrtifolia	1
	Solanum lycopersicum	21
	Lambertia formosa	7
	Grevillea caleyi	4
	Banksia spinulosa	3
	Hakea sericea	1
	Grevillea buxifolia	1
	Hakea microcarpa	1
	Cyperaceae	5
	Gymnoschoenus sphaerocephalus	2
	Poaceae	5
	Zizania	1
	Leptecophylla juniperiana	6
	Cassinia	4
	Olearia viscosa	1
	Exocarpos	2
	Gleichenia dicarpa	2
	Drymophila	1
	Atherosperma moschatum	1
	Logania	1
	Lasiopetalum ferrugineum	1
	Schizomeria	1

	Callitris	1
	Pimelea linifolia	1
	Nothofagus	1
	Xanthorrhoea	1
	Total	**164**
Bathyllus	Undet Poaceae	32
	Lolium	12
	Spinifex	10
	Phalaris	6
	Ehrharta	4
	Cenchrus clandestinus	3
	Gastrolobium calycinum	7
	Medicago sativa	6
	Bossiaea aquifolium	5
	Acacia dealbata	3
	Medicago lupulina	3
	Acacia decurrens	3
	Acacia	3
	Trifolium fragiferum	2
	Fabaceae	2
	Acacia saligna	1
	Genisteae	1
	Daviesia brevifolia	1
	Argyranthemum fructescens	8
	Chrysanthemoides monilifera	4
	Olea europaea	11
	Eucalyptus gomphocephala	3
	Eucalyptus	2
	Metrosideros armillaris	1
	Thryptomene calycina	1
	Chamelaucium uncinatum	1
	Citrus sp. x tangelo	4
	Rutaceae	3
	Citrus	1
	Hypericum perforatum	6
	Cyperaceae	2
	Mesomelaena tetragona	2
	Baumea articulata	1
	Pinus radiata	4
	Pinus elliottii	1
	Lamiaceae	2
	Westringia	2
	Mentha	1
	Grevillea	2

	Grevillea teretifolia	1
	Dianella	2
	Xanthorrhoea	1
	Echium plantagineum	2
	Solanum tuberosum	1
	Lycium ferocissimum	1
	Dietes iridioides	2
	Vitis vinifera	1
	Vitis	1
	Plantago	1
	Cedrela australis	1
	Duma	1
	Leucopogon australis	1
	Prunus persica	1
	Beyeria	1
	Juncus	1
	Disa bracteata	1
	Lomandra	1
	Thomasia	1
	Total	**188**
Interocrea	*Eucalyptus*	1
	Total	**1**
Philagra	*Eucalyptus*	41
	Eucalyptus haemastoma	12
	Melaleuca quinquenervia	4
	Eucalyptus dunnii	3
	Melaleuca linarifolia	2
	Corymbia maculata	2
	Melaleuca	1
	Leptospermum laevigatum	1
	Leptospermum flavescens	1
	Leptospermum	1
	Angophora	1
	Eucalyptus tricarpa	1
	Acacia	37
	Cytisus scoparius	4
	Acacia melanoxylon	4
	Acacia verniciflua	3
	Acacia dealbata	3
	Acacia binervata	2
	Pultenaea villosa	2
	Medicago sativa	1
	Acacia aneura var. intermedia	1
	Acacia mearnsii	1

	Acacia myrtifolia	1
	Casuarina glauca	11
	Casuarina	10
	Casuarina cunninghamiana	3
	Malus domestica	3
	Lantana camara	3
	Lomandra	1
	Hakea gibbosa	1
	Cassinia laevis	1
	Total	**162**
Undet aphrophorid	*Acacia dealbata*	6
	Acacia	1
	Plantago	1
	Paeonia	1
	Casuarina	1
	Bulbine bulbosa	1
	Total	**11**
	Grand Total	537

Table A3.3. Plants associated with Cercopidae, grouped by insect genus. Records with no associated plant were omitted.

Cercopid genus	Plant scientific name	# of records
Aufidus	*Acacia*	5
	Mangifera indica	1
	Total	**6**
Euryaulax	*Cochliasanthus caracalla*	8
	Tephrosia	1
	Terminalia muelleri	5
	Poaceae	2
	Zizania	1
	Zea mays	1
	Dioscorea alata	4
	Ficus coronata	3
	Mangifera indica	1
	Ipomoea batatas	1
	Hibiscus cannabinus	1
	Total	**28**

Petyllis	*Acacia dealbata*	3
	Acacia longifolia	1
	Banksia spinulosa	1
	Persoonia levis	1
	Grevillea caleyi	1
	Eucalyptus	1
	Leptospermum	1
	Eucalyptus ovata	1
	Leucopogon	2
	Leptecophylla juniperiana	1
	Gymnoschoenus sphaerocephalus	1
	Total	**14**
Tonnoiria	*Cyperaceae*	6
	Gymnoschoenus sphaerocephalus	2
	Poaceae	3
	Cyathodes glauca	1
	Total	**12**
Undet cercopid	*Hypericum perforatum*	13
	Senecio quadridentatus	2
	Senecio amygdalifolius	1
	Senecio lautus	1
	Cynoglossum australe	2
	Xanthorrhoea	1
	Tamariaceae	1
	Saccharum	1
	Total	**22**
	Grand Total	82

Table A2.4. Plants associated with Machaerotidae, grouped by insect genus. Records with no associated plant were omitted.

Machaerotid genus	Plant scientific name	# of records
Chaetophyes	*Eucalyptus*	24
	Eucalyptus camaldulensis	8
	Eucalyptus viminalis	6
	Eucalyptus globulus	3
	Eucalyptus moluccana	3
	Eucalyptus amygdalina	2
	Eucalyptus crebra	2
	Eucalyptus nitens	1
	Eucalyptus delegatensis	1
	Eucalyptus ovata	1
	Eucalyptus acaciiformis	1
	Leptospermum	1
	Angophora	1
	Eucalyptus deanei	1
	Quercus suber	2
	Vitis vinifera	1
	Casuarina	1
	Olearia lirata	1
	Acacia decurrens	1
	Total	**61**
Machaerota	*Sida cordifolia*	6
	Sapindus	1
	Total	**7**
Pectinariophyes	*Eucalyptus*	8
	Eucalyptus pulchella	1
	Phyllota phylicoides	2
	Acacia	1
	Solanum lycopersicum	1
	Olearia lirata	1
	Total	**14**
Undet machaerotid	*Eucalyptus*	6
	Eucalyptus leucoxylon	2
	Eucalyptus camaldulensis	2
	Eucalyptus grandis	2
	Eucalyptus robusta	1
	Malvaceae	5
	Total	**18**
	Grand Total	100

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Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (Xylella) in Australia and New Zealand by understanding its potential vectors

Technical Report: Biodiversity and COI barcoding of the Australian spittlebugs (Hemiptera: Cercopoidea).

June 2024

Francesco Martoni, Lea Rako, Duncan Jaroslow, Caitlin Selleck, Pragya Kant, Jessica Hoskins, & Mark Blacket

VICTORIA State Government | Energy, Environment and Climate Action

AGRICULTURE VICTORIA

External Project Code	WA2001 & 4-EWI2BY6
RCT No	AgVic 6162
Project Leader Name	Mark Blacket

Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.



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Executive Summary

Spittlebugs and froghoppers (Hemiptera: Auchenorrhyncha: Cercopoidea) are xylem-feeding insects, some of which can cause economic damage worldwide through vectoring plant pathogenic bacteria such as *Xylella fastidiosa*. Australia does not have *Xylella fastidiosa* nor its primary vector, the spittlebug *Philaenus spumarius*, but it is home to many described and undescribed species of spittlebugs.

Australian cercopoid diversity has been relatively scarcely studied, with the main literature assessment (mostly morphology-based), dating back more than a decade ago. In fact, only a few Australian cercopoid species had been previously characterised using DNA. Such a paucity of DNA records across the main publicly available repositories (e.g., BOLD, NCBI), strongly impacts the application of molecular diagnostic methods and a prompt and accurate DNA-based identification of both native and exotic species, which may not be able to be differentiated from the native ones due to a lack of DNA sequences.

Better understanding of genetic diversity across the superfamily in Australia will allow faster and more precise molecular diagnostic techniques, including DNA barcoding and DNA metabarcoding, to be applied. Furthermore, DNA sequences of the COI gene from multiple individuals per species may also allow exploration of species delimitation across taxa, revealing cryptic taxonomic diversity that may hide undescribed species, among the more than 40 currently described species.

Here we generated 135 new COI sequences for 22 Australian cercopoid taxa, across three families and 10 genera (Figure 9). We also assessed genetic diversity within some of the most widespread Australian species, including *Bathyllus albicinctus* which was shown to be a single species (Figures 9 & 10). This new dataset forms the first comprehensive work on the genetic diversity of the Australian Cercopoidea, and together with the high-resolution morphological images of each taxon that we have provided, will be invaluable tool for improving diagnostic identification of the Australian spittlebugs.

Introduction

Background

The superfamily Cercopoidea (Hemiptera: Auchenorrhyncha) is composed of more than 2600 described species worldwide, across more than 360 genera and five families (Soulier-Perkins 2015; Bartlett et al. 2018). Australia is home to at least 40 described cercopoid species, belonging to at least 16 genera and three families, including taxa with distributions limited to Norfolk, Lord Howe and Christmas Island (Fletcher 2010). Most species are endemic to Australia, with some exceptions: native to Australia but present also in Papua New Guinea, New Zealand and Indonesia (Fletcher 2010). These species are known to be associated with a variety of host plants, including native and introduced species, however no Australian cercopoid species is currently known to be a pest and no exotic species are known to be present in the country. On the other hand, the exotic *Philaenus spumarius* was introduced to New Zealand around 1960 (Archibald et al. 1979) and poses the main threat to Australian plant health due to geographical proximity.

Cercopoid diversity in Australia has been scarcely studied, with the main literature assessment dating back more than a decade (Fletcher 2010) and being mostly morphology-based. In fact, only a few Australian cercopoid species had been previously characterised using DNA. Such a paucity of DNA records across the main publicly available repositories (e.g., BOLD, NCBI), strongly impacts molecular diagnostic methods and a prompt DNA-based identification of both native and exotic species, which cannot be differentiated from the native ones due to a lack of sequences.

Better understanding of genetic diversity across the superfamily in Australia will allow faster and more precise molecular diagnostic techniques, including DNA barcoding (Hebert et al. 2003) and DNA metabarcoding, to be applied. Furthermore, DNA sequences of the COI gene from multiple individuals per species may also allow exploration of species delimitation across taxa, revealing cryptic taxonomic diversity that may currently hide undescribed species.

Project Objectives

A major aim of this project was to improve surveillance methods of potential Australian vectors of *X. fastidiosa*. Including, better understanding of the taxonomic diversity of potential insect vectors present in Australia.

Study Aim

The main aim of this study was to explore the biodiversity and assess genetic diversity within the Australian Cercopoidea superfamily, by extracting DNA and sequencing a fragment of the COI gene (i.e., DNA barcoding) from specimens freshly caught from the field through an array of collection methods, to determine how many species were collected.

Methodology

Sample collection

A total of 131 Cercopoid specimens and 4 Auchenorrhyncha (used as outgroups) were collected from three different states (Victoria, Queensland and Western Australia, Table 1). Specimens were either collected by sweeping or directly by hand from host plants, as well as from a sheet during light trapping events (Supplementary Table 1).

The insect light trap (Figure 1) was set up for a period of 3 to 4 h, from 7-11pm each night. A rope was straightened in the double passage between two trees, at a distance of 3–5 m from each other, following the procedure highlighted in Martoni et al. (2020). A sheet was positioned on one of the lines, while a LepiLED entomological light (Brehm 2017) was fixed on the front line. Individual spittlebugs were collected from the sheet directly into a 5ml vial and preserved in high grade ethanol.

In the laboratory, spittlebugs were morphologically examined and subdivided into morphospecies and identified to the genus-level following available keys (Fletcher 2010). High-resolution auto montage photos of the spittlebugs were taken before DNA extraction using the Leica Application Suite software (version 4.5.0), from 10 to 20 stacked images obtained using a Leica stereo microscope M205C with a DFC450 camera. The software GNU Image Manipulation Program (GIMP) version 2.10.18 was used to collate some of these photos into plates (Figures 2-7).



Figure 1. Cercopoid light trapping near Albany, Western Australia (Nov 2023). Staff: Francesco Martoni (L), Jess Hoskins (R).

DNA extraction, amplification, and sequencing

DNA was destructively extracted from a leg from each individual insect using a DNeasy Blood and Tissue kit (Qiagen, Germany), with the insect specimen retained as a preserved (pinned) morphological voucher. Briefly, individual legs were homogenised using 3mm glass beads and left overnight (~17 hours) at 50 ºC in a 1:9 solution of proteinase K and ATL buffer. The following day, DNA was extracted following the manufacturer's instructions and re-eluted in 100 µL.

We used the DNA barcoding technique (Hebert et al. 2003) to assess taxonomic identity and genetic variability across the different populations. A ~700 bp fragment of the subunit I of the *cytochrome oxidase* (COI) mitochondrial gene was targeted using the primer pair LCO1497-HCO2198 (Folmer et al. 1994) and the following PCR cycling conditions: initial denaturation at 94 ºC for 2 minutes, followed by 30 cycles at 94 ºC for 30 seconds, 50 ºC for 45 seconds, 72 ºC for 45 seconds, and a final extension phase at 72 ºC for 7 minutes. Successful amplification was confirmed running the PCR product on 1% Agarose gels for 30 minutes at 100V. Successful PCR amplicons were sequenced both ways using an external provider (Macrogen Inc, Korea and AGRF, Melbourne). Electropherograms were analysed using the software MEGA v11 (Kumar et al. 2018). Forward and reverse sequences were paired and a consensus sequence was generated for the neighbour joining analysis (Figure 2).

Key Research Findings

Cercopoidea COI diversity

A total of 131 Cercopoid and 4 Auchenorrhynchan (outgroup) COI sequences were generated for this study. The primary aim of this study was to assess interspecific genetic divergence as well as intraspecific genetic divergence across the different taxa collected, especially for those species that were recorded across a broad distributional range.

When applying a similarity threshold of 97%, we could identify 20 morphospecies, while a similarity threshold of 98.5% separated 22 taxa, adding information on genetic diversity within species (Figure 2, Table 1). The 22 taxa recorded here appeared to cluster into 10 genera, with only one taxon not matching the expected morphological identification. This species was *Chaetophyes pulcherrima* which appeared to cluster separately from all other *Chaetophyes* spp. (Figure 2). Except for this taxon, all other genetic distances within genera matched the expected morphology-based taxonomical assignment.

Two genera showed higher than expected species-level variation, *Pectinariophyes* (with 5 taxa) and *Chaetophyes* (with 5-6 taxa *sensu stricto,* 6-7 taxa *sensu latu*) (Table 1). Whenever genetic diversity could be observed (including when applying a similarity threshold of 98.5%), this matched phenotypical changes observed during morphological assessment (Figures 3 to 8).

Intraspecific genetic divergence was analysed for the 30 specimens of *Bathyllus albicinctus,* since this species was recorded both in Victoria and in Western Australia (Table 1), and showed high phenotypical variability, including a clear sexual dimorphism particularly apparent in changes of colouration (Figure 3). However, genetic variation across all 30 specimens was very low, indicative of intraspecific variation.

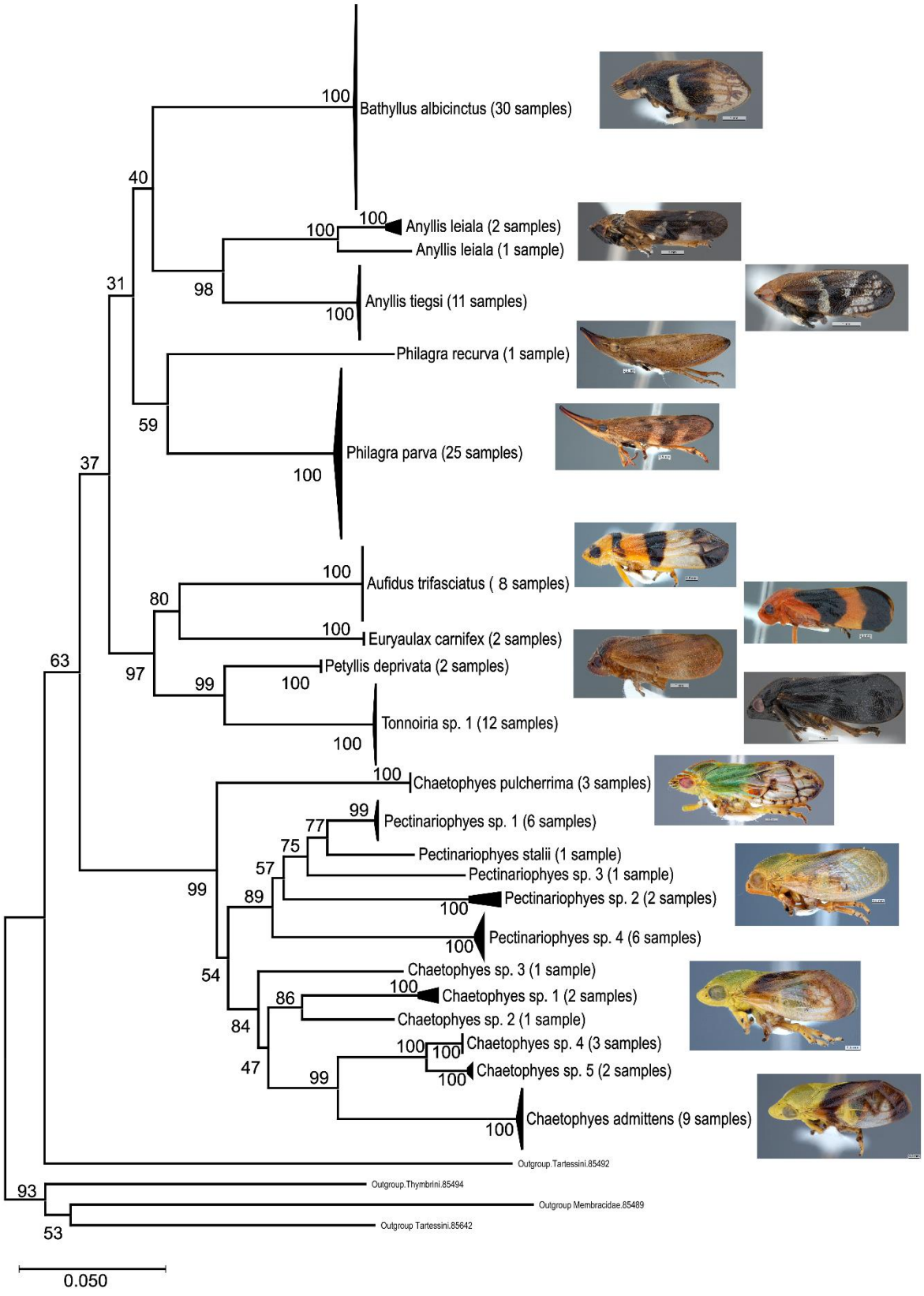


Figure 2: Neighbour-Joining COI tree generated using a total of 135 sequences obtained in this work. The tree separates 20 taxa at 3% minimum divergence and 22 taxa at 1.5% minimum divergence.

Table 1: Summary of the samples collected and sequenced for this study. The table includes information on family, genus, species, number of individuals and distributions for each taxon identified after applying a 98.5% similarity threshold to the COI sequences generated. The star (*) indicates *Chaetophyes pulcherrima* which appeared to cluster separately from all other *Chaetophyes* species (Figure 2), suggesting this may actually be a different genus.

Family	Genus	Species	Samples	States
Aphrophoridae	*Bathyllus*	*B. albicinctus*	30	VIC, WA
Aphrophoridae	*Anyllis*	*A. leiala* “A”	2	VIC
Aphrophoridae	*Anyllis*	*A. leiala* “B”	1	VIC
Aphrophoridae	*Anyllis*	*A. tiegsi*	11	VIC
Aphrophoridae	*Philagra*	*P. parva*	25	QLD, VIC
Aphrophoridae	*Philagra*	*P. recurva*	1	QLD
Cercopidae	*Aufidus*	*A. trifasciatus*	8	QLD
Cercopidae	*Euryaulax*	*E. carnifex*	2	QLD
Cercopidae	*Petyllis*	*P. deprivata*	2	WA
Cercopidae	*Tonnoiria*	*Tonnoiria* sp. 1	12	VIC
Machaerotidae	**Chaetophyes*	**C. pulcherrima*	3	QLD
Machaerotidae	*Chaetophyes*	*C. admittens*	9	QLD
Machaerotidae	*Chaetophyes*	*Chaetophyes* sp. 1	2	VIC
Machaerotidae	*Chaetophyes*	*Chaetophyes* sp. 2	1	VIC
Machaerotidae	*Chaetophyes*	*Chaetophyes* sp. 3	1	VIC
Machaerotidae	*Chaetophyes*	*Chaetophyes* sp. 4	3	QLD
Machaerotidae	*Chaetophyes*	*Chaetophyes* sp. 5	2	VIC
Machaerotidae	*Pectinariophyes*	*P. stalii*	1	QLD
Machaerotidae	*Pectinariophyes*	*Pectinariophyes* sp. 1	6	QLD
Machaerotidae	*Pectinariophyes*	*Pectinariophyes* sp. 2	2	WA
Machaerotidae	*Pectinariophyes*	*Pectinariophyes* sp. 3	1	QLD
Machaerotidae	*Pectinariophyes*	*Pectinariophyes* sp. 4	6	QLD
3	**9 + 1***	**22**	**131**	**3**

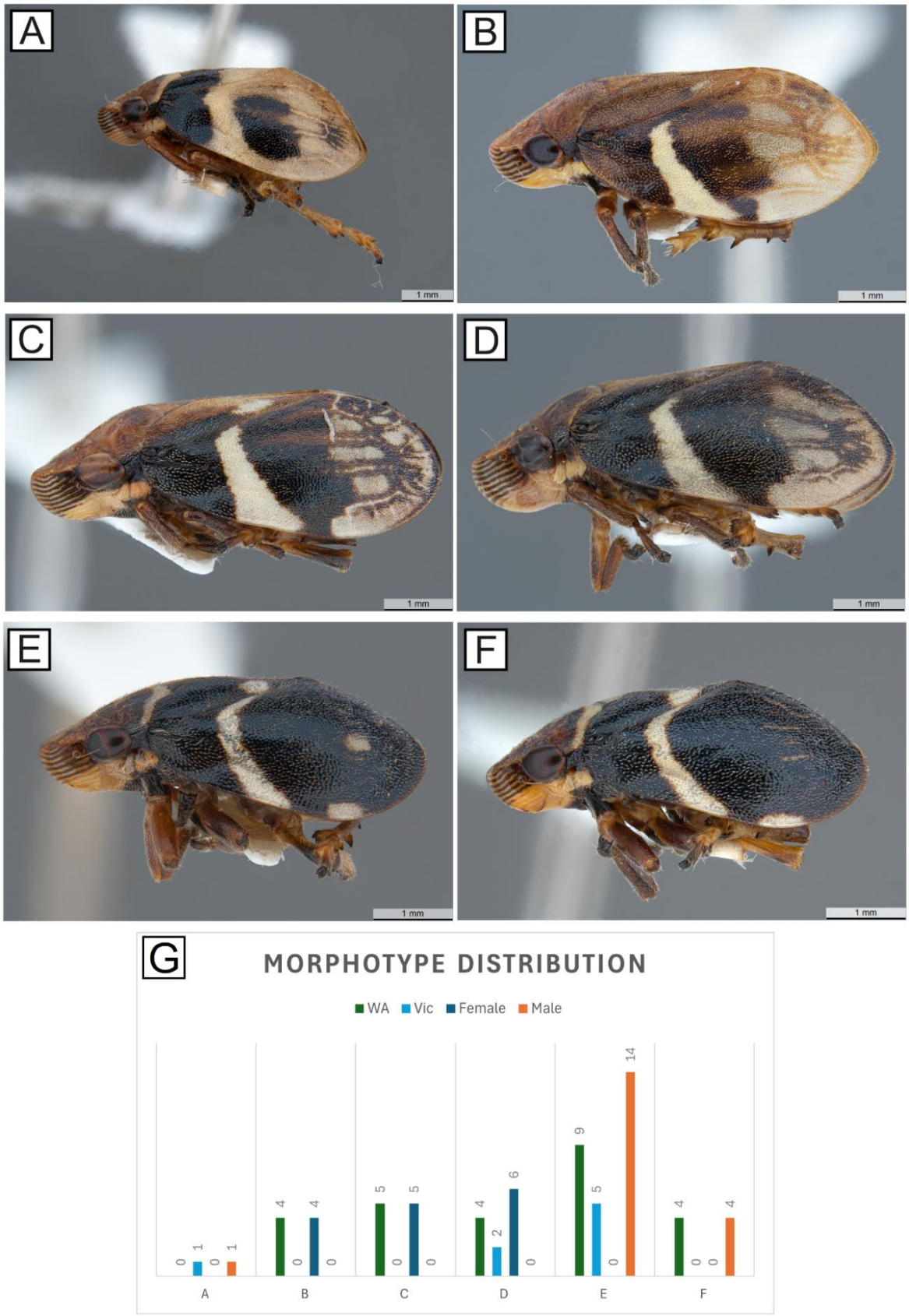


Figure 3: Habitus lateral photos showing examples of the morphological variation across the 30 specimens of Bathyllus albicinctus analysed for this study, grouped in six morphotypes (A-F). Geographical distribution and distribution across sexes for each morphotype is shown in figure (G).

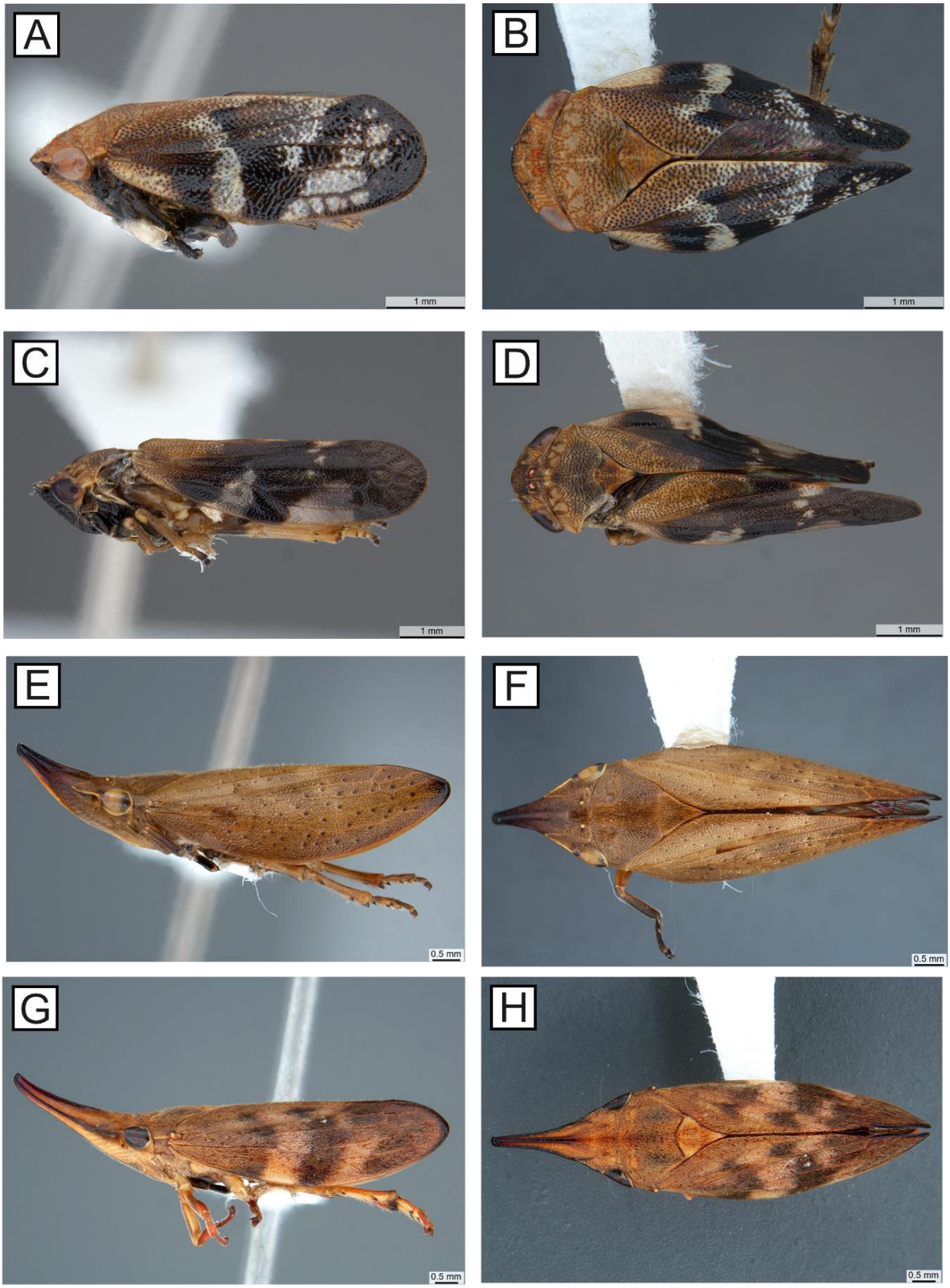


Figure 4: Habitus lateral and habitus dorsal photos showing the morphological diversity within the family Aphrophoridae. *Anyllis leiala* (**A-B**), *Anyllis tiegsi* (**C-D**), *Philagra recurva* (**E-F**), *Philagra parva* (**G-H**). Scale bars are 1 mm (A-D) and 0.5 mm (E-H).

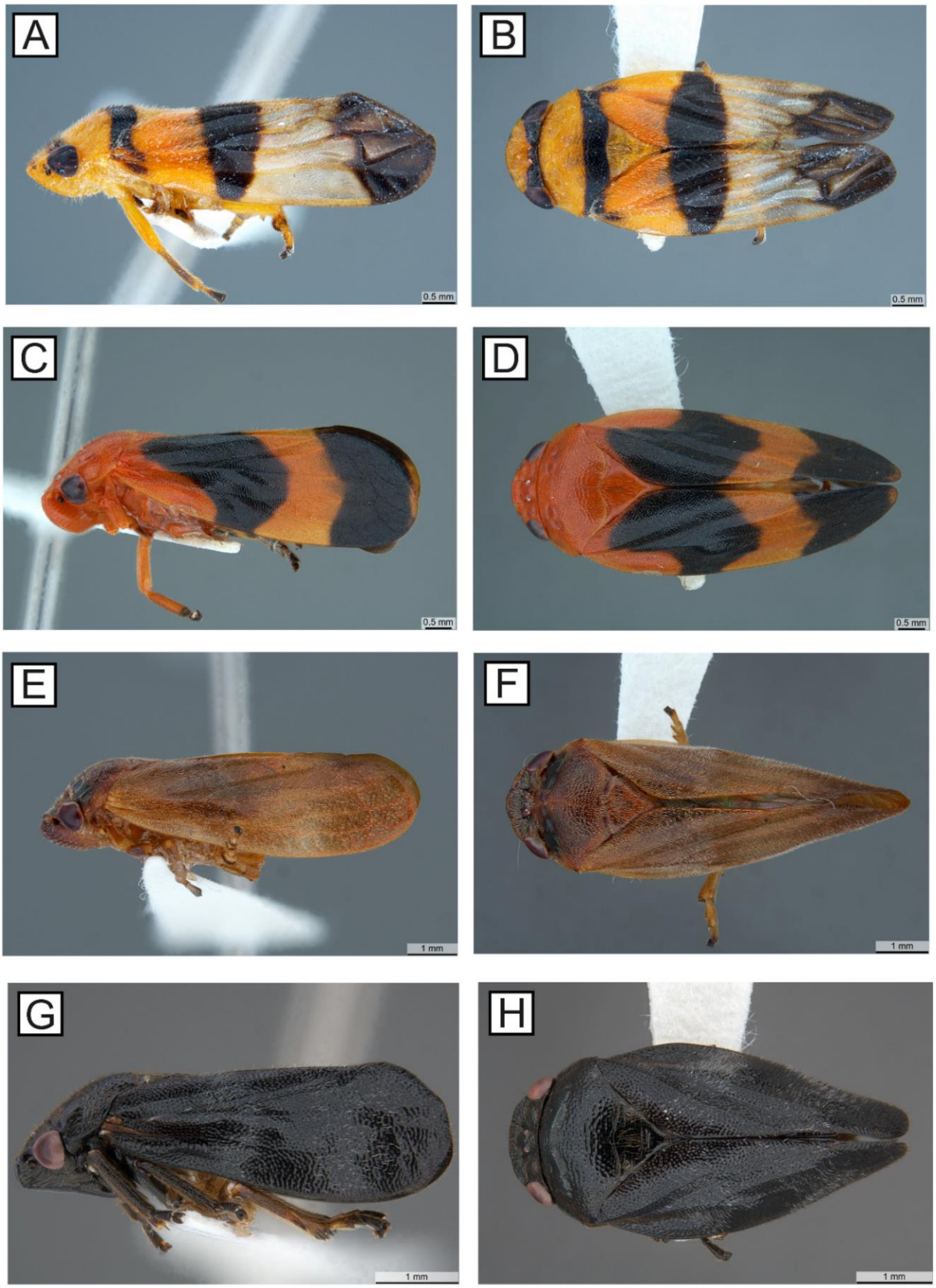


Figure 5: Habitus lateral and habitus dorsal photos showing the morphological diversity within the family Cercopidae. *Aufidus trifasciatus* (**A-B**), *Euryaulax carnifex* (**C-D**), *Petyllis deprivata* (**E-F**), *Tonnoiria* sp. (**G-H**). Scale bars are 0.5 mm (A-D) and 1 mm (E-H).

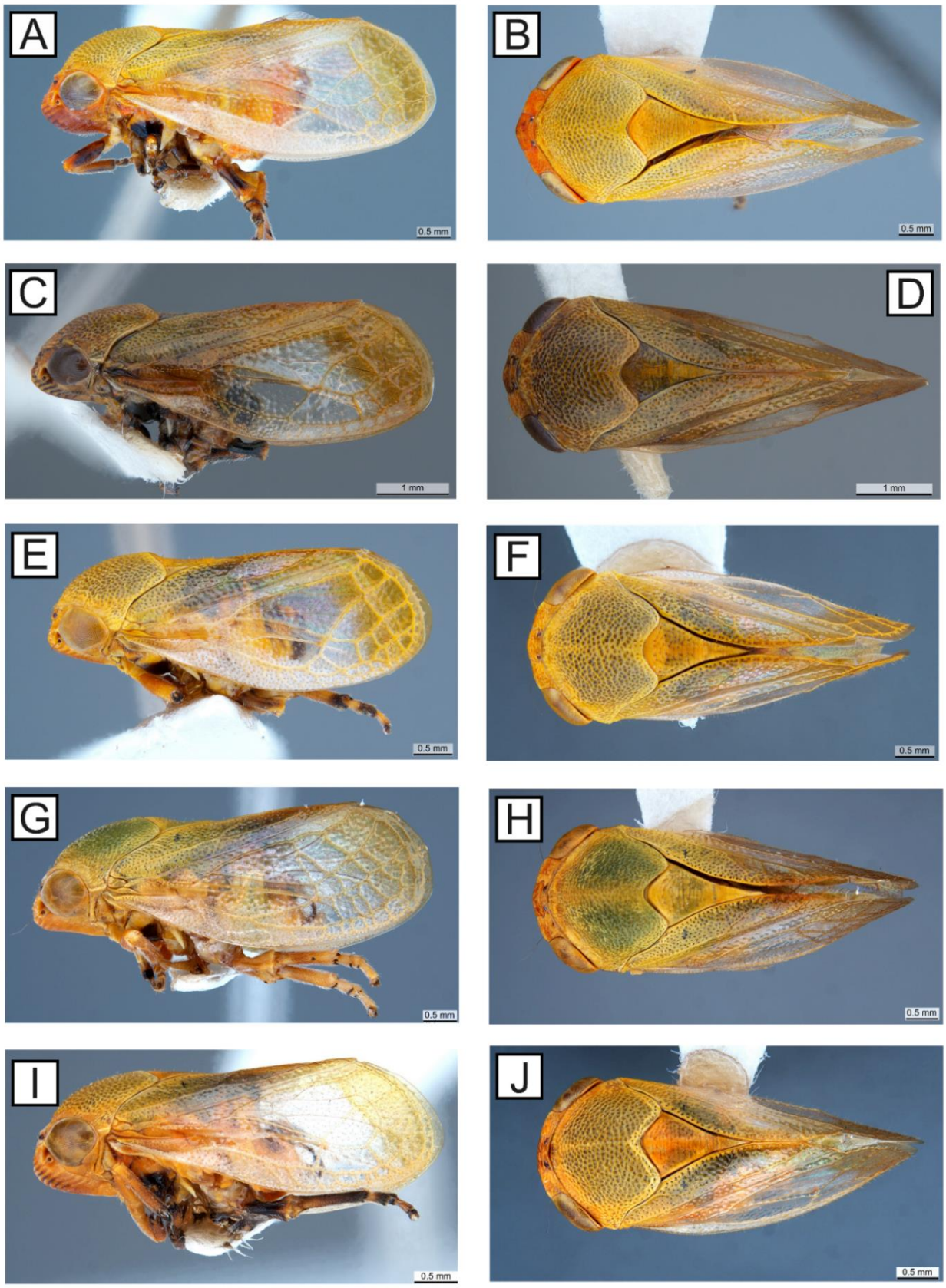


Figure 6: Habitus lateral and habitus dorsal photos showing the morphological diversity within the genus *Pectinariophyes* (Machaerotidae). *Pectinariophyes* sp. 1 (**A-B**), *Pectinariophyes* sp. 2 (**C-D**), *Pectinariophyes* sp. 3 (**E-F**), *Pectinariophyes* sp. 4 (**G-H**), *Pectinariophyes stalii* (**I-J**). Scale bars are 0.5 mm (A-B, E-J) and 1 mm (C-D).

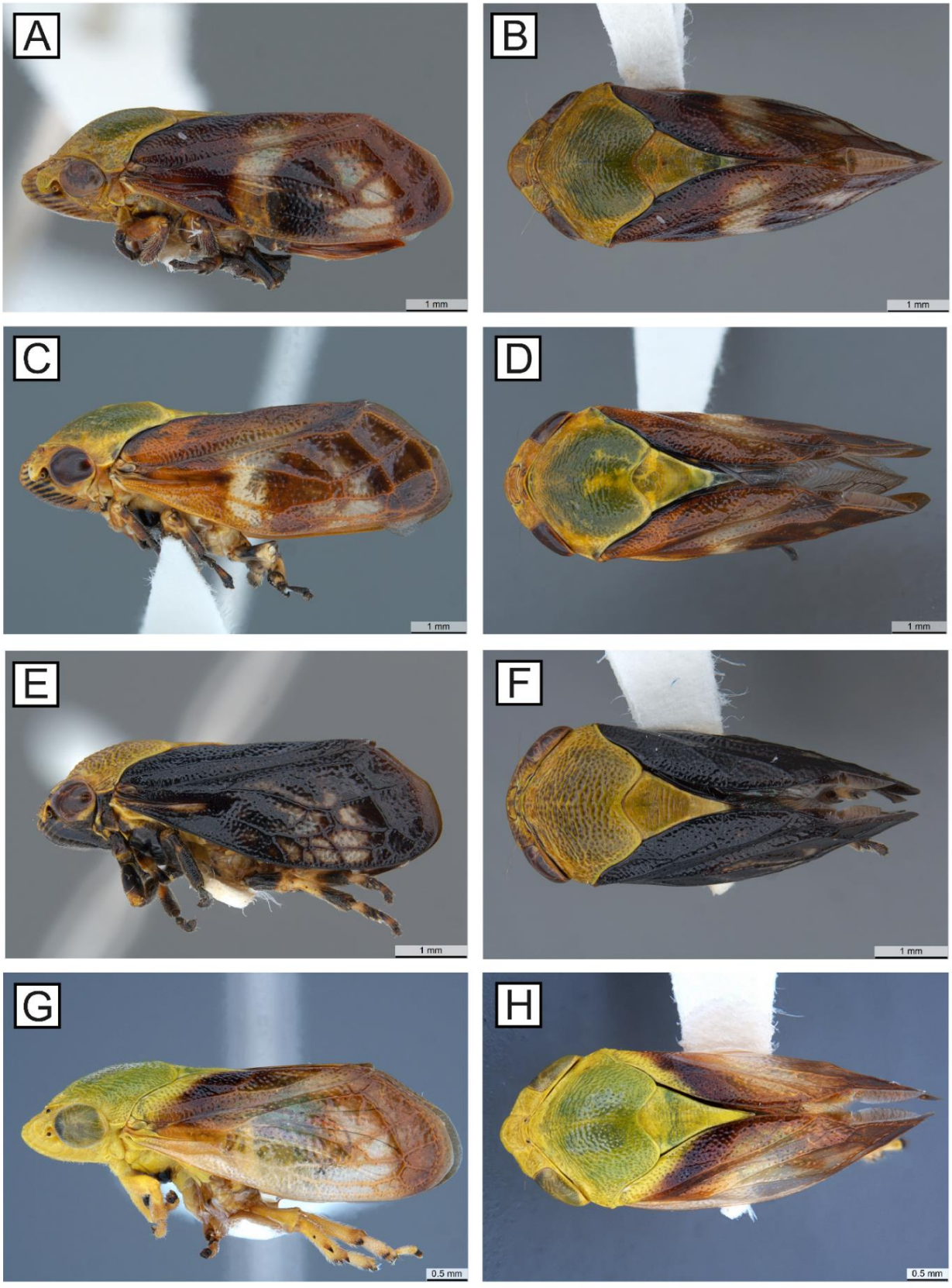


Figure 7: Habitus lateral and habitus dorsal photos showing the morphological diversity within the genus *Chaetophyes* (Machaerotidae) (Continued in Figure 8). *Chaetophyes* sp. 1 (**A-B**), *Chaetophyes* sp. 2 (**C-D**), *Chaetophyes* sp. 3 (**E-F**), *Chaetophyes* sp. 4 (**G-H**). Scale bars are 1 mm (A-F) and 0.5 mm (G-H).

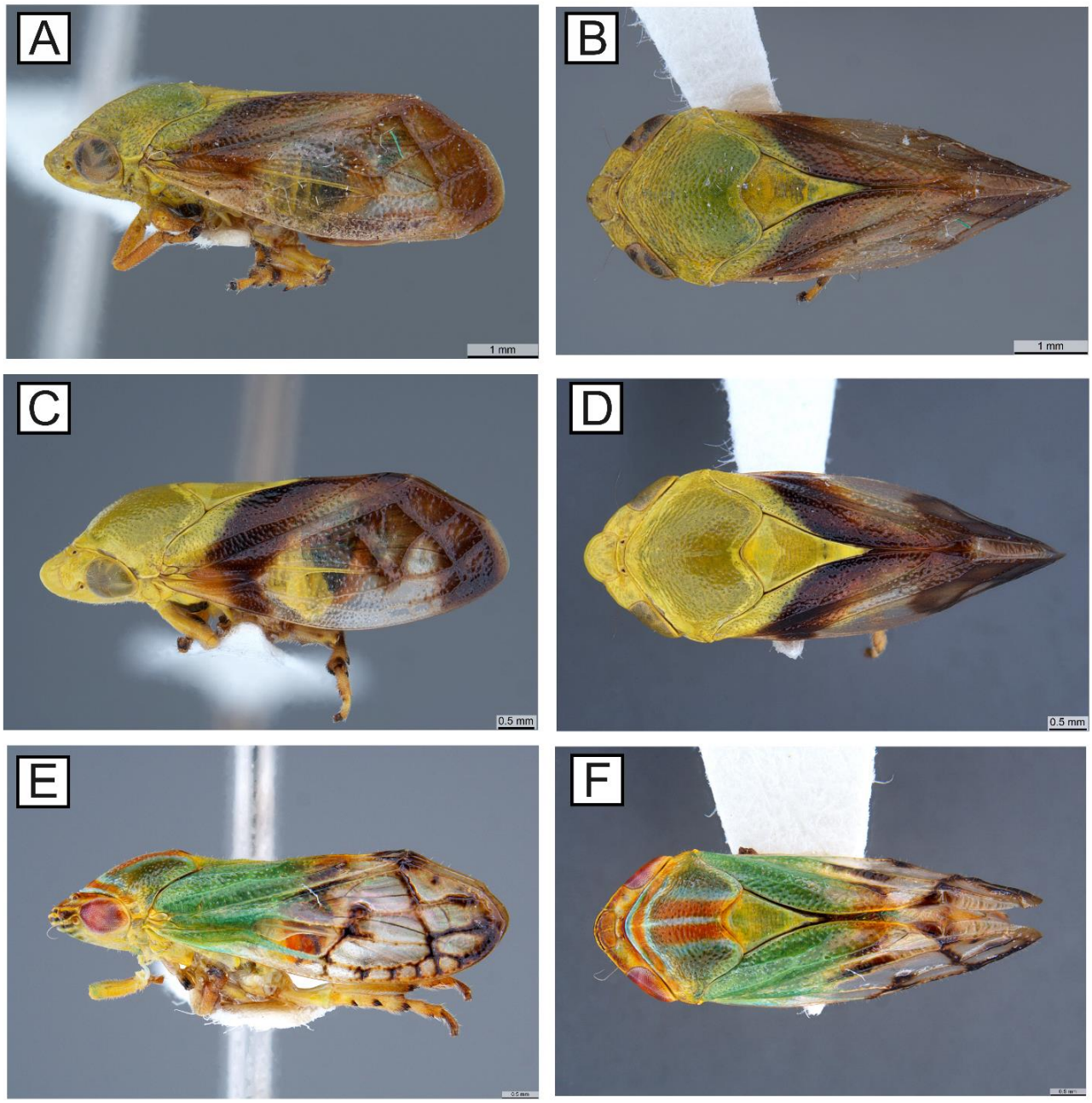


Figure 8: Habitus lateral and habitus dorsal photos showing the morphological diversity within the genus *Chaetophyes* (Machaerotidae) (Continued from Figure 7). *Chaetophyes* sp. 5 (**A-B**), *Chaetophyes admittens* (**C-D**), *Chaetophyes pulcherrima* (**E-F**). Scale bars are 1 mm (A-B) and 0.5 mm (C-F).

Discussion

Here we generated 135 COI sequences for 22 taxa, across three different families and 10 genera. This dataset forms the first comprehensive work on the genetic diversity of the Australian Cercopoidea, and it enables a better insight into the Cercopoid diversity of the country. Highlights include:

- We recorded higher-than-expected diversity for two genera, *Chaetophyes* and *Pectinariophyes,* which were expected to include four and three described species, respectively (Fletcher 2010). Instead, we recorded seven species of *Chaetophyes* and five of *Pectinariophyes,* almost doubling the number of Australian spittlebugs for these genera.
- Our assessment of genetic variation of *Bathyllus albicinctus* revealed that all specimens examined belonged to a single species, despite the large geographic range and morphological diversity observed.
- COI data highlights a strong genetic divergence from the species *Chaetophyes pulcherrima,* which may suggest that this taxon belongs to a separate genus.

The non-destructive DNA extraction approach used here enabled the preservation of reference voucher specimens deposited at the Victorian Agricultural Insect Collection (VAIC), to which our DNA barcodes will be permanently linked. This is of paramount importance for future morphological identification, and it will allow to re-examine each sample in case of taxonomical revisions and changes. This will enable to keep the taxonomical classification updated also for the genetic database we have generated, always allowing to link the genetic data to a specific voucher specimen. Furthermore, preservation of the voucher specimens allowed us to generate the high-resolution plates presented in this report. This will further facilitate morphological identification both in the laboratory and in the field, providing a field tool for growers, agronomists and biosecurity officers.

Overall, this work provides an invaluable tool for molecular-based identification of the Australian spittlebugs. For example, future barcoding and metabarcoding studies will be able to generate more precise taxonomic identification by accessing the genetic sequences we have generated here. This will provide a fast and reliable tool for the prompt identification of native and exotic spittlebugs alike. Indeed, prior to this work, the molecular record of an unidentified spittlebug could not be matched to almost any Australian native spittlebug, forcing the diagnostic identification to be based solely on morphological characters. Thanks to this work, any future molecular-based identification will confidently be able to determine an identification for the 22 taxa presented here, as well as for any additional taxon belonging to the same genera.

Recommendations

Outcomes

Based on the results obtained during this project, we have highlighted the following recommendations:

Additional field collections and molecular characterisation should be conducted to assess the Cercopoid diversity across Australia.

This should focus on the use of light trapping and net sweeping, which proved more effective than trap-based collections. The aim of this should be to collect additional species and make the Cercopoid database more comprehensive.

Successful collection of additional species/population should enable further DNA-based assessment to better understand population-level and species-level genetic diversity across Australian states.

COI-based genetic assessment not only provides additional insight into the biodiversity of the Australian Cercopoidea,but can determine the presence of new/undescribed native species. Characterisation of such “hidden” diversity is of paramount importance in case of the detection of a suspected exotic, since an unknown native species may be misidentified as exotic and lead to an expensive biosecurity response.

Further molecular work including multi-gene phylogenetic assessments and microbiome analysis can provide a powerful insight in the similarities between the Australian spittlebugs and their exotic counterpart.

The generation of a large dataset of Australian Cercopoid species freshly collected can allow additional analyses such as deeper phylogenetic work (using multiple markers in addition to COI) and microbiome analysis that would not be possible using older specimens preserved in collections. Assessing the microbiome diversity of multiple Australian native species allows to compare these with that of exotic species capable of vectoring *Xylella,* and could highlight which native species could be more at risk of transmission of the same bacteria.

Conclusion

Future directions

- The field collection conducted during the past year enabled us to collect 22 taxa, of which at least five are possibly indicative of new/undescribed Australian species. Future works should build on this expertise and further explore the native diversity to improve the genetic characterisation of the Australian Cercopoidea

- Both COI identification (for the insects) and 16S profiling (for the insect microbiome) should be extended to the entirety of the Australian cercopoid fauna. This will enable to compare the Australian native species to the exotic ones and highlight which native insects could be at risk of vectoring *Xylella* in case of an incursion.

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Supplementary Materials

Supplementary Table 1: List of samples collected for this study.

	Species	Coll. Method	State	VAIC	VAITC
1	Anyllis leiala	Hand collected	VIC	085485	11206
2	Anyllis leiala	Hand collected	VIC	085511	
3	Anyllis leiala	Hand collected	VIC	085513	
4	Anyllis tiegsi	Hand collected	VIC		11204
5	Anyllis tiegsi	Hand collected	VIC	085484	11205
6	Anyllis tiegsi	Hand collected	VIC		11207
7	Anyllis tiegsi	Hand collected	VIC		11208
8	Anyllis tiegsi	Hand collected	VIC		11209
9	Anyllis tiegsi	Hand collected	VIC	085495	11232
10	Anyllis tiegsi	Hand collected	VIC		11233
11	Anyllis tiegsi	Hand collected	VIC		11234
12	Anyllis tiegsi	Hand collected	VIC	085509	
13	Anyllis tiegsi	Hand collected	VIC	085510	
14	Anyllis tiegsi	Hand collected	VIC	085512	
15	Aufidus trifasciatus	Hand collected	QLD	072784	
16	Aufidus trifasciatus	Hand collected	QLD	072785	
17	Aufidus trifasciatus	Hand collected	QLD	072786	
18	Aufidus trifasciatus	Hand collected	QLD	085591	
19	Aufidus trifasciatus	Hand collected	QLD	085592	
20	Aufidus trifasciatus	Hand collected	QLD	085594	
21	Aufidus trifasciatus	Hand collected	QLD	085595	
22	Aufidus trifasciatus	Hand collected	QLD	085596	
23	Bathyllus albicinctus	Hand collected	VIC		11224
24	Bathyllus albicinctus	Hand collected	VIC		11225
25	Bathyllus albicinctus	Hand collected	VIC		11226
26	Bathyllus albicinctus	Hand collected	VIC		11227
27	Bathyllus albicinctus	Hand collected	VIC		11228
28	Bathyllus albicinctus	Hand collected	VIC		11229
29	Bathyllus albicinctus	Hand collected	WA	085432	
30	Bathyllus albicinctus	Hand collected	WA	085433	
31	Bathyllus albicinctus	Hand collected	WA	085434	
32	Bathyllus albicinctus	Hand collected	WA	085435	
33	Bathyllus albicinctus	Hand collected	WA	085436	
34	Bathyllus albicinctus	Hand collected	WA	085437	
35	Bathyllus albicinctus	Hand collected	WA	085438	
36	Bathyllus albicinctus	Hand collected	WA	085439	
37	Bathyllus albicinctus	Hand collected	WA	085440	
38	Bathyllus albicinctus	Hand collected	WA	085441	
39	Bathyllus albicinctus	Hand collected	WA	085442	
40	Bathyllus albicinctus	Hand collected	WA	085443	
41	Bathyllus albicinctus	Hand collected	WA	085456	
42	Bathyllus albicinctus	Hand collected	WA	085458	

43	Bathyllus albicinctus	Hand collected	WA	085459	
44	Bathyllus albicinctus	Hand collected	WA	085460	
45	Bathyllus albicinctus	Hand collected	WA	085462	
46	Bathyllus albicinctus	Hand collected	WA	085463	
47	Bathyllus albicinctus	Hand collected	WA	085464	
48	Bathyllus albicinctus	Hand collected	WA	085465	
49	Bathyllus albicinctus	Hand collected	WA	085467	
50	Bathyllus albicinctus	Hand collected	WA	085468	
51	Bathyllus albicinctus	Hand collected	VIC	085475	
52	Bathyllus albicinctus	Hand collected	VIC	072774	
53	Chaetophyes admittens	Light trap	QLD	085610	
54	Chaetophyes admittens	Light trap	QLD	085611	
55	Chaetophyes admittens	Light trap	QLD	085612	
56	Chaetophyes admittens	Light trap	QLD	085613	
57	Chaetophyes admittens	Light trap	QLD	085614	
58	Chaetophyes admittens	Light trap	QLD	085615	
59	Chaetophyes admittens	Light trap	QLD	085617	
60	Chaetophyes admittens	Light trap	QLD	085618	
61	Chaetophyes admittens	Light trap	QLD	085619	
62	Chaetophyes pulcherrima	Light trap	QLD	085633	
63	Chaetophyes pulcherrima	Light trap	QLD	085634	
64	Chaetophyes pulcherrima	Light trap	QLD	085635	
65	Chaetophyes sp. 1	Hand collected	VIC	085490	11221
66	Chaetophyes sp. 1	Hand collected	VIC	085514	
67	Chaetophyes sp. 2	Hand collected	VIC	085493	11230
68	Chaetophyes sp. 3	Hand collected	VIC	085444	
69	Chaetophyes sp. 4	Light trap	QLD	085616	
70	Chaetophyes sp. 4	Light trap	QLD	085600	
71	Chaetophyes sp. 4	Light trap	QLD	085601	
72	Chaetophyes sp. 5	Hand collected	VIC	085482	
73	Chaetophyes sp. 5	Hand collected	VIC	085483	
74	Euryaulax carnifex	Light trap	QLD	085597	
75	Euryaulax carnifex	Light trap	QLD	085598	
76	Pectinariophyes sp. 1	Light trap	QLD	085604	
77	Pectinariophyes sp. 1	Light trap	QLD	085636	
78	Pectinariophyes sp. 1	Light trap	QLD	085638	
79	Pectinariophyes sp. 1	Light trap	QLD	085639	
80	Pectinariophyes sp. 1	Light trap	QLD	085640	
81	Pectinariophyes sp. 1	Light trap	QLD	085641	
82	Pectinariophyes sp. 2	Light trap	WA	085451	
83	Pectinariophyes sp. 2	Hand collected	WA	085452	
84	Pectinariophyes sp. 3	Light trap	QLD	085637	
85	Pectinariophyes sp. 4	Light trap	QLD	085606	
86	Pectinariophyes sp. 4	Light trap	QLD	085602	
87	Pectinariophyes sp. 4	Light trap	QLD	085603	
88	Pectinariophyes sp. 4	Light trap	QLD	085607	
89	Pectinariophyes sp. 4	Light trap	QLD	085608	

90	Pectinariophyes sp. 4	Light trap	QLD	085609	
91	Pectinariophyes stalii	Light trap	QLD	085605	
92	Petyllis deprivata	Hand collected	WA	085454	
93	Petyllis deprivata	Hand collected	WA	085455	
94	Philagra parva	Hand collected	VIC	086823	
95	Philagra parva	Hand collected	VIC	086824	
96	Philagra parva	Hand collected	VIC	086825	
97	Philagra parva	Hand collected	VIC	086826	
98	Philagra parva	Hand collected	VIC	086827	
99	Philagra parva	Hand collected	VIC	086828	
100	Philagra parva	Hand collected	QLD	084733	
101	Philagra parva	Hand collected	QLD	084734	
102	Philagra parva	Hand collected	QLD	084735	
103	Philagra parva	Light trap	QLD	084736	
104	Philagra parva	Hand collected	QLD	086831	
105	Philagra parva	Light trap	QLD	086832	
106	Philagra parva	Light trap	QLD	086833	
107	Philagra parva	Hand collected	QLD	086834	
108	Philagra parva	Hand collected	QLD		11237
109	Philagra parva	Hand collected	QLD	085491	11222
110	Philagra parva	Hand collected	QLD	085445	
111	Philagra parva	Hand collected	QLD	085446	
112	Philagra parva	Hand collected	VIC	085470	
113	Philagra parva	Hand collected	VIC	085480	
114	Philagra parva	Hand collected	VIC	085481	
115	Philagra parva	Hand collected	QLD	085589	
116	Philagra parva	Hand collected	QLD	085590	
117	Philagra parva	Hand collected	QLD	085593	
118	Philagra parva	Light trap	QLD	085599	
119	Philagra recurva?	Hand collected	QLD	085588	
120	Tonnoiria sp. 1	Hand collected	VIC		11210
121	Tonnoiria sp. 1	Hand collected	VIC		11211
122	Tonnoiria sp. 1	Hand collected	VIC		11212
123	Tonnoiria sp. 1	Hand collected	VIC		11213
124	Tonnoiria sp. 1	Hand collected	VIC		11214
125	Tonnoiria sp. 1	Hand collected	VIC		11215
126	Tonnoiria sp. 1	Hand collected	VIC	085486	11216
127	Tonnoiria sp. 1	Hand collected	VIC		11217
128	Tonnoiria sp. 1	Hand collected	VIC	085487	11218
129	Tonnoiria sp. 1	Hand collected	VIC	085488	11219
130	Tonnoiria sp. 1	Hand collected	VIC	085496	11235
131	Tonnoiria sp. 1	Hand collected	VIC		11236
132	Cicadellid - Erythroneurini	Hand collected	VIC	085494	11231
133	Tartessini (Cicadellidae)	Hand collected		085642	
134	Thymbrini: Rhotidoides?	Hand collected		085492	11223
135	Membracidae	Hand collected		085489	11220

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Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (Xylella) in Australia and New Zealand by understanding its potential vectors

Technical Report: Diversity and composition of the bacterial communities (microbiome) in the spittlebugs *Bathyllus albicinctus* and *Philagra parva* (Hemiptera: Aphrophoridae).

June 2024

Francesco Martoni, Lea Rako, Duncan Jaroslow, Caitlin Selleck, Pragya Kant, Narelle Nancarrow, & Mark Blacket

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Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.



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Executive Summary

Spittlebugs and froghoppers (Hemiptera: Auchenorrhyncha: Cercopoidea) are xylem-feeding insects, with some known to cause serious economic damage worldwide through vectoring plant pathogenic bacteria, such as *Xylella fastidiosa*. Australia does not have *Xylella fastidiosa,* nor its primary vector, the exotic spittlebug *Philaenus spumarius*, but it is home to many described and undescribed spittlebug species. Amongst the more than 40 described species of spittlebugs present in Australia, *Bathyllus albicinctus* and *Philagra parva* (belonging to the family Aphrophoridae) are two of the most widely distributed, being present in almost all Australian states and territories.

Should *B. albicinctus* or *P. parva* be able to vector *X. fastidiosa*, they would be widespread biosecurity risks in case of an incursion of the pathogenic bacterium into Australia. Unfortunately, not much is known about these spittlebugs. Identification of *B. albicinctus* and *P. parva* is challenging with adults displaying high morphological diversity, which could possibly indicate cryptic taxonomic diversity, while nymphs appear morphologically similar other Aphrophoridae species and are found on a wide variety of host plants.

Here, we collected and analysed more than eighty Aphrophoridae spittlebug nymphs, from twelve host plants, across five locations in the state of Victoria (Australia) to assess diversity and composition of the bacterial communities (microbiome) harboured by *B. albicinctus* and *P. parva*. The aim of this study was to assess which factors contribute to shaping the bacterial communities harboured by these widespread spittlebug species, including geographic location, insect phylogenetics and host plant associations. We analysed the insect genetic variation using COI barcoding and assessed the bacterial diversity using 16S high throughput sequencing (HTS) metabarcoding, comparing the bacterial communities across a variety of host plants.

This study revealed very low insect genetic divergence within the insects, but a significant divergence in the diversity of the bacterial communities harboured by both *B. albicinctus* and *P. parva* nymphs feeding on different plants, even when these plants were within a few meters of each other (Figures 11 & 12). Such diverse bacterial communities may play an important role in facilitating vectoring and transmission of plant pathogenic bacteria, while the insect-plant relationships highlight the risk of transmission to many plant hosts. These results highlight the importance of understanding insect-plant-bacteria interactions, especially in a biosecurity context.

Introduction

Background

The superfamily Cercopoidea (Hemiptera: Auchenorrhyncha) is composed by more than 2600 described species worldwide, across more than 360 genera and five families (Soulier-Perkins 2015; Bartlett et al. 2018). Many Cercopoidea are known to be associated with nitrogen-fixing angiosperms, with different plant types utilised by each insect family. For example, cercopids are generally associated with nitrogen-fixing grasses, clastopterids with actinorhizal plants, and aphrophorids with legumes (Thompson 1994). In general, most spittlebugs are known to be associated with a variety of host plants but generally play a limited role in plant-pathogen transmission. For example, spittlebugs don't transmit plant viral pathogens (e.g., Nault and Ammar 1989; Bartlett et al. 2018), and are generally poor vectors of bacterial diseases due to the fact they are usually phloem-limited (Weintraub and Wilson 2010). An exception to this rule is the exotic meadow spittlebug, *Philaenus spumarius*, which is known to vector the bacterium *Xylella fastidiosa*, which causes economically important diseases such as the olive quick decline syndrome (Martelli et al. 2016).

Australia is home to at least forty described cercopoid species, belonging to at least sixteen genera and three families, including taxa with distribution limited to Norfolk, Lord Howe and Christmas Islands (Fletcher 2010). Most of these species are endemic to Australia, with some native species also present in Papua New Guinea, New Zealand and Indonesia (Fletcher 2010). These are known to be associated with a variety of host plants, including native and introduced species. Notably, no Australian cercopoid species is currently known to be a pest and no exotic species are known to be present in the country. On the other hand, the exotic *Philaenus spumarius* was introduced to New Zealand around 1960 (Archibald et al. 1979) and poses the main cercopoid threat to Australian plant health due to geographic proximity.

Insect-bacteria associations have been studied broadly, with a particular focus on hemipteran insects hosting primary and secondary symbionts required for the biosynthesis of nutrients (Munson et al., 1991; Moran et al., 1993) or vectoring plant pathogens of economic importance (Hogenhout et al. 2008; Navas-Castillo et al. 2011; Haapalainen 2014; Gilbertson et al. 2015). The obligate endosymbionts, also known as 'primary endosymbionts', supplement the insect hosts' diets through the provision of essential nutrients such as amino acids, and are vertically transmitted from the parental line to the offspring across many hemipteran insects, including aphids, psyllids and white flies (Munson et al., 1991; Thao et al. 2000a; Thao and Baumann, 2004; Baumann, 2005). The facultative endosymbionts, or 'secondary endosymbionts', are not essential for the insect host survival and one or more secondary endosymbionts of the same or different supergroups may provide advantages to their insect hosts (Buchner 1965; Thao et al. 2000b).

This is also the case for hemipteran insects of the superfamily Cercopoidea, for which two obligate symbionts were originally reported: *Candidatus* Sulcia muelleri and *Ca*. Zinderia insecticola (McCutcheon and Moran 2010). While *Ca*. Sulcia species are a common primary symbionts across other hemipteran groups, such as Cicadas and froghoppers (McCutcheon and Moran 2010), *Ca*. Zinderia insecticola was first described in spittlebugs (McCutcheon and Moran 2010). However, some spittlebugs such as the exotic *P. spumarius* have switched one of their obligate symbionts, with *Ca*. Zinderia insecticola replaced by a *Sodalis*-like species (Koga et al. 2013).

As the main vector of *Xylella fastidiosa*, researchers worldwide have recently focused their attention on *P. spumarius* and have been studying its microbial associations in greater detail (Koga et al. 2013; Koga and Moran 2014; Martelli et al. 2016; Nencioni et al. 2023). However, very little is known about the microbial communities (microbiome) harboured by other spittlebugs and what factors may impact their diversity and composition.

Project Objectives

A major objective of this project was to better understand host plant use, including feeding behaviour and physiology, of potential Australian *Xylella* vector insect species.

Study Aim

Here we analysed the bacterial communities associated with fourteen populations belonging to two species of spittlebugs - *Bathyllus albicinctus* and *Philagra parva* (both family Aphrophoridae) - collected from twelve host plants in Victoria, Australia. We compared bacterial communities associated with these insects across different spittlebug populations, different host plants and different geographic areas.

The main aims of this feeding behaviour & physiology research were:

1) To explore bacterial diversity (microbiome) of two widespread Australian spittlebug species and compare their obligate symbionts with those of other spittlebugs recorded worldwide and with those spittlebug species known to vector *Xylella*.
2) To assess whether factors such as geographic location and host plant associations contributed to shaping the bacterial communities associated with each insect population.

Methodology

Sample collection

Immatures (nymphs) were generally found surrounded by “spittle”, as is common for Aphrophoridae species, and were collected using a fine brush to transfer them in a vial containing high grade (100%) ethanol. Adults were collected using entomological aspirators or captured manually into a vial, then immediately killed using high grade ethanol.

A total of six adult and 64 immature specimens of *Bathyllus albicinctus* were collected from three different locations in Victoria and from at least ten host plants (Table 1). Additionally, seventeen immature specimens of a second aphrophorid species, *Philagra* sp., were collected from three populations and two different locations to be used as comparison (Table 1).

DNA extraction, amplification, and sequencing

Insects

DNA was extracted from single individual insects using a DNeasy Blood and Tissue kit (Qiagen, CITY). Briefly, individuals were fully homogenised using a glass beads and left overnight (~17 hours) at 50 ºC in a 1:9 solution of proteinase K and ATL buffer. The following day, DNA was extracted following the manufacturer’s instructions and re-eluted in 100 µL. The same DNA extract was used to amplify both insect and bacterial DNA.

Due to the morphological similarity of aphrophorid nymphs, we used the DNA barcoding technique (Hebert et al. 2003) to assess taxonomic identity and genetic variability across the different populations. A ~700 bp fragment of the subunit I of the *cytochrome oxidase* (COI) mitochondrial gene was targeted using the primer pair LCO1497 / HCO2198 (Folmer et al. 1994) or the newly designed primers Cercopoidea-F 5’-TTYGGDATTTGATCAGGAATAATTGG-3’ / Cercopoidea-R 5’- GAATAAATGTTGRTATAAAATWGGRTC-3’ (640 bp fragment internal to LCO-HCO), and the following PCR cycling conditions: initial denaturation at 94 ºC for 2 minutes, followed by 30 cycles at 94 ºC for 30 seconds, 50 ºC for 45 seconds, 72 ºC for 45 seconds, and a final extension phase at 72 ºC for 7 minutes. Successful amplification was confirmed running the PCR product on 1% Agarose gels for 30 minutes at 100V.

Successful PCR amplicons were sequenced both ways using an external provider (Macrogen Inc, Korea). Electropherograms were analysed using the software MEGA v11 (Kumar et al. 2018). Forward and reverse sequences were paired and a consensus sequence was generated. A COI gene tree was generated using the Neighbour Joining algorithm and 5,000 bootstrap replicates (Figure 1, on the left). The COI sequences

obtained in this study were trimmed to be aligned together with the shortest sequence generated (542 bp) and the software PopART (Leigh and Bryant 2015) was used to perform a Median Joining Network analysis (Bandelt et al. 1999) with ε = 0 (Figure 1, on the right).

Table 1: Aphrophorid populations collected for this study. The table includes spittlebug species, location, and host plant.

Insect Species	Location	Host plant	Individuals	AgVic Sample #
Bathyllus albicinctus	Bundoora, VIC	*Carpobrotis* sp.	*2*	35-36
		Dianella sp.	*2*	40-41
		Myoporum sp.	*5*	29-34
		Poa sp.	*3*	37-39
		Westringia sp.	*9*	21-28
	Grampians, VIC	*Gonocarpus* sp.	*5*	76-80
		Calluna sp.	6	67-72
		Olea sp.	3	73-75
	Horsham, VIC	Lamiaceae	10	11-20
			6	52-57
		Petroselinum	3	64-66
		Westringia sp.	*10*	1-10
			6	58-63
***Philagra* sp.**	Coburg, VIC	*Acacia* sp.	*7*	81-87
	Moolap, VIC	*Acacia* sp.	*6*	42-47
		Casuarina sp.	*4*	48-51

Bacteria

For the analysis of the bacterial communities, we used a 16S metabarcoding approach, similar to that presented in Martoni et al. (2023). Here, we targeted a ~253 bp fragment of the 16S gene using the primers Fwd515 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA) - Rev806 (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT) (Caporaso et al. 2011), modified with partial Illumina adapters (underlined) and the following PCR cycling conditions: initial denaturation at 94 ºC for 2 minutes, followed by 35 cycles at 94 ºC for 30 seconds, 50 ºC for 45 seconds, 72 ºC for 45 seconds, and a final extension phase at 72 ºC for 7 minutes. Successful amplification was confirmed running the PCR product on 1% Agarose gels for 30 minutes at 100V.

The remainder of the Illumina adapter sequences containing 8 bp unique dual indexes were then attached to each PCR product using a real-time PCR (rtPCR) with the Phusion High Fidelity PCR kit (Thermo Fisher Scientific, MA, USA) following Martoni et al. 2023a. Each 50 µL reaction consisted of 32.5 µL of molecular grade water, 10 µL of 5X Phusion HF buffer, 1 µL of dNTP mix, 1 µL of 1/1000 SYBR Green I Mix, 0.5 µL of Phusion DNA polymerase, along with 4 µL of adapter primer mix with indexes unique to each sample (2.5 µM) and 1 µL of PCR product. The real-time PCR began with 30 second at 98 °C, followed by 7 cycles of: 98 °C for 10 seconds, 65 °C for 30 seconds and 72 °C for 30 seconds, with fluorescence measurement conducted in the 65 °C and 72 °C phases. The amplification curve was visually inspected in real time and stopped while still in the exponential phase to prevent over-amplification artefacts. DNA concentrations of each library were normalised using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions, but re-eluting in 15 µL instead of 20 µL, then all samples were pooled together by target barcode. The DNA fragment size and concentration of the three pooled barcode libraries were measured using a High Sensitivity D1000 ScreenTape assay on a 2200 TapeStation (Agilent Technologies, CA, USA). Pooled libraries were then cleaned and concentrated using the ProNex Chemistry Kit, following the manufacturer's instructions to remove fragments > 350 base pairs.
The final 16S library was diluted to a concentration of 7 pM, spiked with 15% PhiX and sequenced using V3 chemistry (2 x 250 bp reads) on a single flow cell on an Illumina MiSeq system (Illumina, CA, USA).

Bioinformatic analysis.

Raw sequence reads were demultiplexed using bcl2fastq allowing for a single mismatch to the expected index combinations, then trimmed of PCR primer sequences using BBDuK v38 (Bushnell, Rood & Singer, 2017), retaining only sequences with primers present in the forward and reverse reads. Sequence quality profiles were then used to remove reads with more than one expected error (Edgar & Flyvbjerg 2015), or those containing ambiguous 'N' bases. Due to the natural length variability in the 16S amplicons, a minimum read length of 50bp was applied with no truncation or maximum length filtering. Filtered sequences were dereplicated and denoised into amplicon sequence variants (ASVs) using DADA2 v1.26 (Callahan et al. 2016), with the error model estimated separately for each amplicon, then chimeras were detected and removed de-novo using the removeBimeraDenovo function in DADA2. Taxonomy was assigned to the

filtered ASVs using the IDTAXA algorithm of Murali et al. (2018) implemented in the DECIPHER v2.26 R package, using a separate reference database for each target marker. The ASVs were taxonomically assigned using the Genome Taxonomy Database v202 (Parks et al. 2018). Species-level taxonomic assignments with IDTAXA were only accepted if they surpassed a bootstrap confidence threshold of 60% or above, otherwise the ASV was classified to a higher taxonomic rank for which this bootstrap criterion could be achieved. Finally, the ASVs with less than 10 reads were discarded from the dataset before generating a phylogenetic tree using the package phangorn v2.11.1 (Schliep 2011), with the Neighbour Joining algorithm and the GTR model.

Statistical analysis

All bioinformatic and statistical analyses were conducted within the R v4.3.1 statistical computing environment (R Core Team, 2024) and RStudio v4.2.2 following Martoni et al. (2023a,b), using amplicon sequence variants (ASVs) as the diversity unit. For α-diversity measures, we used three complementary metrics to account for phylogenetic distance (phylogenetic diversity – pd; Faith, 1992) and abundance (Shannon diversity; Shannon, 1948), as well as simple presence- absence (observed richness). Observed richness and Shannon diversity were calculated using the phyloseq v1.44 R package (McMurdie & Holmes 2013), and phylogenetic diversity was calculated using the picante v1.8.2 R package (Kembel et al., 2010). We then used α-diversity to compare mean observed diversity and standard error across all host plants. For β-diversity analysis, we used three distance metrics (Jaccard, Aitchison and Unifrac). These assessed presence-absence of taxa (Jaccard index; Jaccard, 1912), their relative abundance within a compositional data analysis framework (Aitchison distance; Aitchison et al., 2000) and phylogenetic divergence as well as relative abundance between samples within a similar compositional framework (Unifrac; Lozupone et al. 2011). Principal coordinates analysis (PCoA; Figures 5-6) was used to graphically represent the relationships between samples in multidimensional space using the β-diversity dissimilarity matrices. Finally, we compared β-diversity between host plant and locations method using permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) tests using the adonis2 function from the vegan v2.6.4 R package (Oksanen et al., 2020).

Functional annotation

Bacterial ASVs were assigned to ecological groups utilising the Functional Annotation of Prokaryotic Taxa (FAPROTAX) database v1.2.7 (Louca et al., 2016), following the analysis presented in the work of Vido *et al.* (2024), and using the R package microeco v1.6.0 (Liu et al. 2021). FAPROTAX is a database enabling to match bacterial ASVs to metabolic or ecologically relevant groups by using information collated from published literature.

Key Research Findings

Insect COI diversity

A total of 84 COI sequences were generated for this study. The COI sequences were used to determine the identity of the species included in this work, and 69 sequences matched sequences available on public database for *Bathyllus albicinctus,* while 15 sequences were determined to belong to *Philagra parva* based on match to private datasets (Martoni et al. unpublished).

The COI sequences of both *Bathyllus albicinctus* and *Philagra parva* were used to generate a haplotype network analysis to assess whether genetic variation could be observed across populations on different plants. However, this proved not to be the case (Figure 1). While small genetic variations could be observed, ranging between 0-0.56% for *Bathyllus albicinctus* and between 0-0.18% for *Philagra parva*, these variations did not correspond to collection location or host plant. The intraspecific genetic variation observed for both species was <1%, confirming that all specimens collected across Victoria belonged to the same two species.

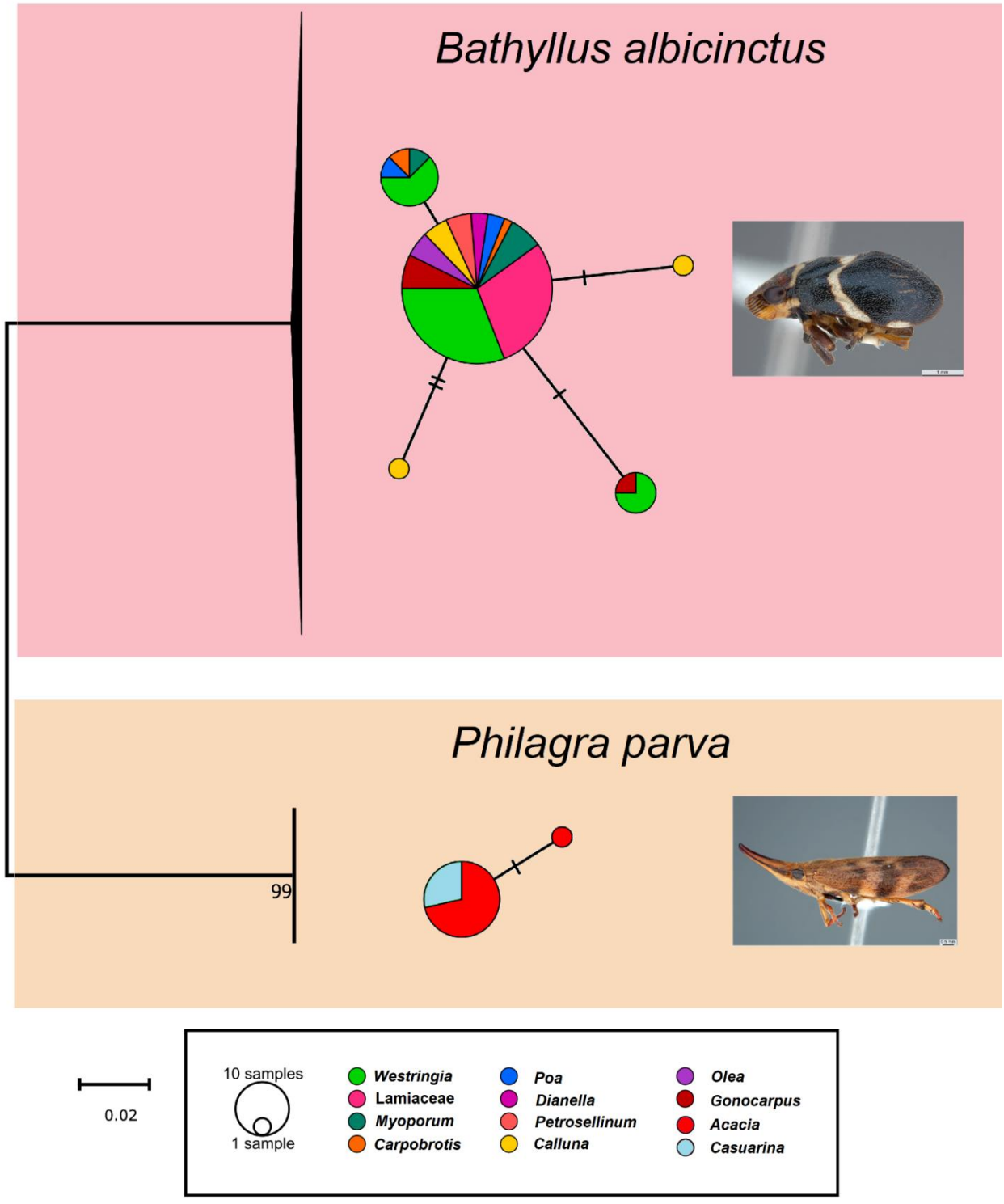


Figure 1: Haplotype network analysis of the 84 COI sequences of *Bathyllus albicinctus* and *Philagra parva* generated for this study. The network was obtained using 84 partial COI DNA sequences. The network is colour-coded based on the host plant from which each specimen was collected. The Neighbour Joining COI tree on the left was generated using 5,000 bootstrap replicates, and the scale bar represents a 2% genetic variation.

Bacterial 16S diversity

The 16S microbiome of all 87 insect samples, belonging to two species (*Bathyllus albicinctus* and *Philagra* sp.), collected from 12 host plants, were successfully sequenced (Figure 2).

A total of 15,736,749 reads were retained after quality control and filtering. These belonged to 1,284 ASVs, belonging to 39 bacterial classes and 84 orders (Figure 2). The top 12 ASVs recorded here contributed 13,602,517 reads (86.44%) and included symbiotic bacteria, plant growth-promoting bacteria as well as potential pathogenic taxa (Table 2).

Of the 1,284 bacterial ASVs retained for this work, 534 matched with at least one of the 92 functional groups present on the FAPROTAX database (Figure 3). Across our dataset, most bacterial ASVs matched the function of aerobic chemoheterotrophy (Figure 3), followed by anaerobic chemoheterotrophy, fermentation, methanol oxidation, methylotrophy, nitrate reduction and animal symbiosis/parasitism. Some of the ASVs that did not match, such as *Ca.* Sulcia muelleri*,* are probably too specialised and are not present in the FAPROTAX database.

Table 2: Top 12 ASVs by number of reads. The table shows the top 12 ASVs recorded in this study by number of reads. These contributed 13,602,517 reads, 86.44% of the total number of reads.

Class	Order	Family	Species	Reads
Bacteroidia	Flavobacteriales	Blattabacteriaceae	*Ca.* Sulcia muelleri	6,776,261
Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	*Ca.* Zinderia	4,125,708
Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas* sp.	545,021
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Agrobacterium* sp.	486,082
Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium* sp.	433,486
Alphaproteobacteria	Rickettsiales	Anaplasmataceae	*Wolbachia pipientis*	332,017
Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	*Ca.* Zinderia insecticola	217,127
Bacteroidia	Flavobacteriales	Weeksellaceae	*Chryseobacterium* sp.	192,723
Gammaproteobacteria	Burkholderiales	Burkholderiaceae	*Pigmentiphaga* sp.	148,379
Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Alkanindiges* sp.	142,490
Gammaproteobacteria	Burkholderiales	Burkholderiaceae	*Janthinobacterium* sp.	107,390
Gammaproteobacteria	Burkholderiales	Burkholderiaceae	*Ralstonia* sp.	95,833

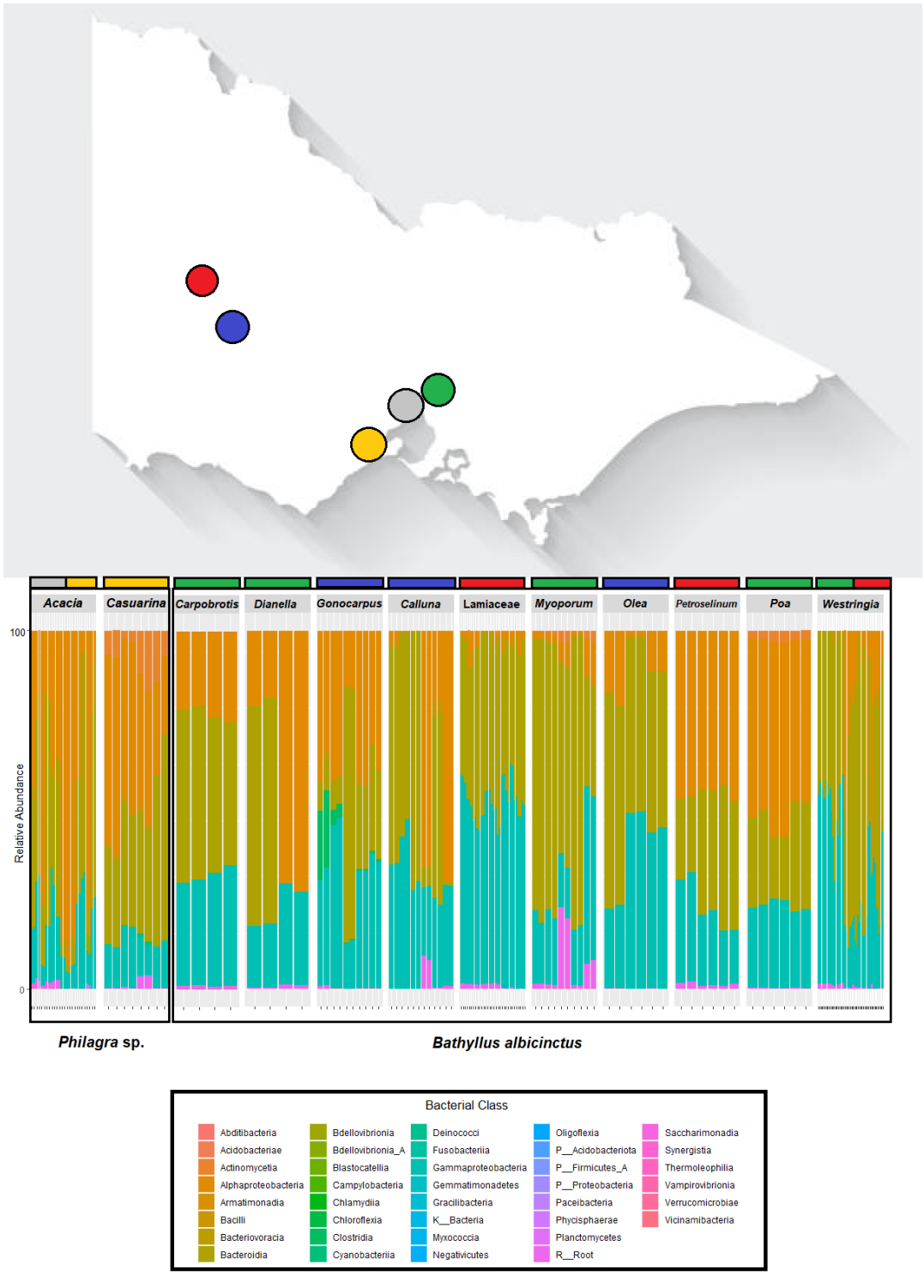


Figure 2: Relative abundance of bacterial classes across *Bathyllus albicinctus* and *Philagra* sp. The plot highlights the diversity of bacterial classes recorded across the 87 samples collected on 12 host plants, from five locations. Locations are highlighted in the map: Horsham (red), Grampians (blue), Bundoora (green), Coburg (grey) and Moolap (yellow).

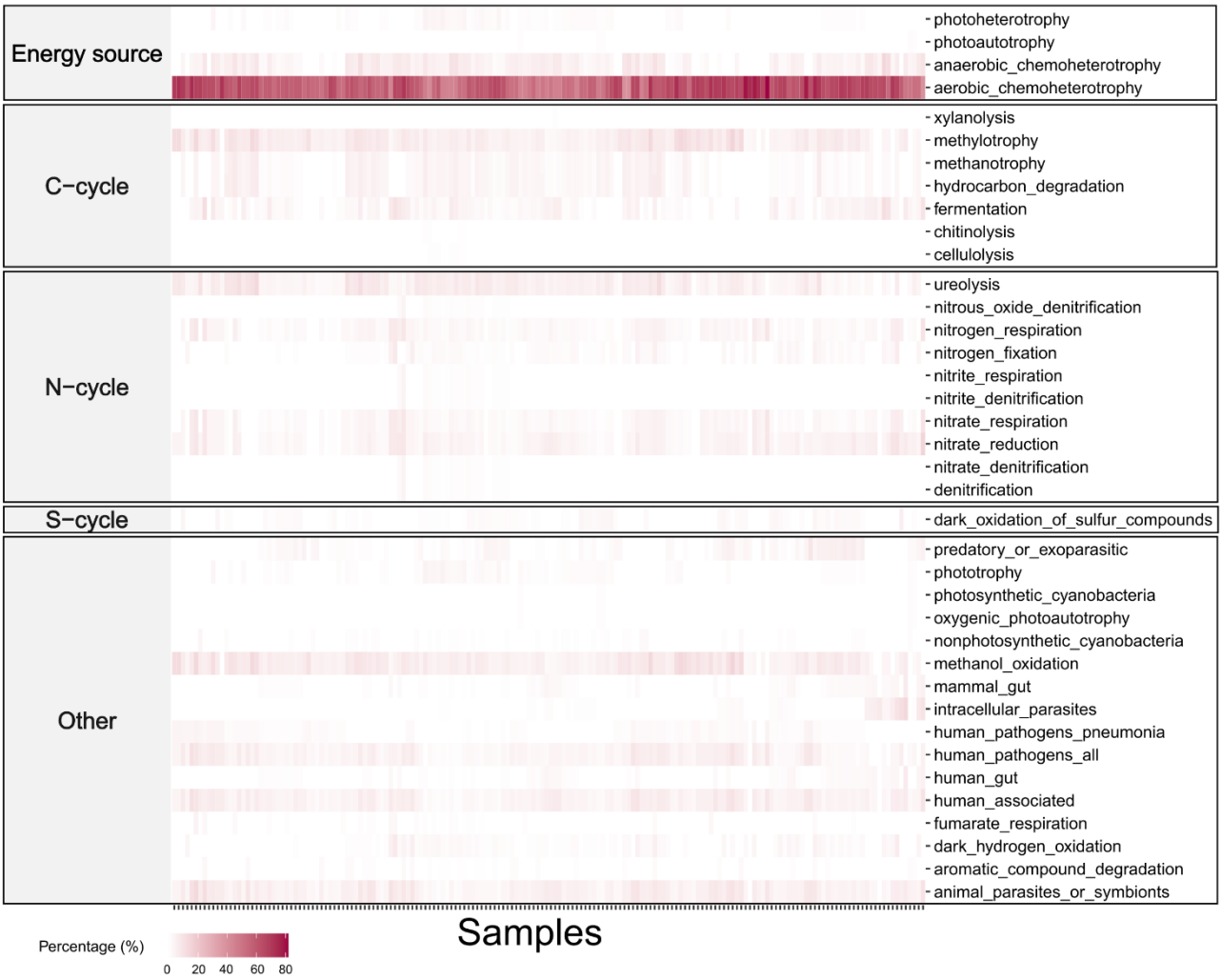


Figure 3: Heat map of functional groups identified for the ASVs of each sample. The main categories (on the left) include different functional groups (on the right). The heat map shows aerobic chemoheterotrophy as the main function across most of the samples analysed here.

Symbionts and beneficial bacteria

The two ASVs with the highest number of reads were identified as *Candidatus* Sulcia muelleri (6,776,261 reads, ~43%) and *Candidatus* Zinderia sp. (4,125,708, ~26.2%). The first ASV matched with >99% similarity other sequences present on GenBank, while the second ASV matched *Ca.* Zinderia insecticola, but only with ~94% similarity. An additional ASV of a *Ca.* Zinderia sp. (217,127 reads, 1.4%) matched with 100% similarity *Ca.* Zinderia insecticola sequences.

Other symbiotic and potentially symbiotic bacteria recorded here, including *Wolbachia pipientis* (332,017 reads, 2.1%), a *Pigmentiphaga* species (148,379 reads, 0.94%), a species from the genus *Alkanindiges* (142,490 reads, 0.91%), and a species of *Brevundimonas* (545,021 reads, 3.5%).

Plant growth-promoting bacteria, environmental bacteria, and potential pathogens

The other two bacteria with the highest number of reads were from another bacterium recorded amongst the top 12, *Janthinobacterium* (107,390 reads, 0.68%), known to be present in the microbiota of many different environments.

Some of the bacteria recorded here could potentially belong to plant pathogenic groups. These include ASVs from the genera *Rhodococcus, Rathayibacter, Ralstonia, Xanthomonas.* However, pathogenicity can only be hypothesised, and not inferred, from analyses of 16S sequencing, as 16S is not considered diagnostic due to not being able to discriminate some bacterial taxa. Further targeted testing conducted on the insect DNA extracts in this study for did not detect any known plant associated *Ralstonia* (Appendix I).

Bacterial composition across populations

When analysing the bacterial composition across samples a number of aspects can be extrapolated from this study. Firstly, both the insect species and the insect-plant association appear to play a role in shaping the bacterial composition of our samples both when looking at the α-diversity and at the β-diversity (Figures 4, 5, 6).

When analysing the observed α-diversity across different populations of *Bathyllus* (Figure 4), observed bacterial diversity in the samples collected from *Myoporum* and *Carpobrotis* appeared considerably higher compared to all other populations, while the population collected from *Westringia* in Horsham showed the lowest α-diversity (mean observed diversity ± SE: *Myoporum* = 165.5 ± 15.42, *Carpobrotis* = 173 ± 14.06, *Poa* = 124.5 ± 4.11, *Gonocarpus* = 95.2 ± 3.16, *Westringia* from Bundoora = 77.1 ± 6.04, *Dianella* = 76.75 ± 7.28, Lamiaceae = 72.09 ± 4.28, *Calluna* = 56.92 ± 8.68, *Petroselinum* = 54.33 ± 4.37, *Olea* = 53.67 ± 10.83, *Westringia* from Horsham = 43.59 ± 3.24).

On the other hand, the samples of *Philagra* from Moolap showed a higher observed α-diversity compared to those from Coburg, even when both populations were collected from the same type of host plant, *Acacia* (Figure 4) (mean observed diversity ± SE: *Acacia* from Moolap = 116.67 ± 6.12, *Casuarina* from Moolap = 130.12 ± 8.86, *Acacia* from Coburg = 37.79 ± 5.89).

The ADONIS test revealed a significant difference in bacterial β-diversity among host plants and locations, with between 39% and 55% of the variance in sample composition explained by host plant (Jaccard R^2 = 0.39, p = 0.001; Aitchison R^2 = 0.55, p = 0.001; Unifrac R^2 = 0.37, p = 0.001), and between 23% and 34% explained by the location (Jaccard R^2 = 0.23, p = 0.001; Aitchison R^2 = 0.34, p = 0.001; Unifrac R^2 = 0.25, p = 0.001). However, since different host plants were often collected at different locations, these results may be correlated. When examining the Jaccard PCoA plot (Figure 5) we observed a clear separation between the beta diversity of the two species (*Bathyllus* and *Philagra*), but we can also highlight a separation of the Lamiaceae and clear clustering between samples of the same host plant (e.g., *Myoporum, Dianella, Carpobrotis*).

In order to better understand the role played by the host plant in shaping the bacterial diversity of the spittlebugs, we selected a subset of 20 samples, all collected in Horsham at the same time of the year, from two host plants: a *Westringia* sp. and a Lamiaceae sp. This dataset enabled us to eliminate any effect caused by the location and focus our attention on the role of the host plant.

The 20 specimens analysed were collected from four different groups: five were collected from a Lamiaceae plant (Figure 6, left), five were collected from a *Westringia* (Figure 6, right), five were collected from a plant of the same Lamiaceae species, but growing intertwined with the *Westringia* (Figure 6, inset), and five from the *Westringia* in the spot where this plant was growing intertwined with the Lamiaceae (Figure 6, inset). When examining both the Jaccard and the Aitchison PCoA plots, we could observe the bacterial communities of the insects collected from the different groups, with the two groups from Lamiaceae clearly separate from the two groups from *Westringia* (Figure 6).

When comparing the bacterial species between the *Westringia* populations and the Lamiaceae populations (Figure 7), some taxa appeared to be equally present across both host plants. This was the case, for example, for both the known symbionts of spittlebugs (*Ca.* Sulcia muelleri and *Ca.* Zinderia). On the other hand, some taxa appeared to be associated with either one or the other plant (Figure 7). For example, *Brevundimonas, Neorhizobium, Massilia* species appeared to be consistently associated with *Westringia* (Figure 7). Amongst the bacteria associated more consistently with Lamiaceae, we recorded *Sphingobacterium, Alkanindiges, Dyadobacter,* Burkholderiaceae species, and *Devosia* (Figure 7).



Figure 4: Bacterial α-diversity (observed bacterial species) across the two spittlebug species (*Bathyllus albicinctus* and *Philagra* sp.), the 12 host plants and the five collection localities (colour-coded). For this plot, α-diversity was measured using observed diversity (number of ASVs).

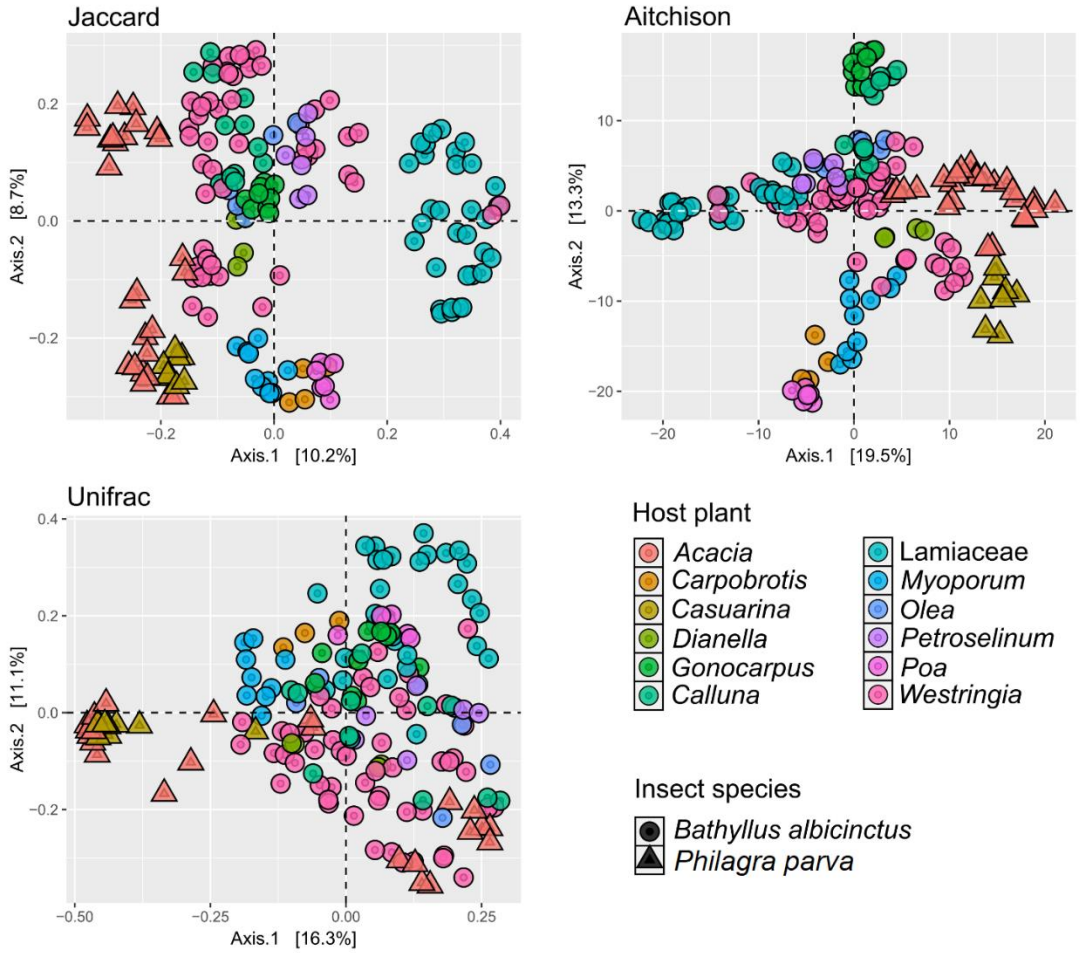


Figure 5: PCoA of the bacterial β-diversity recorded from all samples analysed for this work. The plot shows differences in the β-diversity harboured by insects belonging to different species (*Bathyllus albicinctus* and *Philagra parva*) as well as by populations of the same species feeding on different host plants.

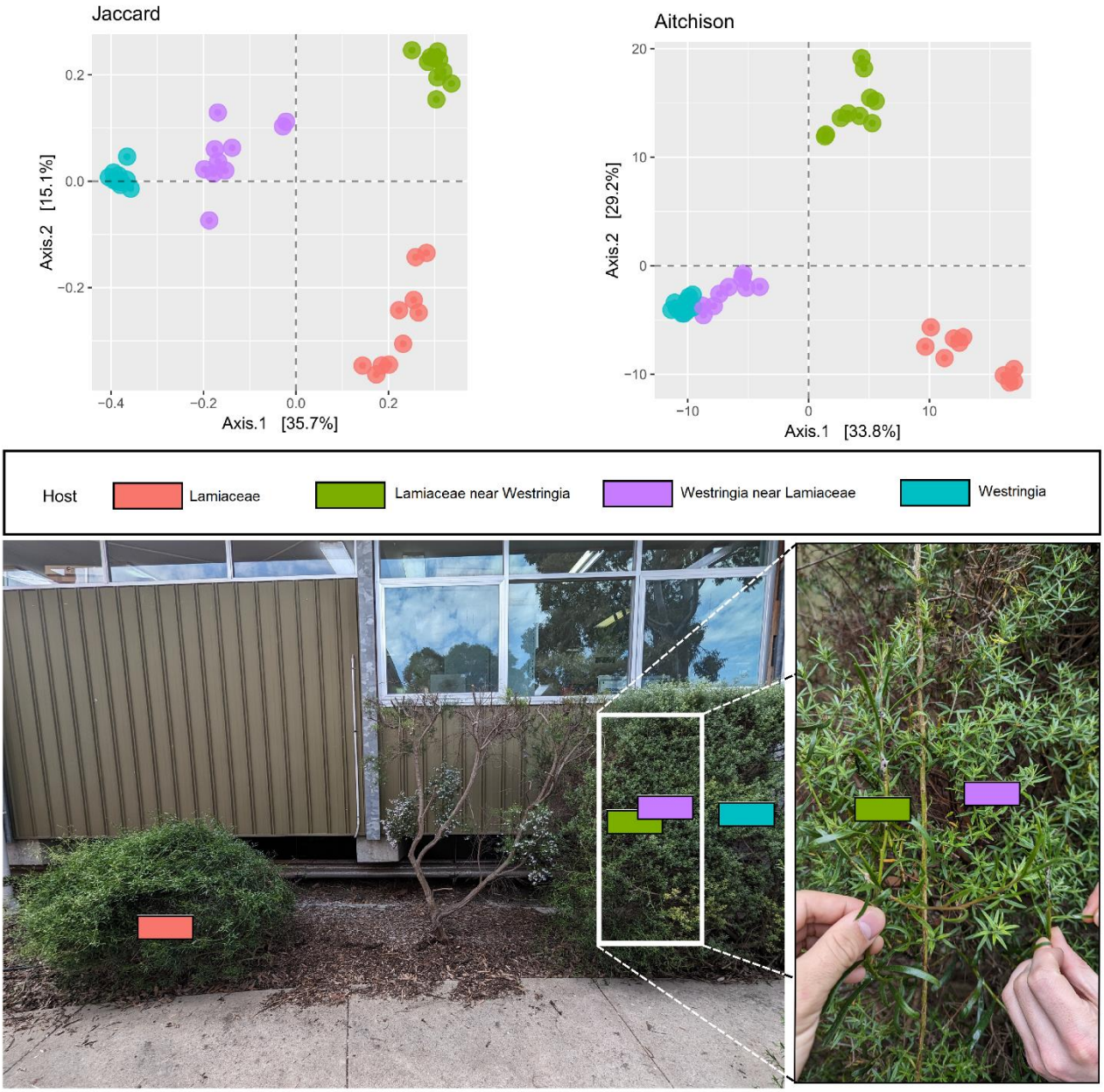


Figure 6: PCoA of the bacterial β-diversity recorded from *Bathyllus albicinctus* specimens collected in Horsham on two plants. The PCoA plot shows differences in the β-diversity harboured by insects feeding on different plants at the same location.

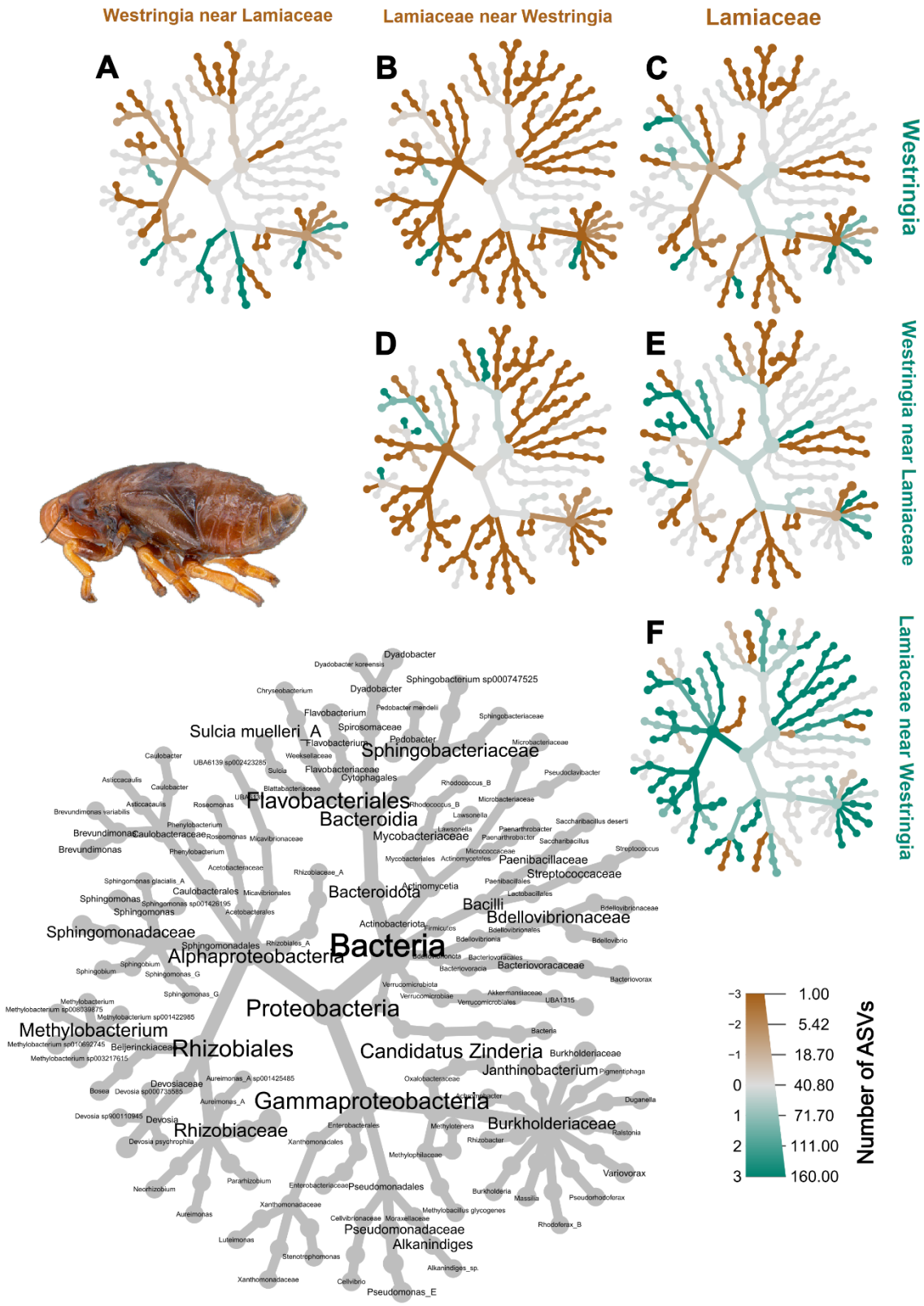


Figure 7: Heat trees comparing the insect diversity recorded by each sample from the Horsham subset, subdivided by host plant. The grey tree on the lower left functions as a key for the unlabelled trees. Each of the smaller trees represent a comparison between two samples, each from a specific plant. A taxon branch coloured brown is more abundant in the sample with the brown label, and a taxon branch coloured green is more abundant in the sample with the green label.

Discussion

Assessing whether insect genetic diversity may impact bacterial communities

Bacterial communities associated with insects have often been found to be strictly associated with the host's systematics, with closely related insect species showing more similar bacterial communities, a phenomenon referred to as 'phylosymbiosis' (Colman et al. 2012; Lim and Bordenstein 2020). This has been shown to be the case for other hemipteran insects, such as psyllids (Martoni et al. 2023), and it is often considered an evolutionary mechanism allowing insects to specialise on feeding from their host plant.

To determine whether the insect genetic diversity played a role in shaping the bacterial communities observed in this study, we compared the insect COI gene across all specimens analysed here. The bacterial communities of *Bathyllus albicinctus* clearly separated from those of *Philagra* sp., suggesting that different species may harbour different bacterial communities. While we included only two Australian spittlebug species in this work, these preliminary results would suggest that a form of phylosymbiosis may be present in Australian spittlebugs.

However, the primary aim of this study was not to assess bacterial communities across species, especially considering the limited number of taxa included here (which should be a topic for further research). Instead, we aimed to assess if bacterial communities could vary across different insect populations hosted across different host plants. In order to explore this, COI variation was assessed across all populations of *Bathyllus albicinctus,* and was reported to vary between 0-0.56% across the different populations collected in Victoria, from 10 different host plants. Therefore, with an intraspecific genetic divergence <1%, all populations were found to belong to the same species. Furthermore, the genetic divergence appeared to be not correlated with host plant association, and was therefore not the main factor shaping the microbial communities across different host plants.

Bacterial diversity in *Bathyllus albicinctus*

Here we recorded the two long-term obligate symbiotic bacteria known to be hosted in spittlebugs: *Ca.* Zinderia insecticola and *Ca.* Sulcia muelleri. Both bacteria have previously been recorded in spittlebugs (McCutcheon and Moran 2010) but had never been recorded in *Bathyllus albicinctus.*

Interestingly, this highlights an important difference between the bacterial microbiome of *B. albicinctus* and exotic *Philaenus spumarius,* the main exotic vector of the plant pathogen *Xylella fastidiosa.* In fact, in *P. spumarius,* the symbiont *Ca.* Zinderia insecticola was found to be replaced by a *Sodalis*-like symbiont (Koga et al. 2013). The symbiont swap from *Ca.* Zinderia to a *Sodalis* species has been hypothesised to favour host functionality, as free-living bacteria possess many more metabolic capabilities than anciently associated obligate symbionts (Koga and Moran 2014).

Another symbiotic bacterium recorded here included *Wolbachia pipientis*, a maternally transmitted, obligatory intracellular bacterium which infects a great number of species of arthropods and nematodes (Werren et al. 2008). Similarly, some of the bacteria recorded here may play a symbiotic role, although this has not yet been demonstrated. This may be the case for *Pigmentiphaga,* a genus of bacteria from the family Burkholderiaceae, that has previously been found on human skin, floral nectar, tree sap, stream sediment and soil, as well as in association with amphibian skin, suggesting it may have a beneficial role (Bletz et al. 2019).

The genus *Alkanindiges* includes bacteria known to contain obligate alkane degraders, implicated in biological foaming in activated sludge systems (Klein et al. 2007). These bacteria have been hypothesised to play a role in foam formation or stabilization in plants, and their presence here may suggest they play the same role for spittlebugs. Interestingly, while no *Alkanindiges* was previously reported in association with spittlebugs, the genus *Brevundimonas* has recently been recorded in association with spittlebug foam and has been hypothesised to play a role in the insect defensive system (Nencioni et al. 2023). Interestingly, the chemical composition of the foam produced by the juveniles of *Aphrophora alni* (Aphrophoridae) has recently been discovered to mainly consist of fucose, a sugar characteristic of carbohydrate polymers produced by marine bacteria, algae and fungi (Szterk et al. 2024). Sterk and colleagues even mentioned that the foam could be produced by symbiotic bacteria producing a polymer similar to some extent to marine fucoidians (Szterk et al. 2024). The results obtained here suggest that *Alkanindiges*-like bacteria might be the symbionts allowing spittlebugs to produce the foam that protects their nymphs.

On the other hand, most of the bacteria recorded here are almost certainly linked to the host plant, as is the case for *Agrobacterium* and *Rhizobium,* two plant growth-promoting rhizobacteria (PGPR), known to facilitate plant growth (Lugtenberg and Kamilova 2009; Naqqash et al. 2020).

Bacterial communities of *B. albicinctus* are shaped by host plant associations

The bacterial communities recorded here from different populations of *Bathyllus albicinctus* proved to be significantly different across populations. Variation was highlighted to be due to the insect-host plant association rather than to the geographic location, since it was recorded from populations collected in the same locality. This may be due to a number of factors, including bacterial transmission from the plant to the insect during feeding, but also to the presence of environmental bacteria on and around the insects, especially during their nymphal stage characterised by limited motility.

Since this study examined the overall bacterial diversity, and was not limited to the gut content, it is not possible to determine which bacterial species were present inside the insect and which taxa were recorded from the environment. Nonetheless, the fact that the bacterial communities showed such a significant variation across different plants would suggest that even the “environmental” bacteria were circumscribed to

a localised distribution that could be defined as a “plant environment” as opposed to a more general “soil environment” or “air environment”.

Here we explored for the first time the bacterial communities across populations of *Bathyllus albicinctus,* and we recorded large variation across different populations. Such a strong pattern, together with the record of a number of bacterial lineages closely related to pathogenic bacteria, may suggest a potential risk for *Bathyllus albicinctus* to vector plant pathogens should these be present in the plant. Ultimately, transmission of plant pathogens cannot be determined without further studies, including *in planta* and *in silico* testing (e.g. Morris et al. 2017) as well as other mechanical techniques, such as using electrical penetration graphs (EPG) (e.g. Mustafa et al. 2015).

Nonetheless, the results obtained here suggest spittlebugs may harbour a number of environmental bacteria associated with the plants they feed on and this should warrant further studies to better understand transmission risks linked to native spittlebug species in case of the establishment in the country of exotic plant pathogenic bacteria.

Recommendations

Outcomes

As a result of the data collected here, the following recommendations are highlighted:

Importance of 16S microbiome analysis for native spittlebug species.

The 16S bacterial metabarcoding analysis performed here was extremely effective in highlighting bacterial biodiversity patterns across different species and populations. The 16S fragments generated here, despite the limitations due to their relatively short length, still enabled a bacterial biodiversity assessment capable of providing a valuable insight into the environmental factors shaping bacterial communities.

Comparison of the main primary symbionts across multiple native species and similarities with exotic pests.

The analysis conducted here showed that both native spittlebug species examined harboured the “traditional” primary symbionts of spittlebugs (*Candidatus* Sulcia and *Ca.* Zinderia), as opposed to the *Sodalis*-like species harboured by *Philaenus.* It would be valuable for future studies to aim to determine if this is the case for other Australian native species or not.

Conclusion

Future directions

Based on the recommendations above, the future research directions should focus on:

Broader assessment of Australian native spittlebug microbiome.

This should focus on a large-scale assessment comparing different species from different locations and host plant associations. This would provide an important dataset to assess and characterise the bacterial diversity already present in Australia.

Assessment of the main primary symbionts across multiple native species and similarities with exotic pests.

Due to the fact *Philaenus spumarius,* the main exotic vector of *Xylella fastidiosa*, is known to have switched one of its primary symbionts, an assessment should be conducted across Australia to determine if any species native to the country underwent the same symbiont switch. The identification of any such species would suggest the insect could be more likely to vector similar bacteria, including *Xylella.*

Collaborations with New Zealand researchers to assess the microbiome changes within *Philaenus spumarius* and *Bathyllus albicinctus* in a different country.

To assess whether the microbiome composition of *Philaenus spumarius* and *Bathyllus albicinctus* in New Zealand show modifications from that of populations of the same species in their native range.

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Appendices

Appendix I. Testing for the presence of *Ralstonia solanacearum* in the insect DNA extracts.

Introduction

Ralstonia solanacearum, a soil-borne bacterial pathogen, causes bacterial wilt in a wide range of host plants and is present in Australia. It primarily affects solanaceous crops like potatoes and tomatoes, but its distribution and impact are managed through strict biosecurity measures. The species complex includes several strains, some of which are considered high-risk due to their potential to cause significant agricultural damage. Despite these efforts, the threat of *Ralstonia solanacearum* remains significant, necessitating ongoing vigilance and innovation in biosecurity practices.

The *Ralstonia solanacearum* species complex, as described by Fegan and Prior (2005), consists of four genetic groups known as phylotypes. Phylotype I includes strains mainly from Asia, Phylotype II from the Americas, Phylotype III from Africa, and Phylotype IV from Indonesia and other Asian countries. Different species within this complex have been defined by these phylo groups: *R. syzygii* (Phylotype IV), *R. solanacearum* (Phylotype II), and *R. pseudosolanacearum* (Phylotypes I and III). The pathogen overwinters in diseased plant debris and propagative organs such as tubers, rhizomes, suckers, or seeds of some crops, posing a persistent threat to agriculture.

Samples: All samples were tested with PCR to rule out the presence of *R. solanacearum*.

Molecular testing of *Ralstonia solanacearum*

Ralstonia solanacearum (Smith 1896) Yabuuchi et al., 1996, a regulatory pest listed in the European and Mediterranean Plant Protection Organization (EPPO) A2 List, requires precise molecular detection methods. The Crop Health Services Laboratory employs protocols outlined in EPPO standards for detecting *Ralstonia* phylogroups.

Endpoint PCR assays were performed following the procedures detailed in EPPO Standard PM 7/21 (3) (EPPO 2022), which are recommended for regulating quarantine pests in the European and Mediterranean regions. The primers p759 and p760, specific to all four phylogroups, were designed from the conserved lpxC gene, coding for an in planta expressed protein sequence (Opina et al., 1997).

PCR conditions included an initial denaturation at 96°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 15 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. All amplicons were separated on a 2% agarose gel in 0.5% Tris-borate-EDTA buffer.

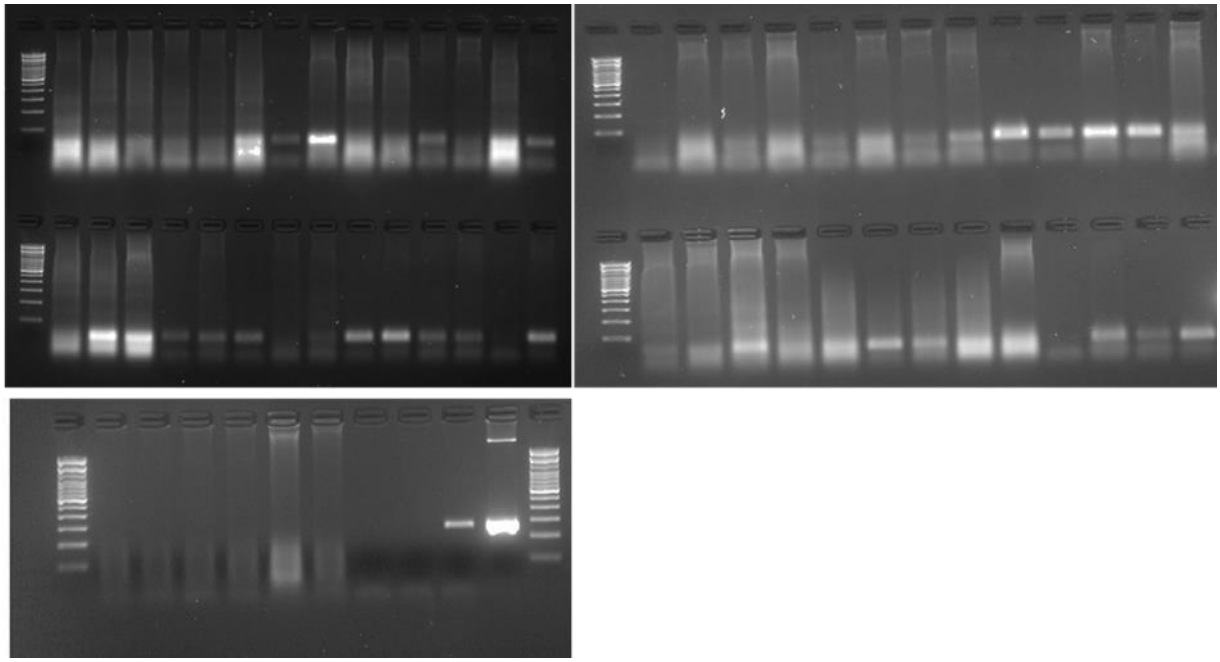


Figure. 1 Gel pictures of the insect samples tested for the *Ralstonia* species complex (Phylo group I- IV). All samples were negative. The band in the last two lanes on the bottom gel is the 280 bp positive control. The ladder is 100bp Invitrogen.

Primer sequences:

p759: 5′-GTCGCCGTCAACTCACTTTCC-3′

p760: 5′-GTCGCCGTCAGCAATGCGGAATCG-3′

Results:

All samples tested negative, with no amplification of the target PCR product, confirming that *R. solanacearum* was not detected.

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Other Ralstonia

Testing did not detect any known plant associated *Ralstonia*

Ralstonia mannitolilytica is a Gram-negative bacteria, widely present in the environment such as soil and plants [1]. It is a frequent contaminant of water supplies [2]. It rarely causes clinical infections, mainly affecting immunosuppressed individuals.

Ralstonia insidiosa and its close relative, *Ralstonia pickettii*, are betaproteobacteria that are common in water systems and other parts of the built environment [3, 4] and can be found infrequently as opportunistic pathogens in human infections.

Ralstonia pickettii is a Betaproteobacteria species found in moist environments such as soils, rivers, and lakes. It has also been identified in biofilms in plastic water pipes. It is an oligotrophic organism, making it capable of surviving in areas with a very low concentration of nutrients. Several strains have shown an ability to survive in environments highly contaminated with metals. The ability to persist in these harsh conditions makes *R. pickettii* a candidate for bioremediation [3].

Ralstonia pickettii and *R. insidiosa* are emerging pathogens in hospital settings.[4]

Ralstonia syzygii is a species of bacteria in the family Burkholderiaceae . This bacterium is the plant pathogen responsible for Sumatra disease that affects the cloves (*Syzygium*) in Indonesia. It can be transmitted by Hemiptera insects of the spittlebug group.

References:

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Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (*Xylella*) in Australia and New Zealand by understanding its potential vectors

Technical Report: Diagnostic testing of potential insect vectors for the presence of *Xylella* species

June 2024

Pragya Kant, Fiona Constable & Mark Blacket

VICTORIA State Government | Energy, Environment and Climate Action

AGRICULTURE VICTORIA

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Project Leader Name	Mark Blacket

Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.



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Executive Summary

In Australia, the presence of diverse native spittlebugs raises concerns about their role as potential vectors for *Xylella* species, similar to their counterparts overseas. While Australia currently remains free of *Xylella fastidiosa*, the wide geographic ranges and polyphagous feeding habits of some spittlebugs, such as those in the genus *Bathyllus* and *Philagra*, could potentially facilitate the spread of this bacterium if it were introduced to Australia. Research and monitoring are essential to understand the possible vector capacity of Australian spittlebugs. Identifying and studying these native species to develop proactive measures to prevent and manage possible *Xylella* incursions, will help ensure protection of Australia's agricultural and native flora health.

In this study spittlebug adults and nymphs were collected from Victoria and tested for the presence of *Xylella* species using molecular assays from the recently updated *Xylella* National Diagnostic Protocol (NDP). This is the first instance of the NDP assays being applied to insect extracts, with no cross-reactivity observed, validating the use of these assays on insects. This work enhances *Xylella* preparedness through validation of reliable molecular tests which can be applied to potential *Xylella* insect vectors in Australia.

The key findings of this study were:

1. Application of the standard *Xylella* NDP assays designed to detect exotic *Xylella* species in a qPCR, as well as using endpoint PCR assays, as described in the recently updated *Xylella* (draft) NDP (Figure 13).
2. Applying these key diagnostic assays on a range of spittlebug insect DNA samples, including multiple species and life stages, has validated and refined the updated NDP for use on insects.
3. Importantly, this testing confirmed the specificity of the *Xylella* diagnostic assays, as no cross-reactivity was observed.
4. *Bathyllus albicinctus* and *Philagra parva* spittlebug adults (n=20) and nymphs (n=87) collected from multiple sites and host plants in Victoria were tested and all found to be negative for exotic *Xylella* species (Figure 13).

Introduction

Background

Xylella fastidiosa is a bacterium that resides within the xylem tissue of plants. It is the number one exotic National Priority Pest (NPP) for Australia due to its broad host range and the significant threat it poses to the environment and agricultural and food production industries. *X*. *fastidiosa* can infect over 550 plant hosts and causes a range of symptoms (severe to symptomless).

The genus *Xylella* comprises two species: *fastidiosa* and *taiwanensis*. The NDP for *Xylella* was recently updated to address its outdated status, which was originally designed specifically to target *X. fastidiosa* in grapevines. The identification of *Xylella taiwanensis* in 2016 led to recognition as a distinct *Xylella* species. The revised NDP now incorporates generic assays capable of detecting both *Xylella* species. A duplex quantitative PCR (qPCR) has been developed, integrating the qPCR developed by Harper et al in 2010 for *X. fastidiosa* and a newly designed probe which is specific to *X. taiwanensis* in one assay. In addition, a newly designed generic endpoint PCR can detect both species as a single PCR product. These assays were developed in the Nationally coordinated project "Improving preparedness for *Xylella fastidiosa*" funded by Horticulture Innovation MT17006 (CON001470). These diagnostic assays were developed by NSW-DPI and validated by key diagnostic laboratories in different states, including AVR 's Crop Health Services (CHS) before being an updated NDP. These two tests would be the priority assays for detecting *Xylella* spp at the time of incursion. The new NDP is under review, and the assays however have not yet been validated on insect DNA.

Sap-sucking vectors, particularly spittlebugs and sharpshooters, have been instrumental in the spread of *Xylella fastidiosa* in various regions overseas. In Europe, the meadow spittlebug (*Philaenus spumarius,* family Cercopoidea) has significantly contributed to the *Xylella* outbreak devastating olive groves in Italy, leading to severe economic losses. Similarly, in the United States, the glassy-winged sharpshooter (*Homalodisca vitripennis*) has facilitated the spread of *Xylella*, affecting vineyards and other important crops. These vectors transmit the bacteria by feeding on the xylem sap of infected plants and then moving to healthy plants, making their management crucial for controlling the spread of *Xylella*. In a recent outbreak in Apulia, Italy, *P. spumarius* has been identified as the key vector in spreading the disease in olive plants. Cercopoidea generally are the dominant group of potential *X. fastidiosa* insect vectors (Cornara et al 2019).

Project Objectives

A major objective of this project was to better understand host plant use, including feeding behaviour and physiology, of potential Australian *Xylella* vector insect species.

Study Aim

The main aims of this feeding behaviour & physiology research were to utilise available molecular assays designed to detect *Xylella* species to:

1) Validate the updated *Xylella* National Diagnostic Protocol (NDP) molecular assays on two widespread potential *Xylella* vectors, *Bathyllus albicinctus* and *Philagra parva* (nymph and adult specimens).
2) Rule out the presence of exotic *Xylella* species in the insects collected in Victoria during field surveillance in this project.

Methodology

Insect samples

Twenty *Bathyllus albicinctus* adult specimens (10 males and 10 females) collected from Horsham in 2022 were tested for the presence of *Xylella* species. Additionally, a second batch of 87 spittlebug nymphs (immature insects) collected from multiple locations and host plants throughout Victoria in 2023 and 2024 were also tested.

Molecular assays

Insect DNA extractions:
DNA extraction was carried out by crushing each whole insect individually, using either the Tissue Lyser (Qiagen) or a plastic pestle in a 1.5 ml Eppendorf tube. The DNA was extracted using the Blood and Tissue (Qiagen) kit, following the manufacturer protocol. DNA was eluted with AE buffer or water and quantified using a nanodrop.

Insect COI amplification and DNA barcoding:
All insect specimens were identified to species using standard DNA barcoding protocols, by DNA sequencing amplicons of the mitochondrial COI locus, with universal LCO/HCO primers (Folmer et al. 1994); as reported

in Martoni et al. (2024). Insect COI PCR's was performed with an initial denaturing step at 94°C for 2min, followed by 35 cycles of 30s steps at 94, 48 and 72°C and a final extension step of 72°C for 2min.

***Xylella* species testing:**

Generic qPCR assay: The duplex qPCR assay, utilizing FAM specific for *X. fastidiosa* (Harper et al 2010) and VIC specific for *X. taiwanensis* (newly designed for the updated NDP) labelled probes for species differentiation was tested. The cycling and annealing temperature profile was used as described in Harper et al 2010.

Generic endpoint PCR: The endpoint PCR (newly designed for the updated NDP) that targets both species of *Xylella* as a single band of 650bp. The profile used was denaturing step at 95°C for 2min, followed by 35 cycles of 30sec steps at 95, 60 and 72°C and a final extension step of 72°C for 5min. All Amplicons were separated on 2% agarose gel in 0.5% Tris-borate-EDTA buffer.

Key Research Findings

DNA quality and COI identification of insect samples

Twenty adult insect samples were amplified using universal primers for the COI gene (~750 bp) from the extracted DNA. This PCR indicated that all insect samples had DNA which could be amplified (Figure 1).

Likewise, the batch of 87 nymph DNA samples were used to amplify COI, to confirm DNA quality, and for DNA barcoding identification. These nymphs were identified as being *Bathyllus albicinctus* and *Philagra parva* (both family Aphrophoridae); see Martoni et al. (2024).

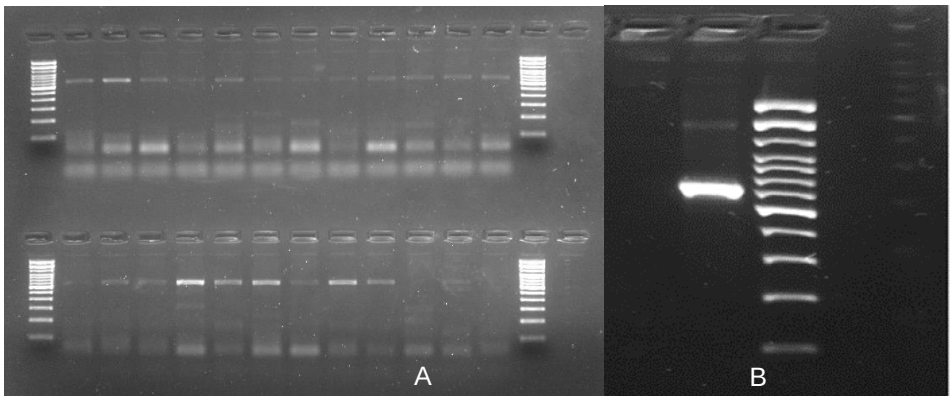


Figure 1. PCR amplification of the universal COI of 750p from insect DNA. **A**, adult insect samples from Horsham. **B**, Lane 1 is a negative control water and Lane 2 is a positive control healthy insect DNA. Ladder is 100 bp (Invitrogen).

Xylella testing on adult insect DNA samples

The duplex qPCR assay utilized FAM specific for *X. fastidiosa* and VIC specific for *X. taiwanensis* labelled probes for species differentiation. No amplification curves and *Xylella* PCR bands were recorded for *X. fastidiosa* or *X. taiwanensis* in the insect samples (Table 1 and Figure 2). The positive controls exhibited amplification below 25 CT.

Spiked *Xylella* DNA in insect DNA showed no interaction with the background DNA. The pure *X. taiwanensis* DNA had 20.8 and spiked *X. taiwanensis* DNA had 21.2 CTs, respectively. Similarly, *X. fastidiosa* pure DNA and spiked DNA did not show variation in the CTs suggested the accuracy of these assays.

The assays had healthy plant and insect DNA as a negative control to test any cross reaction, as expected no amplification was recorded. The late amplification after 34 cycles was threshold and were considered as negative this might be due to the background noise. To further confirm, a generic endpoint PCR was tested on these DNA and confirmed all the insect samples didn't not have *Xylella* DNA (Figure 2A).

Table 1. Duplex qPCR resulted negative for the presence of *Xylella* in the 20 *B. albicinctus* and six overseas sharpshooter samples. UD: undetermined (negative results).

Sample	Species	Location	Male/female	CT-XF probe	CT-XT probe
M1	*B. albicinctus*	Horsham	Male	UD	UD
M2	*B. albicinctus*	Horsham	Male	UD	UD
M3	*B. albicinctus*	Horsham	Male	UD	UD
M4	*B. albicinctus*	Horsham	Male	UD	UD
M5	*B. albicinctus*	Horsham	Male	UD	UD
M6	*B. albicinctus*	Horsham	Male	UD	UD
M7	*B. albicinctus*	Horsham	Male	UD	UD
M8	*B. albicinctus*	Horsham	Male	UD	UD
M9	*B. albicinctus*	Horsham	Male	UD	UD
M10	*B. albicinctus*	Horsham	Male	UD	UD
M11	*B. albicinctus*	Horsham	Female	UD	UD

M12	*B. albicinctus*	Horsham	Female	UD	UD
M13	*B. albicinctus*	Horsham	Female	UD	UD
M14	*B. albicinctus*	Horsham	Female	UD	UD
M15	*B. albicinctus*	Horsham	Female	UD	UD
M16	*B. albicinctus*	Horsham	Female	UD	UD
M17	*B. albicinctus*	Horsham	Female	UD	UD
M18	*B. albicinctus*	Horsham	Female	UD	UD
M19	*B. albicinctus*	Horsham	Female	UD	UD
M20	*B. albicinctus*	Horsham	Female	UD	UD
Insect DNA healthy				UD	UD
Positive	XF ATCC35879 spiked in insect DNA		Spiked DNA	23.3	UD
Positive	XT NCPPB4612		Pure culture DNA	UD	20.8
Plant DNA	Olive healthy DNA			UD	UD
Positive	XF ATCC35879		Pure culture DNA	22.4	UD
Positive	XT NCPPB4612 spiked in insect DNA		Spiked DNA	UD	21.1
Negative	Non- template Control		water	UD	UD
Negative	Non- template Control		water	UD	UD
Negative	Non- template Control		water	UD	UD

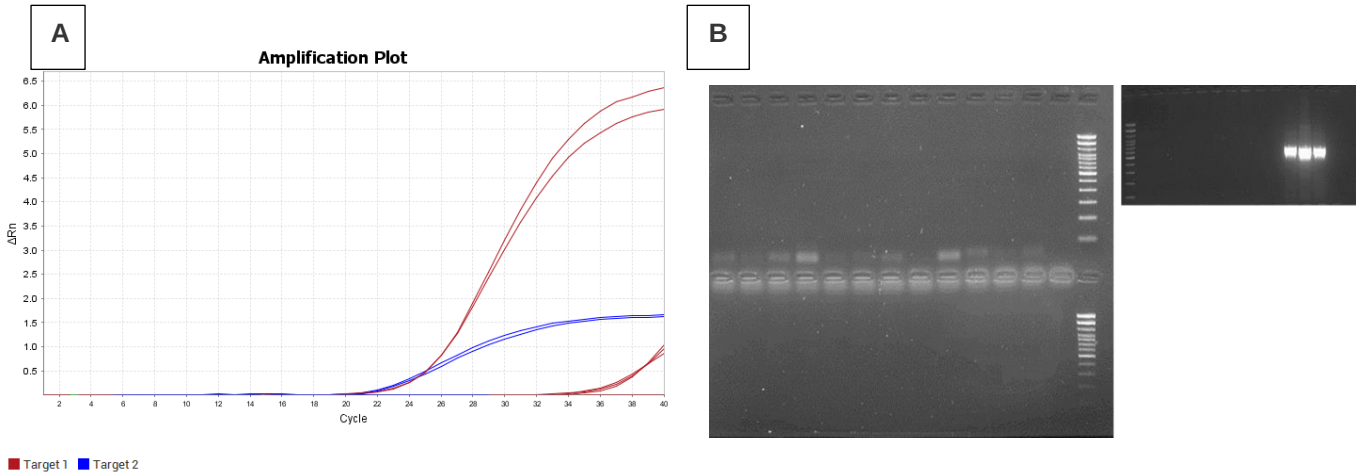


Figure 2. Adult samples (*B. albicinctus* from Victoria) testing for *Xylella* species. (A) Results of the duplex qPCR for *Xylella fastidiosa* (Red) and *Xylella taiwanensis* (Blue) were negative. The early red and blue amplification plots were positive controls for *X. fastidiosa* and *X. taiwanensis*, respectively. The late

amplification after 34 cycles was threshold and flat lines were considered as negatives. (B) The gel picture of generic endpoint PCR) were reconfirmed negative. The three bright bands of 650 bp (right) are positive controls from *X. fastidiosa*, *X. taiwanensis* and ICMP 15179 *X. fastidiosa* spiked in insect DNA.

Xylella testing on nymph insect DNA samples.

The 87 nymph samples, each identified to species using DNA barcoding, underwent qPCR testing, and yielded negative results for the presence of *Xylella* (Table 2 and Fig. 3). In Table 2 no amplification indicated the absence of *X. fastidiosa* or *X. taiwanensis* in the insect samples. At the same time, positive *Xylella* control DNA displayed robust amplification plots. The *X. taiwanensis (*NCPPB4612) control showed CT of 23.0 and two *X. fastidiosa* control samples, non-spiked ATCC35879 and spiked ATCC35879 had Ct of 9.8 and 10.0 respectively. The healthy insect DNA showed a flat line suggesting no cross-reactivity (Fig.3A). The generic endpoint PCR also resulted in negative results in all insects tested (Fig.3B)

Table 2. Duplex qPCR results for the presence of *Xylella* in the 43 *B. albicinctus* samples. All samples yielded negative results. "UD" denotes undetermined, indicating no amplification. CT-threshold cycles, XF- *Xylella fastidiosa*, XT- *Xylella taiwanensis*.

Insect Sample	Species	Location (Victoria)	CT XF	CT XT
1	*Bathyllus albicinctus*	Horsham	UD	UD
2	*Bathyllus albicinctus*	Horsham	UD	UD
3	*Bathyllus albicinctus*	Horsham	UD	UD
4	*Bathyllus albicinctus*	Horsham	UD	UD
5	*Bathyllus albicinctus*	Horsham	UD	UD
6	*Bathyllus albicinctus*	Horsham	UD	UD
7	*Bathyllus albicinctus*	Horsham	UD	UD
8	*Bathyllus albicinctus*	Horsham	UD	UD
9	*Bathyllus albicinctus*	Horsham	UD	UD
10	*Bathyllus albicinctus*	Horsham	UD	UD
11	*Bathyllus albicinctus*	Horsham	UD	UD
12	*Bathyllus albicinctus*	Horsham	UD	UD
13	*Bathyllus albicinctus*	Horsham	UD	UD
14	*Bathyllus albicinctus*	Horsham	UD	UD
15	*Bathyllus albicinctus*	Horsham	UD	UD
16	*Bathyllus albicinctus*	Horsham	UD	UD
17	*Bathyllus albicinctus*	Horsham	UD	UD
18	*Bathyllus albicinctus*	Horsham	UD	UD
19	*Bathyllus albicinctus*	Horsham	UD	UD

20	*Bathyllus albicinctus*	Horsham	UD	UD
21	*Bathyllus albicinctus*	Bundoora	UD	UD
22	*Bathyllus albicinctus*	Bundoora	UD	UD
23	*Bathyllus albicinctus*	Bundoora	UD	UD
24	*Bathyllus albicinctus*	Bundoora	UD	UD
25	*Bathyllus albicinctus*	Bundoora	UD	UD
26	*Bathyllus albicinctus*	Bundoora	UD	UD
27	*Bathyllus albicinctus*	Bundoora	UD	UD
28	*Bathyllus albicinctus*	Bundoora	UD	UD
29	*Bathyllus albicinctus*	Bundoora	UD	UD
30	*Bathyllus albicinctus*	Bundoora	UD	UD
31	*Bathyllus albicinctus*	Bundoora	UD	UD
32	*Bathyllus albicinctus*	Bundoora	UD	UD
33	*Bathyllus albicinctus*	Bundoora	UD	UD
34	*Bathyllus albicinctus*	Bundoora	UD	UD
35	*Bathyllus albicinctus*	Bundoora	UD	UD
36	*Bathyllus albicinctus*	Bundoora	UD	UD
37	*Bathyllus albicinctus*	Bundoora	UD	UD
38	*Bathyllus albicinctus*	Bundoora	UD	UD
39	*Bathyllus albicinctus*	Bundoora	UD	UD
40	*Bathyllus albicinctus*	Bundoora	UD	UD
41	*Bathyllus albicinctus*	Bundoora	UD	UD
42	*Philagra parva*	Moolap	UD	UD
42	*Philagra parva*	Moolap	UD	UD
43	*Philagra parva*	Moolap	UD	UD
44	*Philagra parva*	Moolap	UD	UD
45	*Philagra parva*	Moolap	UD	UD
46	*Philagra parva*	Moolap	UD	UD
47	*Philagra parva*	Moolap	UD	UD
48	*Philagra parva*	Moolap	UD	UD
49	*Philagra parva*	Moolap	UD	UD
50	*Philagra parva*	Moolap	UD	UD

51	*Philagra parva*	Moolap	UD	UD
52	*Bathyllus albicinctus*	Horsham	UD	UD
53	*Bathyllus albicinctus*	Horsham	UD	UD
54	*Bathyllus albicinctus*	Horsham	UD	UD
55	*Bathyllus albicinctus*	Horsham	UD	UD
56	*Bathyllus albicinctus*	Horsham	UD	UD
57	*Bathyllus albicinctus*	Horsham	UD	UD
58	*Bathyllus albicinctus*	Horsham	UD	UD
59	*Bathyllus albicinctus*	Horsham	UD	UD
60	*Bathyllus albicinctus*	Horsham	UD	UD
61	*Bathyllus albicinctus*	Horsham	UD	UD
62	*Bathyllus albicinctus*	Horsham	UD	UD
63	*Bathyllus albicinctus*	Horsham	UD	UD
64	*Bathyllus albicinctus*	Horsham	UD	UD
65	*Bathyllus albicinctus*	Horsham	UD	UD
66	*Bathyllus albicinctus*	Horsham	UD	UD
67	*Bathyllus albicinctus*	Grampians	UD	UD
68	*Bathyllus albicinctus*	Grampians	UD	UD
69	*Bathyllus albicinctus*	Grampians	UD	UD
70	*Bathyllus albicinctus*	Grampians	UD	UD
71	*Bathyllus albicinctus*	Grampians	UD	UD
72	*Bathyllus albicinctus*	Grampians	UD	UD
73	*Bathyllus albicinctus*	Grampians	UD	UD
74	*Bathyllus albicinctus*	Grampians	UD	UD
75	*Bathyllus albicinctus*	Grampians	UD	UD
76	*Bathyllus albicinctus*	Grampians	UD	UD
77	*Bathyllus albicinctus*	Grampians	UD	UD
78	*Bathyllus albicinctus*	Grampians	UD	UD
79	*Bathyllus albicinctus*	Grampians	UD	UD
80	*Bathyllus albicinctus*	Grampians	UD	UD
81	*Philagra parva*	Coburg	UD	UD
82	*Philagra parva*	Coburg	UD	UD

83	*Philagra parva*	Coburg	UD	UD
84	*Philagra parva*	Coburg	UD	UD
85	*Philagra parva*	Coburg	UD	UD
86	*Philagra parva*	Coburg	UD	UD
87	*Philagra parva*	Coburg	UD	UD
Insect healthy DNA	N/A	N/A	UD	UD
Positive XT NCPPB4612	N/A	N/A	UD	23.0
Positive XF ATCC35879	N/A	N/A	9.8	UD
Positive XF ATCC35879, spiked in insect DNA	N/A	N/A	19.5	UD
Non- template Control	UD	UD	UD	UD
Non- template Control	UD	UD	UD	UD

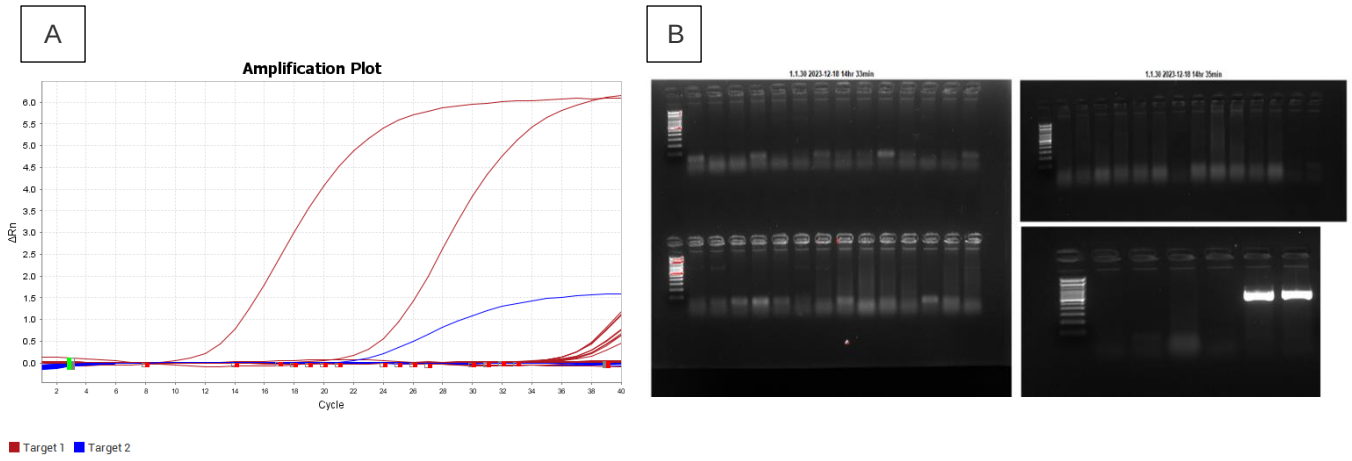


Figure 3. Testing of nymph samples (87 immature spittlebugs from Victoria) for *Xylella* species. A) Example of results of the duplex qPCR for *Xylella fastidiosa* (Red) and *Xylella taiwanensis* (Blue). The early red and blue amplification plots were positive controls for *X*. *fastidiosa* and *X*. *taiwanensis*, respectively. The late amplification after 34 cycles and flat lines were considered as negatives. B) Example gel picture showing no bands in insect DNA samples and two bands are of positive controls of 650bp.

Recommendations

Outcomes

The application of National Diagnostic Protocol (NDP) assays for insect testing significantly enhances our surveillance capabilities for exotic *Xylella* pathogens. By applying these key assays to insect samples, including multiple spittlebug species and lifestages, we validated and refined the NDP for use on insects. The testing confirmed the specificity of the assays, with no cross-reactivity observed. This successful validation ensures that these assays can be reliably used to detect *Xylella* in insect vectors.

Conclusion

Improved diagnostics of *Xylella* in vectors has recently been achieved through the implementation of the National Diagnostic Protocol (NDP). This protocol provides a standardized, reliable method for detecting *Xylella* species in various insect vectors. By applying these assays, we could accurately identify the presence of *Xylella* in spittlebugs, sharpshooters, and other potential carriers. The NDP's specificity ensures that even low levels of the pathogen should be detected, thereby enabling early intervention and more effective management of the disease. This advancement significantly strengthens our biosecurity measures and preparedness, reducing the risk of *Xylella* spread through insect vectors.

Future directions

1. Optimising sensitivity and specificity of the assays using the spiked plant and insect samples.
2. Application of the assays to insect samples (e.g., from traps), testing non-destructive DNA extraction methods for feasibility to detect *Xylella* in insects.
3. Optimization efforts focused on near-field diagnostic assays, such as Loop-Mediated Isothermal Amplification (LAMP) and Recombinase Polymerase Amplification (RPA). These techniques would provide triage tools to test larger numbers of insects in and away from a laboratory setting. LAMP assays, specifically designed for *Xylella* detection, are available through GeneWorks. Further validation of these assays is required to ensure their efficacy and reliability in diverse field conditions. This work will enhance the accuracy and efficiency of *Xylella* detection in vectors, supporting broader surveillance and biosecurity efforts.

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Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (Xylella) in Australia and New Zealand by understanding its potential vectors

Technical Report: Feeding behaviour of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa*.

June 2024

Piotr Trebicki[1,2], Clara Lago[3], Cait Selleck[1], Francesco Martoni[1], & Mark Blacket[1]

[1] Agriculture Victoria, AgriBio, Bundoora Victoria
[2] Applied BioSciences, Macquarie University, Sydney, NSW
[3] Institute of Agricultural Science, Spanish Research Council, Madrid, Spain

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External Project Code	WA2001 & 4-EWI2BY6
RCT No	AgVic 6162
Project Leader Name	Mark Blacket

Acknowledgment

We acknowledge and respect Victorian Traditional Owners as the original custodians of Victoria's land and waters, their unique ability to care for Country and deep spiritual connection to it. We honour Elders past and present whose knowledge and wisdom has ensured the continuation of culture and traditional practices.

We are committed to genuinely partner, and meaningfully engage, with Victoria's Traditional Owners and Aboriginal communities to support the protection of Country, the maintenance of spiritual and cultural practices and their broader aspirations in the 21st century and beyond.



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Executive Summary

The movement of plant material across national borders enables the rapid spread of pests and diseases worldwide. *Xylella fastidiosa* (*Xylella*) is one of the most harmful plant pathogenic bacteria worldwide and is at the top of Australia's exotic National Priority Plant Pests (NPPP) list. *Xylella* is transmitted by xylem-feeding insects, and accordingly any xylem feeder could act as a potential vector. Fortunately, *Xylella*, as well as known exotic insect vectors are not currently present in Australia.

The present study was conducted to investigate feeding physiology of a common native Australian spittlebug, *Bathyllus albicinctus*, to assess its ability to serve as a potential *Xylella* vector. We monitored the feeding behaviour of *B. albicinctus* on grapevines, olives and oranges using the electrical penetration graph (EPG) technique (Figure 14). Performing 4-hour continuous recordings on the three host plants, we identified all stages of feeding previously described for spittlebugs. Our results showed that *B. albicinctus* continuously feed on these three plant species and the feeding patterns obtained match those previously described for exotic xylem feeding spittlebugs known to vector *Xylella*. In addition, Xe (xylem egestion) patterns were observed in insects feeding on all of these susceptible plants (Figure 15), suggesting that *B. albicinctus* should be considered a likely vector of *Xylella* if this devastating bacterial disease was to be introduced in Australia.

An additional project activity involved conducting an EPG training course, at AgriBio in December 2022, associated with an international Hemipteran-Plant Interactions symposium. This course included lectures on EPG background and application, as well as hands-on practical training using Faraday cages, wiring and mounting insects and plants, monitored signal recording (data acquisition), signal analysis, data processing, and statistical analyses. Additional feeding behaviour studies were conducted during this course, using nymphs collected from *Acacia decurrens* and EPG tested on capsicum, confirming the *B. albicinctus* feeding behaviour patterns we had previously observed.

Introduction

Background

Xylella fastidiosa (Xylella) is an exotic destructive plant pathogenic bacterium, known to affect a wide range of plants. Being xylem limited, it is transmitted overseas to plants by leafhoppers, froghoppers, and spittlebugs. Xylella and its exotic vectors have been identified by the Plant Health Committee as the number one National Priority Plant Pest (NPPP) for Australia in 2019, by New Zealand MPI as an 'unwanted organism', and by the European Commission as one of most dangerous plant bacteria worldwide. This project aimed to identify potential Xylella vectors in Australia to provide tools and knowledge to mitigate the potential spread of Xylella by understanding the biology and population dynamics of potential insect vectors.

Project Objectives

A major aim of this project was to support the development of effective vector monitoring, surveillance, and control strategies for Xylella and to understand the feeding behaviour and plant host range of potential vector species. Based on project surveillance efforts, the Australian spittlebug *Bathyllus albicinctus* is considered a likely vector of Xylella if *Xylella fastidiosa* established in Australia, with populations of this spittlebug seasonally reaching high levels, feeding on a wide range of plant species, both native and introduced.

Study Aim

The main aim of this feeding behaviour & physiology research was:

1) To assess the feeding behaviour of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa.*

Methodology

Insects and host plants

Insect feeding experiments were conducted at the Grain Innovation Park at Agriculture Victoria (Horsham, Victoria, Australia) and at Macquarie University (Sydney, Australia), from December 2022 to May 2023. Adult females of *Bathyllus albicinctus* were collected by sweep netting from a variety of plant species in Victoria and South Australia. Adult *B. albicinctus* were maintained inside BugDorm cages in the laboratory on Asteraceae plants, kept at 22℃, under natural lighting.

Electrical Penetration Graph (EPG) recordings (Figure 1) were performed on three different plant species known to be susceptible to *Xylella fastidiosa*:

1. Olive (*Olea europaea* L.),
2. Grapevine (*Vitis vinifera* L.), &
3. Orange (*Citrus x sinensis* (L.).



Figure 1. Electrical Penetration Graph (EPG) setup with the electrode terminal coupled to a live *Bathyllus albicinctus* adult.

Electrical Penetration Graph (EPG) recording

The feeding behaviour of *B. albicinctus* was monitored using the electrical penetration graph (EPG) method, with all EPG assays conducted under controlled environmental conditions, natural light and temperature at 22℃

Adults of *B. albicinctus* were anaesthetized by placing them in the fridge at 4ºC for 1 hour, immobilized with a vacuum device and connected to an EPG insets electrode as previously described by Trebicki et al 2012. Each individual insect was placed on the petiole of one leave and were free to move to other parts of the leaf.

The feeding behaviour of spittlebugs was monitored continuously for 4 hours with two Giga 8-DC (EPG Systems, Wageningen, The Netherlands) devices placed inside a Faraday cage. EPG data acquisition and analysis were conducted using Stylet+Software for Windows (EPG Systems, Wageningen Netherlands).

To study *B. albicinctus* feeding behaviour, we referred to waveforms described by Cornara *et al.*, (2018 and 2020):

- np (non-probing);
- C (pathway; stylet tip in plant tissue);
- R (resting);
- Xc (xylem contact);
- Xi (xylem ingestion);
- Xe (xylem egestion); &
- N (interruption within xylem phase).

We also noted the:

- Proportion of escaped individuals during the recording time (4 hours) (i.e., insect no longer on the plant);
- Total probe events (i.e., stylets inside the plant tissues: C+Xc+Xi+N+R); &
- Successful probe events (i.e., probes where the insect reached the xylem).

We studied the effect of the treatments on nonsequential EPG variables:

- Number of waveforms events per insect and total waveforms duration per insect (minutes);

As well as the following sequential variables:

- Mean time to 1st probe;
- Mean time from 1st np to 1st Xc; &
- Mean time from 1st np to 1st Xi.

We also record the number of insects that produced a given waveform (PPW) from the total insects analyzed. A recording was considered valid and included in the analysis only when the insect performed at least one probe, i.e., inserted the stylets into the plant tissues at least once during the recording. When insects escaped during the EPGs recording, the data retrieved was only included to calculate the sequential variables.

EPG waveforms were manually marked with 'Stylet+a' software (EPG-Systems, Wageningen, Netherlands), and the behavioural variables were calculated using an Excel Macro template designed for xylem feeders.

Key Research Findings

Data analysis of EPG inoculation tests

Bathyllus albicinctus feeding on the three plants species and the waveforms obtained were generally the same as previously described for other species of spittlebugs. The proportion of valid recordings from the total insects wired were 8/14 in grapevine; 7/23 in olive and 7/17 in orange (Table 1). In the valid recordings, 100% of the insects reached the xylem. Furthermore, the first probe occurred in less than 10 minutes, and on average reached the xylem in less than 25 minutes (1st Xc and Xi occurred in less than 25 minutes) in all the susceptible plants (Figure 2).

One of the most interesting results is that Xe waveform (i.e., egestion correlated with *X. fastidiosa* transmission) was observed on insects feeding in all the susceptible plants (Figure 3). The proportion of insects that performed Xe waveform was 4/8 in grapevine, 3/7 in orange, and 2/7 in olive (Table 1).

Finally, all the insects were able to perform sustained xylem ingestion (longer than 5 minutes). And as shown in figure 4, the duration of xylem ingestion (Xi and Lf waveforms) either surpassed or neared 150 minutes for all the plant species, which represents 62.6% of the total duration of the recordings.

Table 1. Frequency of recorded *B. albicinctus* feeding behaviours.

	Grapevine	Olive	Orange
Number of replicates tested	14	23	17
Number of valid recordings	8	11	8
Proportion of valid recordings	57%	48%	47%
Proportion of valid recordings reaching the xylem	100%	100%	100%
Proportion of valid recordings reaching xylem egestion (Xe)	50%	29%	43%

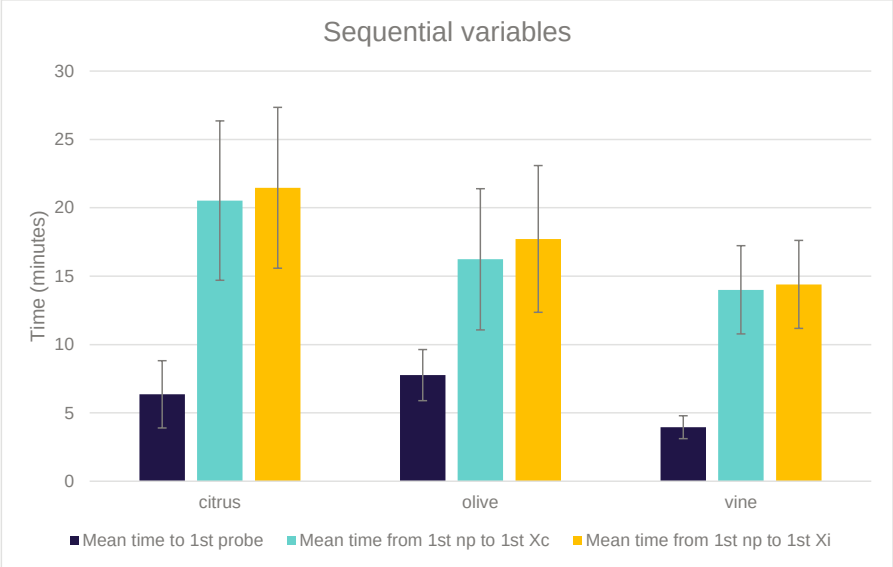


Figure 2. Mean time (minutes) of *B. albicinctus* from the beginning of the recording until the first probe, mean time from 1st non probing until first xylem contact (Xc) and xylem ingestion (Xi).

Commented [MJB(1]: Mark to do - Match order of plants between graphs and text.
E.g., citrus / olive / grapevine etc

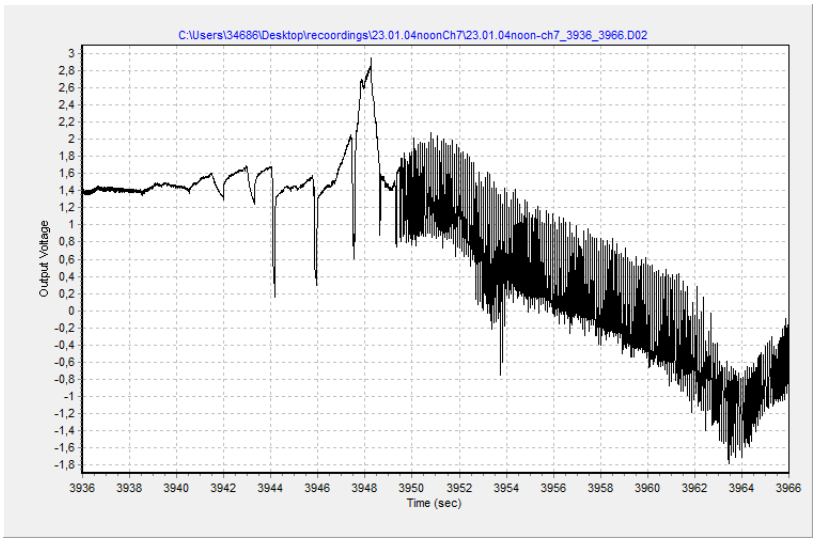


Figure 3. Xylem egestion (Xe) observed in *B. albicinctus* feeding on grapevine.

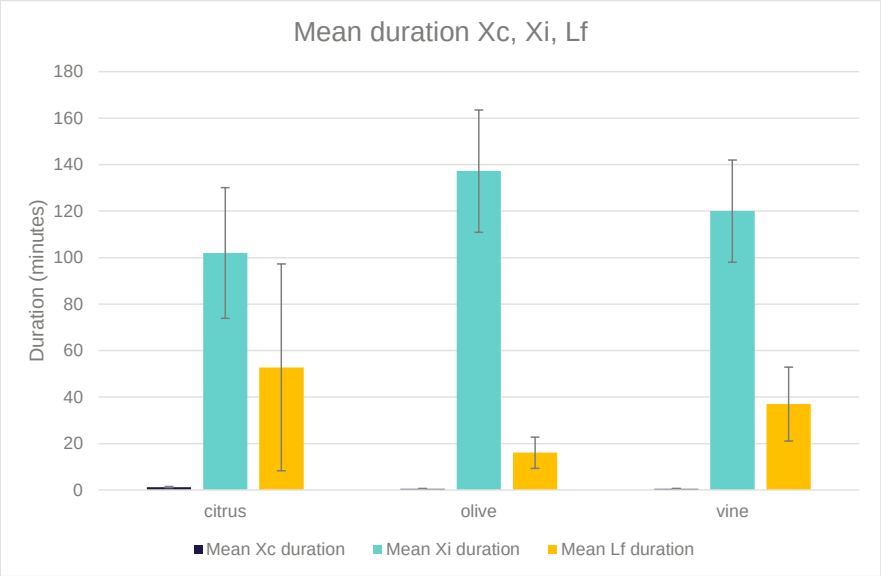


Figure 4. Mean duration (minutes) of *B. albicinctus* for xylem contact (Xc), xylem ingestion (Xi) and Lf (low frequency xylem ingestion) on the different susceptible plants.

EPG training course, AgriBio, December 2022

An additional project activity involved running an EPG training course, held at AgriBio in December 2022. (Appendix A). This training was conducted as a satellite event following the International Hemipteran-Plant Interactions Symposium (HPIS 2022) and was locally organised by Piotr Trebicki and Isabel Valenzuela (Agriculture Victoria).

The EPG training course included lectures on EPG background and application, and hands-on practical training using Faraday cages, wiring and mounting insects and plants, monitored signal recording (data acquisition), signal analysis, data processing, and statistical analyses.

This training course included additional EPG feeding trials were conducted on *B. albicinctus*, with local specimens collected from *Acacia decurrens* and EPG tested on capsicum plants. These additional EPG experiments confirmed the *B. albicinctus* feeding behaviours we had previously observed (Figure 5).



Figure 5. Top left: Narelle Nancarrow (AVR) and Precious Mavende (AVR) placing prepared insects onto plants to conduct an EPG recording. Top right: Fred Tjallingii (Netherlands) calibrating the EPG system to ensure accurate readings are collected. Bottom left: a psyllid (*Acizzia solanicola*) prepared for EPG recording by using conductive silver glue to connect a fine gold thread to the insect's dorsal side. Bottom right: two *Bathyllus albicinctus* nymphs post EPG recording (residual silver glue is still visible on the closer nymph).

Recommendations

Outcomes

A major aim of this project was to support the development of effective vector monitoring, surveillance, and control strategies for Xylella and to understand the feeding behaviour and host plant range of potential vector species. This study was conducted to investigate feeding physiology of a common native Australian spittlebug, *Bathyllus albicinctus*, to assess its ability to serve as a potential Xylella vector.

Bathyllus albicinctus was demonstrated to be able to feed on all three plant types – grapevine, olive, and orange – rapidly accessing the xylem within 25 minutes. Xylem was ingested for hours at a time and insects exhibited xylem egestion (Xe) feeding behaviour. Considering that this has been correlated with transmission of *Xylella fastidiosa* in exotic spittlebugs, these behavioural findings together with other aspects of the biology of *B. albicinctus*, including large seasonal peaks of populations which can feed on a wide range of plant species (Trebicki & Blacket 2023), suggest that *B. albicinctus* should be considered a likely vector of Xylella if this devastating bacterial disease was to be introduced in Australia.

Conclusion

Future directions

This research highlighted key questions:

- Future research could involve experimental studies to determine if *B. albicinctus* can transmit *Xylella fastidiosa* to susceptible host plants. Note, these studies would need to be conducted overseas, as *Xylella fastidiosa* is exotic to Australia.

- Investigate developing more reliable and feasible methods of rearing *B. albicinctus* to maintain colonies for testing *X. fastidiosa* transmission potential.

- Investigate whether *B. albicinctus* displays preferential feeding behaviours on different host plants, including potential sub-optimal hosts.

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Appendix A: EPG Training Course Announcement

Source: https://www.plantbiosecuritydiagnostics.net.au/app/uploads/2022/10/Announcement_2022-EPG-course-Melbourne.pdf

EPG training course (satellite event of HPIS 2022, 7-9 December)
December, Mo.12-We. 14, Melbourne, Australia

Local Organizers:
Piotr Trebicki & Isabel Valenzuela (Australia)
Program organizers:
Freddy Tjallingii (Netherlands), Elisa Garzo & Alberto Fereres (Spain)

Dear colleague,

We are happy to announce a new EPG training course. Though scheduled as a 'satellite', there is no formal relation between these two events. The course will be held at La Trobe University Campus, Bundoora, AgriBio Centre (close to Melbourne).

What is the EPG (Electrical Penetration Graphs) technique?
A plant and a (mostly) Homopteran insect are made part of an electrical circuit by using a soil electrode for the plant and gluing a very thin gold wire to insect's dorsum. Upon plant penetration by the insect, electrically caused waveforms indicate tissue positions of the stylet tips and insect activities (mechanical movements, ingestion, or salivation). Typically, 8 replicates can be recorded for periods up to 8h (more experimental configurations are possible).

EPG recording is commonly used to study plant-insect interactions as involved plant resistance, host plant recognition, plant pathogen transmission, electrophysiological aspects of plant defenses, etc.

Course program: see attachment. This includes lectures on backgrounds and applications (morning sessions) and practical training (afternoon sessions) in Faraday cage use, wiring and mounting insects and plants, monitored signal recording (data acquisition), signal analysis, data processing, and directions for statistical analysis.

There will be a maximum of 16 participants due limited equipment and lab facilities, and assistance. When more people sign up a waiting list will be used.

Participants fee: AUS $500
Registration deadline: November 15

Registration and further information:
Please reply to this e-mail to both local organizers
Piotr.trebicki@agriculture.vic.gov.au
Isabel.valenzuela-gonzalez@agriculture.vic.gov.au

This information is also available on
https://www.epgsystems.eu/events-downloads-presentations

Best regards,

The organizers.



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Tools and knowledge to mitigate the potential spread of *Xylella fastidiosa* (Xylella) in Australia and New Zealand by understanding its potential vectors

Technical Report: Host plant preferences and survival of the native Australian spittlebug, *Bathyllus albicinctus*, a potential vector of exotic *Xylella fastidiosa*

June 2024

Duncan Jaroslow[1], Lea Rako[1], Narelle Nancarrow[3,], Piotr Trebicki[1,2], Cait Selleck[1], Francesco Martoni[1], & Mark Blacket[1]

[1] Agriculture Victoria, AgriBio, Bundoora, Victoria
[2] Applied BioSciences, Macquarie University, Sydney, NSW
[3] Agriculture Victoria, Grains Innovation Centre, Horsham, Victoria

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RCT No	AgVic 6162
Project Leader Name	Mark Blacket

Acknowledgment

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Executive Summary

The xylem feeder *Bathyllus albicinctus* (Aphrophoridae: Cercopoidea: Hemiptera) has been identified as one of the most abundant and widely distributed Cercopoidea insects in Australia and might be a likely vector of the exotic *Xylella fastidiosa* if this were to arrive in Australia. Previous work (this project) has indicated that *B. albicinctus* populations seasonally reach high levels, and are associated with a wide range of plants. However, it is unknown if all of these associated plants are equally viable hosts. Due to the variety of plant records and distribution across the southern half of Australia, it has been predicted that *B. albicinctus* is likely to be able to successfully feed and survive on a broad range of plants, from diverse taxonomic groups.

In the present study, we investigated the feeding physiology and longevity of *B. albicinctus* by monitoring nymphal (immature) and adult insect development in three independent experiments:

1. The first experiment involved monitoring the maturation of size-segregated *B. albicinctus* nymphs on *Westringia* (coastal rosemary; Lamiaceae), a previously documented viable host, under laboratory conditions. The main goal was to document the development from immature nymphs to fully developed adults and determine the persistence of *B. albicinctus* adults on this host. It was expected that larger nymphs would reach maturity and likely perish first, being further along in their development. However, size appeared strongly related to sex, with large female insects found to generally survive longer than smaller males in the same cohort. Interestingly, some adult *B. albicinctus* females were shown to survive on *Westringia* for up to three months after maturation (Figure 16).
2. The second experiment included adult *B. albicinctus* of mixed ages contained in cages with four single species of plant, or with a mixture of these plants, in a screenhouse (Figure 17). The goal was to determine if *B. albicinctus* had a preference in host plant, and if adult survival differed between host plants. *B. albicinctus* males survived in cages with capsicum, basil, and *Westringia* (Figure 18). Contrastingly, female insects only survived the study period on *Westringia*. Notably, neither sex survived the study period when only supplied with barley (Figure 18).
3. The third experiment assessed development and longevity of *B. albicinctus* nymphs on three nursery-grown (olive, grapevine, orange) and one field-transplanted (thistle) plant species susceptible to *X. fastidiosa*. This was done by monitoring survival and maturation of *B. albicinctus* nymphs and comparing these between host plant groups. The host suitability and seasonal timing of insect development differed between plant species, with olives appearing suitable for allowing *B. albicinctus* to survive (Figure 19).

These three studies have greatly increased our understanding of *B. albicinctus* plant host preferences, insect development, and longevity (Figures 16 to 20) which provides vital background knowledge relevant to monitor for and prevent establishment and potential spread of *Xylella* if it were to arrive in Australia.

.

Introduction

Background

Xylella fastidiosa is a threat to plants, in both agricultural and environmental systems, and has been recorded to infect at least 439 species of plants across 69 families (EFSA et al 2023). *Xylella* is spread by xylem-feeding Auchenorrhyncha within Hemiptera, notably Cercopoidea (spittlebugs) and a small group of Cicadellidae (leafhoppers). *Xylella* is transmitted from insect stylets into xylem during feeding (Daugherty and Almeida 2009; Kapantaidaki et al 2021), and specifically during ‘egestion’ (insect backwash when tapping xylem) (Roddee et al 2023). Understanding the community of potential *Xylella* vectors in Australia is of great benefit to the pre-emptive and preparative management efforts for the possibility of *Xylella* arriving in Australia.

Bathyllus albicinctus (Figure 1) is among the most common species of Cercopoidea, which may be likely to vector *Xylella* if it were introduced to Australia. *B. albicinctus* is widespread, seasonally abundant and commonly found across Australia having been recorded in association with 48 plant genera across 26 families (Selleck et al 2024).



Figure 1. *Bathyllus albicinctus*, lateral view of (A) adult male and (B) adult female, and dorsal view of (C) nymph (immature insect) with spittle.

Transmission and establishment of *Xylella* requires a capable herbivore vector and viable host plant for that herbivore (Daugherty and Almeida 2009). An improved understanding of *B. albicinctus*, and Australian Cercopoidea in general, would greatly improve the ability for management efforts for surveillance and to predict when and where *Xylella* is likely to establish. Documenting the suitability of putative host plants for *B. albicinctus* can provide an indication of which plants this insect is most likely to infect with *Xylella*. Investigating *B. albicinctus* insect development, and longevity provides vital background knowledge relevant to monitor for and prevent establishment and potential spread of Xylella if it were to arrive in Australia.

Project Objectives

A major aim of this project was to support the development of effective vector monitoring, surveillance, and control strategies for Xylella and to understand the feeding behaviour and plant host range of potential vector species. Based on project surveillance efforts, the Australian spittlebug *Bathyllus albicinctus* is considered a likely vector of Xylella if *Xylella fastidiosa* established in Australia, with populations of this spittlebug seasonally reaching high levels, feeding on a wide range of plant species, both native and introduced.

Study Aims

The three main aims of the host plant preferences and potential vectors survival research were:

1) To assess longevity of *B. albicinctus* nymphs maintained on *Westringia*, a known host plant.
2) To compare survival of *B. albicinctus* adults on a range of common potential host plants.
3) To compare maturation and longevity of *B. albicinctus* nymphs on specific host plants known to be susceptible to *Xylella*.

Methodology

Experiment 1: Longevity of *Bathyllus* on *Westringia*, a known host plant

Laboratory plants and conditions

On 2nd October 2023, three 1-year-old *Westringia fruticosa* hybrid 'WES01' plants (coastal rosemary, purchased from Bunnings) were transferred into individual BugDorm cages (24.5 × 24.5 x 63 cm) covered in 1 × 1 mm nylon mesh, for secure containment under controlled conditions within the insect laboratory at AgriBio. *Westringia* were kept in 2 L pots in standard soil mix. These plants were watered three times per week. Although artificial lighting within the laboratory was periodically turned on, plants were kept on south-facing windowsills and therefore exposed to a natural sunlight and day-night light cycle. The relative humidity within the laboratory ranged from 17 to 20%, and temperature ranged from 23.3°C to 23.8°C (both recorded by Honeywell International Inc., Honeywell TH1100 Thermostat). Plants were watered as needed until the end of the (>3 month) monitoring period on January 15th, 2024.

Insect development and longevity

Field collected cercopoid Aphrophoridae nymphs were collected on October 2nd, 2023, from established ornamental *Westringia fruticosa* plants located adjacent to the AgriBio insect laboratory in Bundoora, Melbourne. *Westringia* were visually inspected for signs of Aphrophoridae foam excretion, indicative of xylem feeding activity (Ponder et al 2002). Twenty-four nymphs were collected from within foam using a paint brush. Nymphs were suspected of being *Bathyllus albicinctus* (Cercopoidea: Aphrophoridae), due to their general morphological shape, their host plant, and the presence of adult *B. albicinctus* on the same *Westringia* plants. All nymphs that developed into adults over the course of this experiment were confirmed to be *B. albicinctus*.

The eight smallest / shortest nymphs were separated into one petri-dish and categorised as 'small'. The eight largest / longest nymphs were separated into another petri-dish and categorised as 'large'. The remaining moderately sized eight nymphs were placed into a third petri-dish and categorised as 'medium'. Groups of nymphs (small, medium, and large) were transferred using a paint brush onto single potted *Westringia* plants housed in individual mesh cages. *Westringia* plants were inspected when watered and several metrics were recorded from the small, medium, and large colonies.

The number of nymphs, and the number of adult females and adult males were recorded for each of the three colonies until all *B. albicinctus* had perished by January 15th, 2024. The emergence of individual adults and persistence of insects were recorded separately for each colony. From this information, the number of days each insect spent alive, and as a juvenile or adult, was inferred and recorded for statistical analysis.

Experiment 2: Survival of *Bathyllus* on a range of common potential host plants

Screenhouse plants

Five plants from four different species were maintained in five BugDorm cages in a screenhouse for the second experiment (from Oct 24, 2023). These consisted of capsicum (*Capsicum annuum*, variety Giant Bell; Solanaceae), barley (*Hordeum vulgare*; Poaceae), Greek basil (*Ocimum basilicum* var. minimum; Lamiaceae), and coastal rosemary (*Westringia fruticosa* hybrid 'WES01'; Lamiaceae). Costal rosemary and basil plants were purchased from Bunnings store plant nursery, whilst barley and capsicum plants were reared from seed in AgriBio Victoria. All plants were kept in 2 L pots containing standard soil mix. Five large mesh cages (either 24.5 × 24.5 x 63 cm or 32.5 x 32.5 x 32.5 cm) were prepared with four plants (Figure 2).

Cages A – D contained four plants of a single plant species (i.e., capsicum, barley, basil, or costal rosemary) while cage E contained a mix of one of each of the plant species (Table 1). These plants were watered as needed (generally three times per week) until the end of the (~2 ½ month) monitoring period (to Dec 12, 2023).

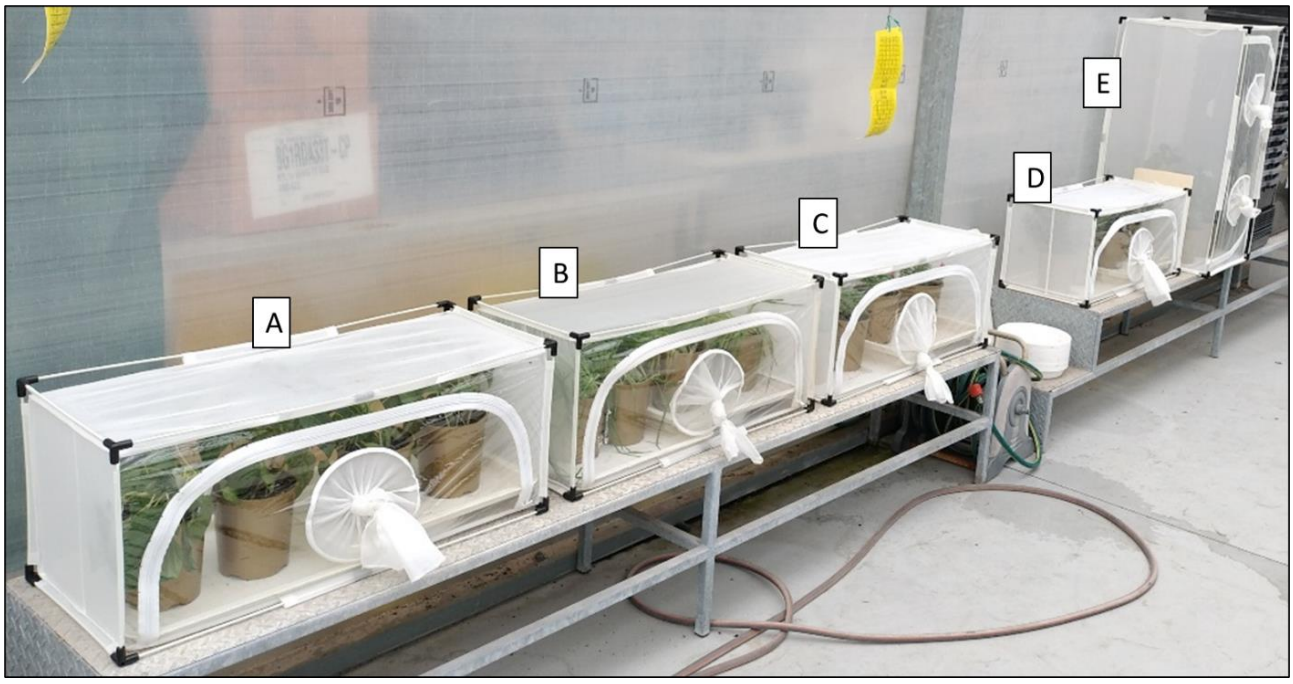


Figure 2. Plants with *Bathyllus albicinctus* in BugDorm cages within screenhouse; capsicum (A), barley (B), basil (C), coastal rosemary (D), mix (E).

Table 1. Summary of plants used for screenhouse cage experiments.

Cage ID	Plant composition
A	4 × Capsicum
B	4 × Barley
C	4 × Basil
D	4 × Coastal Rosemary
E	1 × Capsicum, 1 × Barley, 1 × Basil, 1 × Coastal Rosemary

Screenhouse conditions

Temperature within the screenhouse was recorded by data loggers present prior to this experiment (Honeywell International Inc.), providing the daily maximum and minimum temperature values to the nearest whole number, and the absolute minimum, maximum and mean temperature to one decimal place. The daily maximum value was retained for statistical analysis. The screenhouse had an overall average temperature of 23.9°C and ranged from an absolute minimum of 12.8°C to an absolute maximum of 42.5°C, although temperatures only exceeded 40°C on four days across the study period. Plants maintained in the screenhouse experienced sunlight and a natural day-night light cycle.

Host preferences of *Bathyllus*

At the start of the experiment, *B. albicinctus* adults were field collected from ornamental *Westringia* plants around the AgriBio building courtyard and garden beds. These insects were separated into groups of eight insects, consisting of four female and four males, and each group was transferred to one of the five cages which contained either one or a mix of potential host plant species. Three times a week, when plants were watered, the cages were inspected. The number of deceased *B. albicinctus* adults, and the number of remaining male and female insects were recorded. Dead *B. albicinctus* that were found during inspections were removed from the cages.

Possible confounding interactions with other insects

Despite the careful handling of the cages containing plants, an infestation of aphids was detected in the cage containing a mix of plants on the 30th of October. On November 8th, more aphids were detected in the cage containing only barley, with aphids observed in the mixed cage mostly on barley and virtually absent from basil and coastal rosemary. By November 15th, aphids were also detected in the capsicum cage. On November 22nd, lady beetles (*Harmonia conformis*) were added to the aphid-affected cages to control their numbers. It was, however, unknown if the lady beetles attempted to feed on *B. albicinctus* adults in the cage, or if the presence of aphids influenced *B. albicinctus* feeding.

Experiment 3: Maturation and longevity of *Bathyllus* on host plants susceptible to *Xylella*

Two trials were conducted for this experiment at The Grains Innovation Park, Horsham, Victoria: Trial 1 began in October 2022, and Trial 2 began in December 2022.

Insect collection and host plants

B. albicinctus 2nd to 4th stage instars were field collected from self-propagated plant vegetation in and around Horsham, Victoria. To assess the development and longevity, *B. albicinctus* nymphs were collected from coastal rosemary (*Westringia fruticosa*), sow thistle (*Sonchus sp.*), and an unidentified Lamiaceae plant around Horsham. Insects were individually placed on the petiole/leaf of an orange tree (*Citrus x sinensis* L.), two varieties of olive (*Olea europaea* L), four varieties of grapevine (*Vitis vinifera* L.), and a field transplanted sow thistle (Table 2). A clip cage was then placed over the top of individual nymphs. Instar development, presence of spittle excretions, adult emergence, and insect mortality were recorded as metrics for comparing the development and longevity of *B. albicinctus*. The weight and sex of insects that reached adulthood were also recorded as indicators of development or sex-based divergence in insect-plant relationships.

Table 2. The number of plants and insects examined in Trial 1 (October start) and Trial 2 (December start) to evaluate host use of *Bathyllus albicinctus*.

Host	Variety	Number of plants	Number of insects per plant	Number of observations per variety
Grapevine	Dawn Seedless	1	2	2
	Early muscat	6	2	12
	Fiesta	1	2	2
	Thomuscat	1	2	2
Olive	Manzanillo	4	2	8
	Kalamata	6	2	12
Orange	Washington Navel	10	2	20
Other	Thistle	1	4	4

Growth conditions

Sow thistle, olive, grapevine, and orange plants were grown in a laboratory, which was kept at 22°C and had access to a natural light cycle. Sow thistle plants found with *B. albicinctus* around the Horsham region were initially carefully transported from the field to laboratory conditions and were intended for use as a "positive control host plant" group. Despite taking great care when transplanting sow thistles, it is likely that these plants experienced elevated stress levels. Indeed, the herbaceous plants exhibited symptoms of low leaf water pressure for several hours after transplantation.

Statistical analyses

Statistical analyses were performed using RStudio (version 4.1.0) and Microsoft Excel (version 2403). Differences between treatments within each experiment were reported descriptively, notably using metrics that provide an indication of insect development rate, longevity, and host suitability.

Experiment 1 analyses

The changing numbers of juvenile and adult *B. albicinctus* were documented for each size cohort and reported descriptively. The maximum possible number of days each insect spent as a juvenile was compared between insect size cohorts. This experiment had a small number of insects in each group. Differences among size cohorts were tested via an Analysis of Variance (ANOVA) test ($\alpha = 0.05$). Least Significant Difference test (LSD) was used as a post-hoc analysis where differences were detected among groups. Independent samples t-tests were used to determine if metrics differed between male and female insects.

Experiment 2 analyses

Recorded insect death was converted to a proportion of the original caged *B. albicinctus* population, and from this the proportion of surviving *B. albicinctus* for each inspection was inferred. Daily maximum temperature and cumulative daily temperature were used in regression analysis to explain the occurrence of insect mortality and changes to the proportion of surviving *B. albicinctus* across cage treatments. The model transformation that produced the highest correlation strength is reported in the results.

Experiment 3 analyses

The maximum and mean number of days before *B. albicinctus* in clip-cages matured into adults, or perished, were compared descriptively between host plant groups. Data from Trial 1 and 2 were analysed separately.

Key Research Findings

Results: Longevity of *Bathyllus* on *Westringia*, a known host plant

Twenty-two (92%) of the 24 nymphs initially placed on *Westringia* plants in the insect lab did not survive past 21 days (Oct 23, 2023), with the remaining two adult females (8%) eventually perishing between 101 and 105 days respectively (Jan 11 and Jan 15, 2024). The changing numbers of *B. albicinctus* nymphs, adult males, and adult females, over the initial three weeks (Oct 2023), was visualised in Figure 2. In the small and medium cohorts, adult male *B. albicinctus* were in higher abundance than adult females, emerged earlier, and typically lived longer (Figure 2A and 2B). The large cohort differed in having adult females emerging before males, and with adults persisting far longer (Figure 2C).

Our expectation that insect maturation and longevity would vary with nymph size cohort was partially supported. The number of days an insect spent as a nymph varied among size cohorts (ANOVA: $p = 0.046$, $F_{2,21} = 3.586$; Figure 3). The small *B. albicinctus* cohort were nymphs for longer compared to both the large cohort (LSD: $p = 0.037$) and medium cohort (LSD: $p = 0.026$). This supports the initial expectation that *B. albicinctus* longevity can vary depending on the size of nymphs collected. Nymphs in the large cohort were found to more often be female compared to the small cohort (Non-parametric T-test: $t = 2.256$, $p = 0.021$). These results suggest there may be a subtle interplay between insect development, size, and sex. Additionally, larger nymphs may have been further along in their development and, by association, closer to their adult stage.

Across cohorts, males accounted for two-thirds of individual *B. albicinctus* and one-third female (Table 3). Within cohorts, male insect accounted for 87.5% of the small cohort, and 75% of the medium cohort. The higher proportion of females (62.5%) in the large cohort may indicate they grow larger or emerge as adults earlier than male conspecifics.

The development of *B. albicinctus*, or at least adult longevity, appears driven by environmental conditions at seasonal temporal scales, rather than daily weather conditions. Selleck et al (2024) found *B. albicinctus* abundance was greatest between October and January for Victoria, closely matching the timing of this experiment (Figure 2 and 4).

Table 3. Proportion of female and male *Bathyllus albicinctus* within, and across, insect size-based cohorts.

Cohort	Sex	Count	Percent of cohort
Small	Female	1	12.5%
	Male	7	87.5%
Medium	Female	2	25%
	Male	6	75%
Large	Female	5	62.5
	Male	3	37.5
All	Female	8	33.3%
	Male	16	66.6%

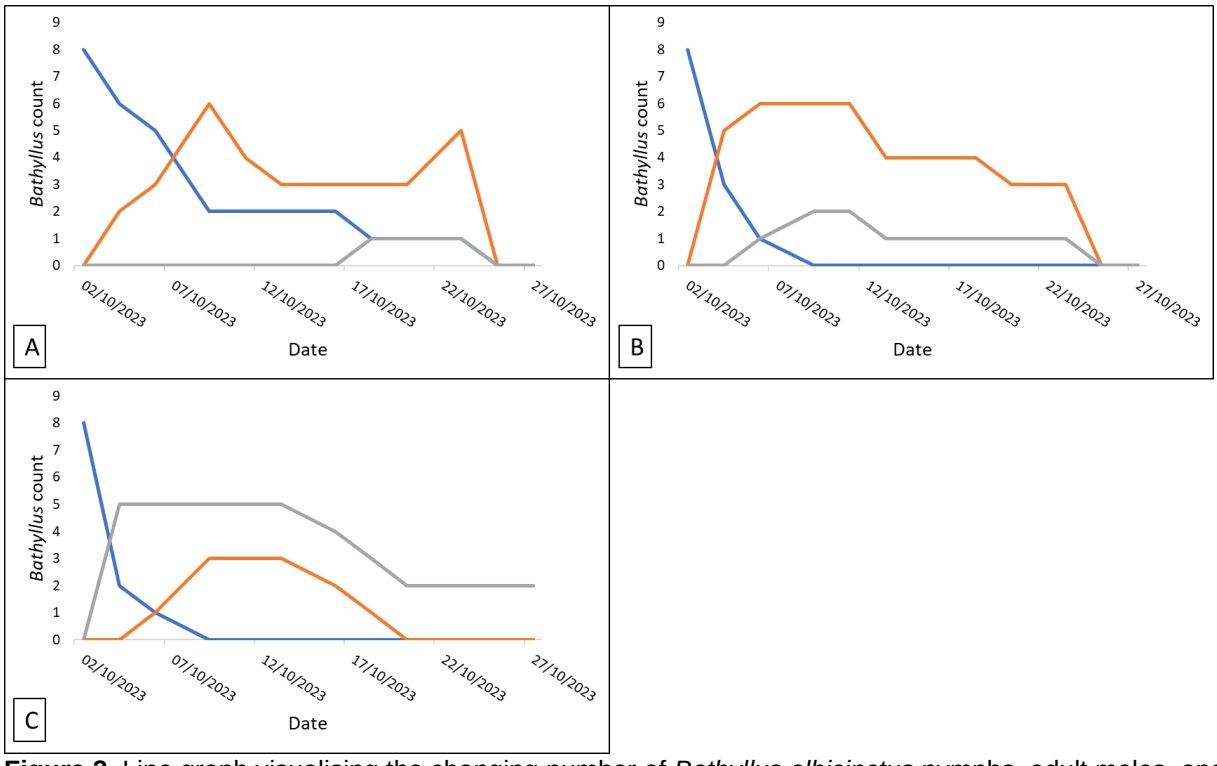


Figure 2. Line graph visualising the changing number of *Bathyllus albicinctus* nymphs, adult males, and adult females in Experiment 1. Changes over initial three weeks (Oct 2023) are represented for the (A) Small Cohort, (B) Medium Cohort, and (C) Large Cohort. Lines are colour coded by insect life stages; nymph (blue line), adult male (orange line), and adult female (grey line). Note: the two adult females in the large cohort persisted for three months until mid-January 2024, which is not plotted.

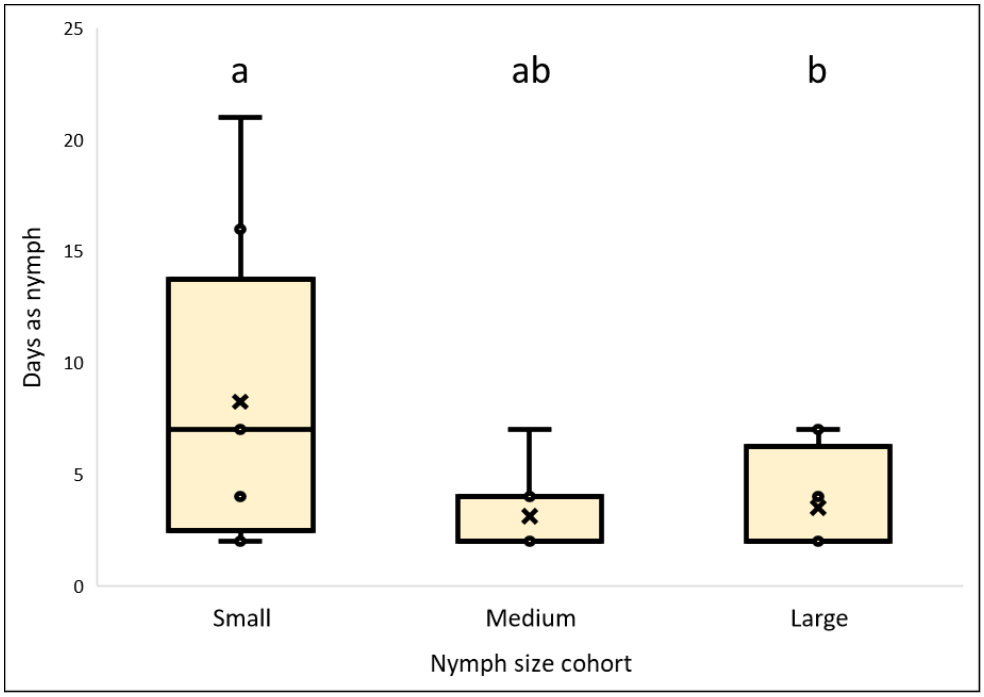


Figure 3. Histogram visualising the longevity of *Bathyllus albicinctus* across three cohorts based on the size of nymphs when collected. Size cohorts that do not have the same floating letter are significantly different ($\alpha < 0.05$), based on LSD post-hoc analysis.

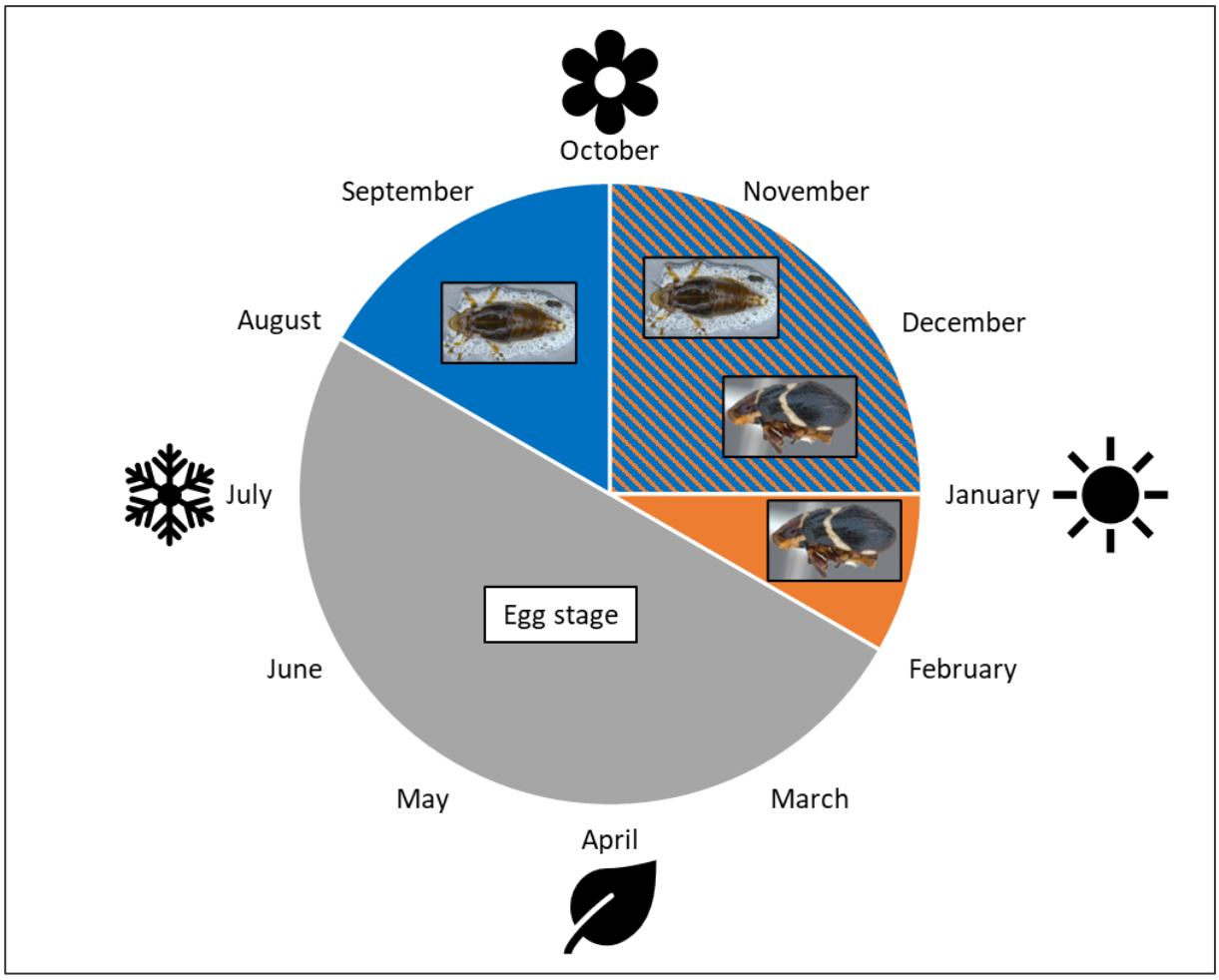


Figure 4. Pie-chart visualising the estimated seasonal phenology of *Bathyllus albicinctus* life-stages across one calendar year in southeastern Australia. Colours indicate the calendar months when mature *B. albicinctus* adults (orange), immature stages (blue), or both (orange and blue stripes), are likely to be encountered, and when insects can be inferred to be in an egg stage (green). Although eggs were not directly observed, it may be inferred that immature *B. albicinctus* are in an egg stage during the months when neither nymph or adults are present.

Results: Survival of *Bathyllus* on a range of common potential host plants

In the screenhouse experiment, some *B. albicinctus* in cages A, C, D, and E survived the monitoring period lasting from October 24, 2023, until December 12, 2023 (Table 4; Figure 5). However, all *B. albicinctus* in cage B (barley) perished before the end of the experiment monitoring period. Survival of insects also differed between female and male *B. albicinctus*, with all female adults perishing before the end of monitoring in the capsicum, barley, and basil cages, while male adults only went extinct in the barley cage. The rosemary cage had the highest rate of survival, with a survival rate of 50% for females and 75% of males. This was followed by the mix-host cage (which notably also contained coastal rosemary), with 25% of females and 50% of males surviving the monitoring period.

The daily maximum temperature explained virtually no variation in the proportion of surviving *B. Albicinctus* (Linear regression: $p = 0.650$, $F_{1,44} = 0.208$, $r^2 = 0.005$; Figure 6A), while the number of surviving insects was strongly associated with the cumulative temperature experienced in the screenhouse (Logarithmic regression: $p < 0.001$, $F_{1,48} = 208.9$, $r^2 = 0.813$; Figure 6B), and the number of days spent in the cage (Logarithmic regression: $p < 0.001$, $F_{1,44} = 208.8$, $r^2 = 0.813$; Figure 6C). These models indicate that more than 80% of the variation in *B. albicinctus* survival could be explained by cumulative temperature or time.

Our results indicated that of the range of plants tested *Westringia* was a preferred host for female and male *B. albicinctus*. It appears that capsicum and basil may not be viable host plants to maintain *B. albicinctus*, as females did not survive. Furthermore, most *B. albicinctus* that did not survive on basil perished within the first two-days of the experiment. This may have been due to possible systemic pesticides applied to the store-bought basil prior to its arrival in-store, whereas capsicum was seed-grown and was not exposed to pesticides. *Westringia* was also store-bought, yet its suitability as a host may have assisted *B. albicinctus* to better tolerate the potential impact of pesticides on *Westringia* applied prior to its arrival in-store.

Neither adult female or male *B. albicinctus* survived the study duration on barley, although it was unknown if the insects attempted to feed on barley. *B. albicinctus* has been recorded in association with a variety of Poaceae host plants (Selleck et al 2024), making it unexpected that it cannot support a *B. albicinctus* population. Furthermore, the two Lamiaceae plants in this study (*Westringia* and Basil) demonstrated very different insect survival rates.

Westringia was found to be able to maintain *B. albicinctus* due to the survival of both male and female adults. This is not unexpected as many field populations have been recorded on ornamental *Westringia*. It is also probable that female insects surviving in the mixed host plant cage were feeding on *Westringia* because females *B. albicinctus* only survived to the end of the experiment in cages containing *Westringia,* both in mixed and single plant type cages.

Table 4. Summary of the *Bathyllus albicinctus* counts, and percent survival of, adult female and adult male *B. albicinctus* among cages with different host plants. Cages initially contain four male and four female *B. albicinctus* adults.

Cage:	A. Capsicum	B. Barley	C. Basil	D. Coastal Rosemary	E. Mix	Total
Females assessed:	3	2	2	2	1	10
Female survival:	0	0	0	2	1	3
Female survival (%):	0	0	0	50	25	15
Males assessed:	3	1	3	3	2	12
Male survival:	2	0	1	3	2	8
Male survival (%):	50	0	25	75	50	40

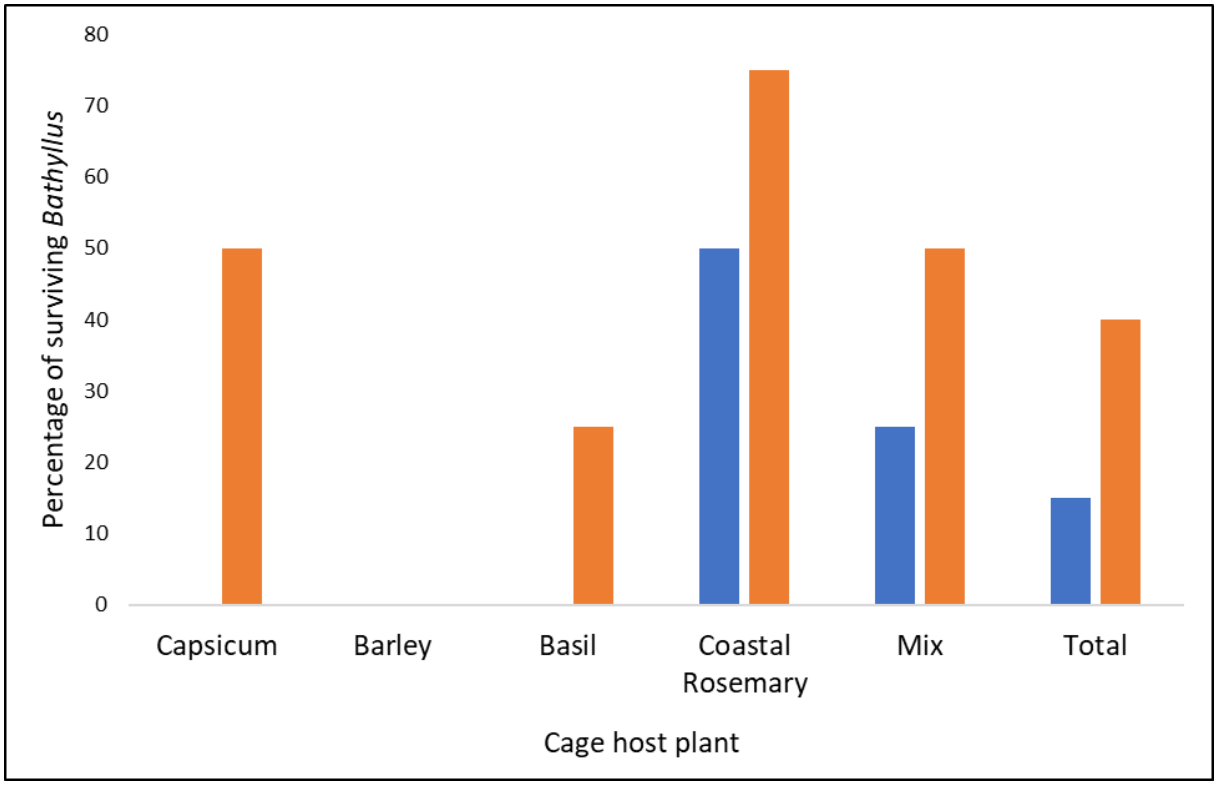


Figure 5. Proportion of *Bathyllus albicinctus* that survived 50 days, across host plant species, and adult male (orange) and female (blue) insects.

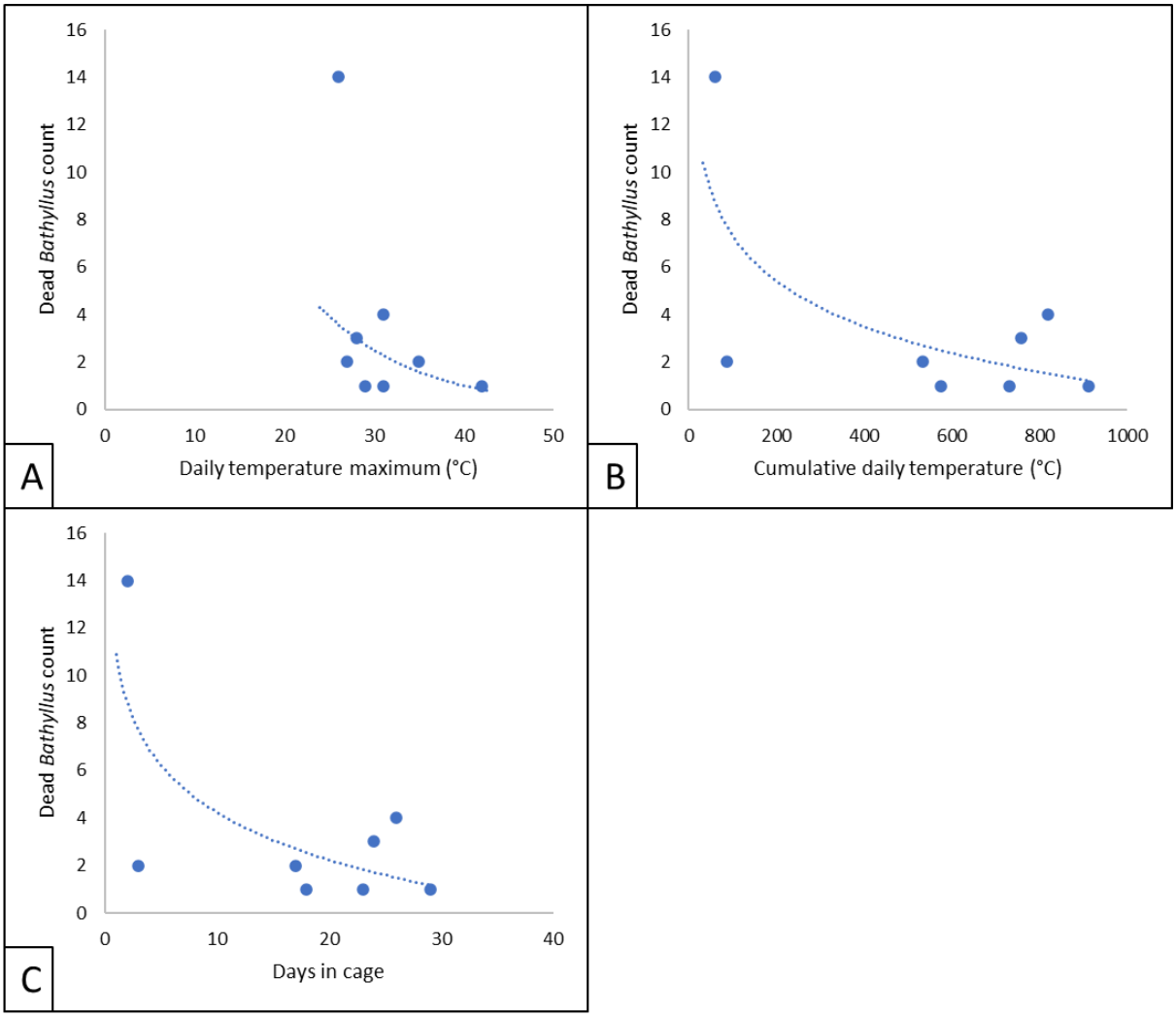


Figure 6. Scatterplot visualising relationships between the proportion of surviving *Bathyllus albicinctus* across total cage treatments and: (A) daily maximum temperature (°C), (B) cumulative daily maximum temperature (°C), and (C) the number of days since *B. albicinctus* were transferred to their cage treatments.

Results: Maturation and longevity of *Bathyllus* on host plants susceptible to *Xylella*

High mortality rates were recorded in the first few days of Trial 1 (starting in October 2022) and 2 (starting in December 2022), but maximal survival duration reached 20 days in Trial 1 and 25 days in Trial 2 (both on grapevine) (Figure 7). Maximal survival of *B. albicinctus* ranged from 11 to 16 days for olive and orange, respectively, across both experiments. Maximal survival was lowest on sow thistle compared to grapevine, olive, and orange in both trials, despite the fact that some of these insects were initially collected from sow thistle.

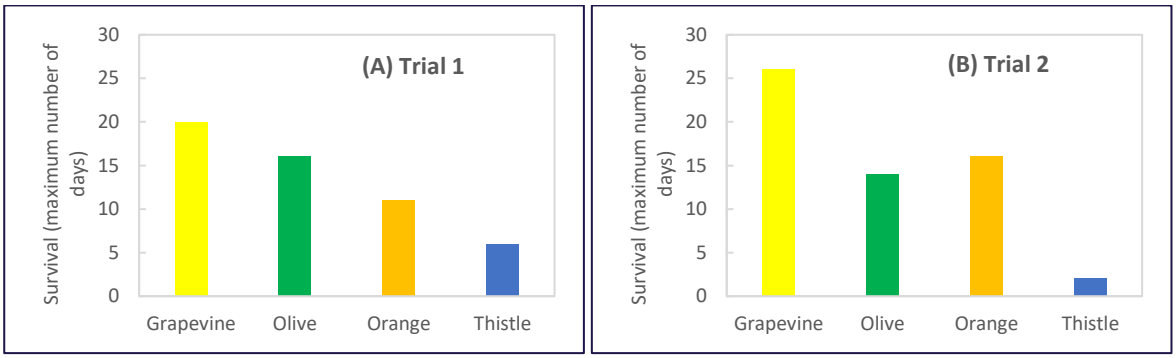


Figure 7. The maximum number of days that *Bathyllus albicinctus* survived on each host type in (A) Trial 1 and (B) Trial 2.

Mean survival time of *B. albicinctus* on grapevine, olive and orange reached 6.1 days (on olive) in Trial 1 and 5.8 days (on grapevine) in Trial 2 (Figure 8). In Trial 1, there were no clear differences between grapevine, orange, and sow thistle (Figure 8A). For Trial 2, *B. albicinctus* survived on sow thistle for approximately half the number of days insects survived on grapevine, olive, or orange (Figure 8B). *B. albicinctus* feeding on grapevine had the shortest survival time in Trial 1 and the longest in Trial 2.

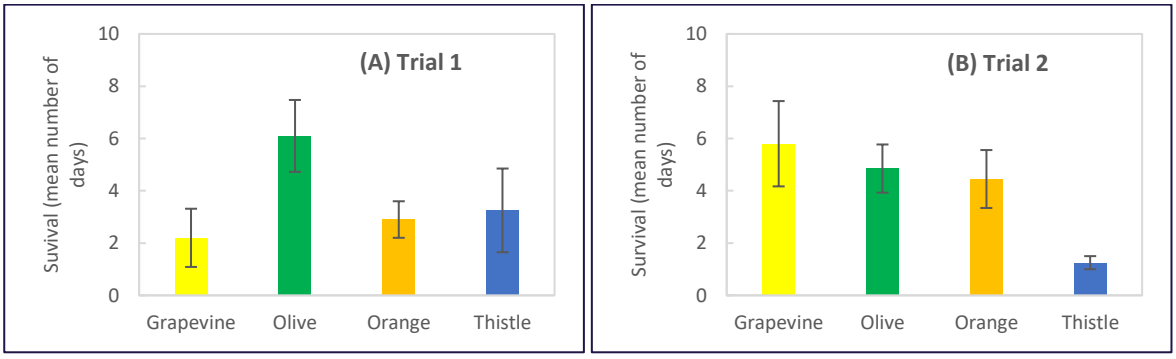


Figure 8. The mean number of days that *B. albicinctus* survived on each host type in (A) Trial 1 and (B) Trial 2.

B. albicinctus in Trial 1 only reached adulthood on olives and grapevine. Adults in Trial 2 were recorded for grapevine, olive, and orange. In both trials, olive had the most nymphs that reached adulthood. *B. albicinctus* did not reach adulthood on oranges in Trial 1 and sow thistle in both trials (Figure 9). All stages of *B. albicinctus* were observed on thistle in the field, and a great amount of care was taken when attaching the insects to the plants. However, due to transplanting from the field to pots, symptoms of stress were observed on the sow thistle plants soon after they were transplanted, therefore, the lack of survival on sow thistle may be due to a lack of turgor pressure in the leaves, which may have made it difficult for the nymphs to feed.

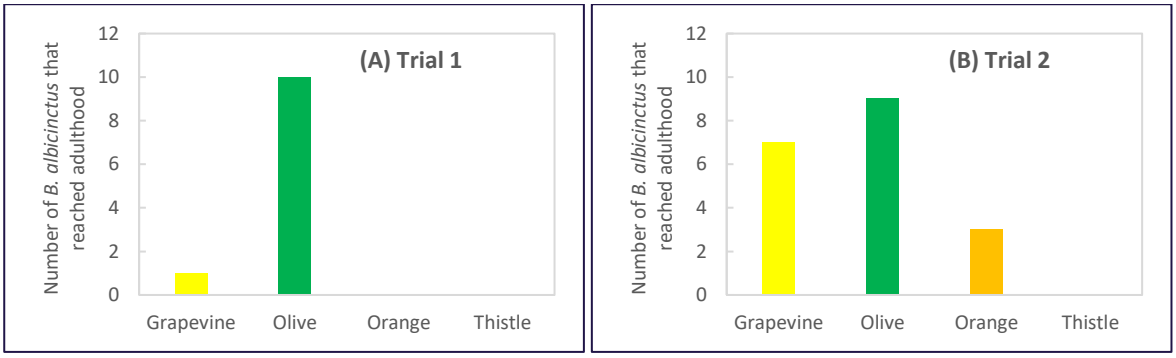


Figure 9. The number of that *Bathyllus albicinctus* reached adulthood on each host type in (A) trial 1 and (B) trial 2.

Recommendations

Outcomes

1. Experiment 1.

In this experiment we have identified variation in the maturation timing of *B. albicinctus* nymphs, feeding on *Westringia*, and compared the longevity of resulting adults between insect size cohorts. Nymphs matured into adults in October, similar to observations from previous field studies in Victoria. Although most insects perished within October 2023, two adult females persisted until January 2024. This demonstrates that the *B. albicinctus* life span, and therefore its opportunity to potentially transmit *X. fastidiosa,* is highly variable between nymph size, sex, and season when insects are abundant.

2. Experiment 2.

Here we identified variation in the survival of *B. albicinctus* adults between a range of host plant species. Insects were most likely to survive on coastal rosemary (*Westringia*). Insect reproduction would only be possible where *Westringia* was available, as females did not survive elsewhere. Even without the ability to reproduce, the other host plants may still be susceptible to *X. fastidiosa* if infected *B. albicinctus* insects attempted to feed on these plants. This highlights the significance *Westringia* may have as a potential reservoir source of *B. albicinctus* insects, and potentially *X. fastidiosa* if it were to arrive in Australia.

3. Experiment 3.

The timing of insect collection was important to insect longevity, with October-collected insects surviving up to 20 days and December-collected insects surviving up to 25 days. Feeding by *B. albicinctus* on grapevine, olive, oranges, and sow thistles indicated that these plants might be susceptible to *X. fastidiosa* infection by *B. albicinctus*. *B. albicinctus* feeding on olives were the most likely to reach adulthood, indicating that olive orchards could act as a source of *B. albicinctus*. On sow thistle, however, *B. albicinctus* did not reach adulthood or survive longer than 10 days, possibly due to the impact of transplanting on thistle health and suitability as a host. The low survival and low maturation count on Experiment 3 host plants was contrasting to Experiment 1, in which all nymphs reached adulthood on an alternative host plant, *Westringia*.

Conclusion

Future directions

This research highlighted several key questions for future research:

1. The results from the laboratory and screenhouse experiments provide useful insight into the seasonal occurrence of *B. albicinctus*. The length of time adults persisted, however, varied between nymph size cohorts. To generalise these findings to field conditions, these experiments should be repeated under field conditions, to understand *B. albicinctus* development on host plants in the field, compared to laboratory or nursery-grown plants.

2. Insect survival was associated with screenhouse conditions, specifically the cumulative temperature experienced by *B. albicinctus*, although only *Westringia fruticosa* successfully sustained both female and male *B. albicinctus*. Due to their common use as ornamental and commercial crops in Australia, and documented susceptibility to *X. fastidiosa* elsewhere (EFSA et al 2023; Figure 10), *W. fruticosa* should be considered at high risk of *X. fastidiosa* infection potentially vectored by *B. albicinctus*, should the pathogen arrive in Australia. As such, follow-up research should focus on documenting the development and actual vector capacity of *B. albicinctus* feeding on *W. fruticosa*, or similarly attractive and common host plants, under field conditions. Our knowledge of optimal conditions for *B. albicinctus* populations could be improved by documenting the relationship between *B. albicinctus* abundance, reproductive success, and field conditions.



Figure 10. *Westringia fruticosa* infected with *Xylella fastidiosa* and exhibiting visible symptoms. Note the chlorotic and desiccated vegetation. Photo copyright held by Donato Boscia, CNR – Institute for Sustainable Plant Protection, UOS, Bari (Italy), European and Mediterranean Plant Protection Organisation.

3. Across the three experiments, *Westringia,* olives, and, to a lesser extent grapevines and oranges, were found to potentially be able to support a reproductively viable *B. albicinctus* population. The success of *B. albicinctus* did not neatly align with the taxonomic relationship of host plants (i.e., plant families) and could differ depending on the seasonality. Environmental conditions (Jarošík 2019; Jaroslow et al 2023), host health and species characteristics (Andersen et al 1992; Taylor et al 2011), or an interplay of these factors (Wong and Daniels 2017), contribute to the suitability and attractiveness of a host to insect herbivores (For review: Awmack and Leather 2002; Knolhoff and Heckel 2014), and this is likely the case for *B. albicinctus*. Further investigations into the relationship between *B. albicinctus* and environmental conditions, such as temperature, would allow for predictions of when and where this insect's activity is greatest and, by association, the risk of potentially spreading diseases such as *X. fastidiosa*.

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AGRICULTURE VICTORIA

Xylella vectors project

Technical Report: Identification of Auchenorrhyncha in Victorian Olive orchards and vineyards (2020-2021)

Agriculture Victoria Research

Apr/2022

AGRICULTURE VICTORIA

VICTORIA State Government | Jobs, Precincts and Regions

Author: Cait Selleck and Linda Semeraro

Project RDC Number: 106162

Project CMI Number: 150665

Department of Jobs, Precincts and Regions
1 Spring Street Melbourne Victoria 3000
Telephone (03) 9651 9999

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INTRODUCTION

This report includes a summary to date, of the species/ genera/ families of Auchenorrhyncha collected from Olive Orchards in the Grampians region (between 2020 and 2021) which have been identified. Its intention is to highlight which groups have been identified as potential xylem-feeders, and confirms the presence of at least one xylem feeding species, the spittlebug *Bathyllus albicinctus*.

A number of non-xylem feeding Auchenorrhyncha were also identified. Further identification of these species may provide baseline data for future work in these orchards.

Samples collected between November 20th 2020 and May 7th 2021 from Grampians Olive near the Grampians, consisted of 33 vials containing Auchenorrhyncha or aphids. The Auchenorrhyncha from these samples were examined and specimens were further sorted to morphospecies. Another 9 unsorted samples were received in November 2021, collected between 10th of September and 5th of November 2021 from Grampians Olive or Norton Estate Vineyard near the Grampians. Five of the nine samples contained Auchenorrhyncha, though no new Auchenorrhyncha species were represented in the more recent samples.

Project outcome

The primary outcome of the identification work is to identify and have a better understanding of the species which are known xylem feeders that currently occur in orchards (Olive and Citrus) and vineyards in south-eastern Australia/ Victoria. This data is currently missing from reference collections and online databases, or is at least incomplete.

Through the identification of all Auchenorrhyncha collected, we can determine which species are potential threats as *Xylella* vectors and which are non-threats. This study also provides, more generally, a baseline of what species occur in the orchards and vineyards. Representatives of each species will be retained in the Victorian Agricultural Insect Collection (VAIC).

This value adds, as many Auchenorrhyncha species are known vectors of other plant diseases and all the data acquired in this study may be useful for future projects.

Outputs:

1) List identified species/genera/families of xylem-feeding Auchenorrhyncha known to occur in olive orchards and vineyards in Victoria (from both recent field collections and previously collected reference material).
2) Highlight a list of xylem-feeding insects currently known to occur in the targeted orchards and vineyards near the Grampians.
3) Provide a summary about the distribution, biology, and host preferences of identified xylem-feeders.
4) Provide DNA barcode sequences for each genus of Auchenorrhyncha from recent field trips, where possible. In some cases, these will be the only sequences available for the species.
5) Acquire identification images representing each of the species collected.

Project background

Xylella fastidiosa is a xylem-limited bacteria that is transmitted by xylem-feeding insect vectors in the Auchenorrhyncha (Hemiptera) suborder, including Cicadellidae (leafhoppers) and Cercopoidea (spittlebugs). The disease seriously effects several crop plants such as grapevines (causing Pierce's Disease), citrus (causing citrus variegated chlorosis), almond (causing leaf scorch) and other stone fruit crops (causing peach phony disease and plum leaf scald) and olives (causing leaf scorch), resulting in serious economic losses to these industries. To date, neither the main vectors known overseas or the bacteria are known to occur in Australia. However, there are many species of Auchenorrhyncha in Australia that are closely related to those vectors overseas and are known xylem-feeders.

In Europe the aphrophorid spittlebug, *Philaenus spumarius*, a native of the region, became the main vector of *X. fastidiosa* once this disease was introduced there. Thus, a spittlebug native to Australia may have the same vectoring potential. It is worth preparing for this situation by investigating the main xylem-feeding Auchenorrhyncha species found in susceptible orchards, so we can be prepared in the event of a *X. fastidiosa* incursion. The research included in this project will allow us to limit the extent of a possible incursion and contain the bacterium.

Project objectives

- The primary aim of the research being conducted at AgriBio is to examine potential *Xylella* vectors trapped during this project, to identify which species are being trapped and which of these may be potential xylem-feeders, based on what is known about their biology.
- Identification of insects may be based on morphological features and/or DNA barcoding.
- High quality images of identified insects will be generated to provide a resource for future identifications.
- Additional work may also be conducted on potential *Xylella* vectors preserved as reference specimens in the Victorian Agricultural Insect Collection (VAIC).

RESULTS

Species Identified

Insect specimens were collected from 20th Nov 2020 to 7th May 2021, from an olive orchard in Laharum, Victoria. Collections were made by Piotr Trebicki and Eva Carreras Navarro.

A list of species identified, along with their higher-level classification, is provided in Table 1.

Morphological identifications were conducted by Cait Selleck and Linda Semeraro using an online Auchenorrhyncha key (Murray et al. 2009). Species that were further validated using descriptions in Evans 1966 and original descriptions are indicated by 1. However further confirmation is required for some identifications.

Species where DNA sequences (COI gene – barcoding gene) were acquired (Figure 1) are marked with an asterisk. However, not all sequences could be matched in the online BOLD or GenBank databases with an identified species. Identifications which could be matched genetically to species level with high confidence, are marked with a 2. Molecular tests were performed and analysed by Cait Selleck and Lea Rako.

The collection information for each vial and ID information for each specimen has been recorded in an excel file titled "*Xylella* vectors survey list", in the AgriBio *Xylella* vectors folder on OneDrive.

Table 1. Species identified collected from 20th Nov 2020 - 7th May 2021. The species highlighted in green is a known xylem-feeder. Taxa marked with * have corresponding COI barcodes. Species marked with [1] were validated with descriptions from Evans 1966. Species marked with [2] were matched genetically to species level with high confidence.

Membracoidea

CICADELLIDAE

Deltocephalinae

Athysanini - *Arawa pulchra*

Chiasmini – *Chiasmus*[1] nr *varicolor*

Deltocephalini – *Maiestas* sp.

Eupelicini - *Paradorydium viridis* [1*]

Goniagnathini - *Goniagnathus* [1] *?osmelaki*[*]

Macrostelini - *Balclutha* [1] spp.

- *Nesoclutha phryne* [1] (Kirkaldy) (the Australian grass leafhopper)

Opsiini - *Orosius argentatus* [1,2 *] (Evans) (the common brown leafhopper)

Iassinae

Batracomorphini – *Batracomorphus*[1] sp.

Megophthalminae

Agalliini – *Austroagallia torrida* [1] Evans (the spotted leafhopper)

Typhlocybinae

Erythroneurini – *Anzygina zealandica*

Empoascini – *Austroasca* sp.[*]

Ulopinae

Cephalelini - undetermined

MEMBRACIDAE

Centrotinae

Terentiini - *Sextius ?virescens*[*]

Cercopoidea

APHROPHORIDAE

Bathyllus albicinctus [1,2*]

Fulgoroidea

FLATIDAE

Flatinae

Siphanta sp.[*]

Figure 1: The above phylogenetic tree was built using sequences from 13 specimens collected at the Grampians Olive site in 2020 and 2021 and compared to reference sequences obtained from BOLD or GenBank (BIH614-08, KMPIF193, GMCWJ827, BIH581, HMAS1720, MN345252).

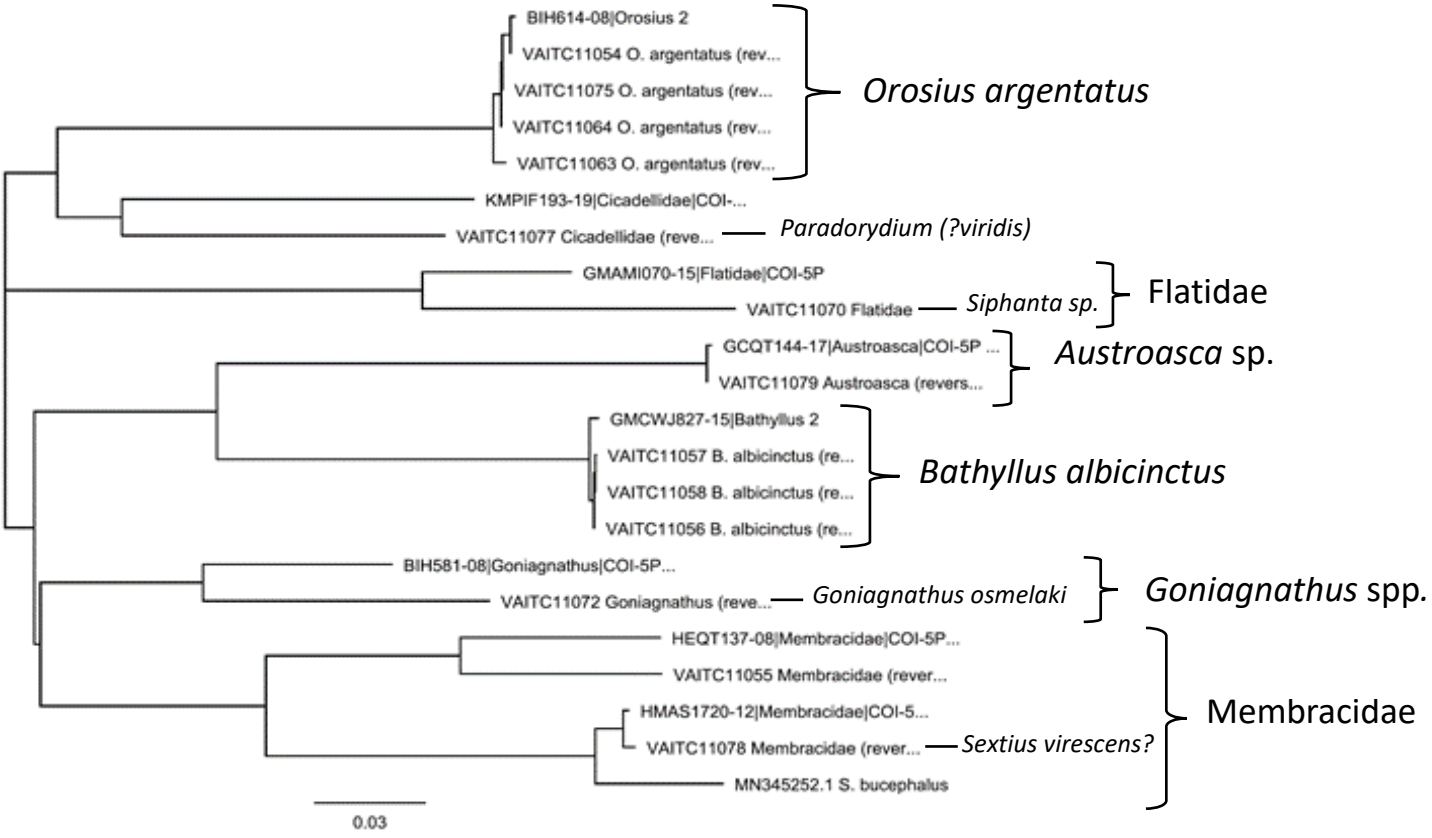


Table 2. Specimens identified in this project through molecular methods.

Specimen	Morphological Identification	Molecular Identification	% Match on database (BOLD/GenBank)
VAITC 11054	*Orosius* sp.	100	*Orosius argentatus*
VAITC 11055	*Sextius virescens?*	90	Membracidae
VAITC 11056	*Bathyllus albicinctus*	99.69	*Bathyllus albicinctus*
VAITC 11057	*Bathyllus albicinctus*	99.69	*Bathyllus albicinctus*
VAITC 11058	*Bathyllus albicinctus*	99.69	*Bathyllus albicinctus*
VAITC 11063	*Orosius* sp.	99.85	*Orosius argentatus*
VAITC 11064	*Orosius* sp.	100	*Orosius argentatus*
VAITC 11070	*Siphanta* sp.	88.64	Flatidae
VAITC 11072	*Goniagnathus osmelaki*	88.04	*Goniagnathus*
VAITC 11075	*Orosius* sp.	100	*Orosius argentatus*
VAITC 11077	*Paradorydium viridis*	84.56	Cicadellidae
VAITC 11078	*Sextius virescens?*	99.54	Membracidae
VAITC 11079	Empoascini	100	*Austroasca* sp.

Species of Interest – *Bathyllus albicinctus*

Bathyllus albicinctus (Erichson) was the only Cercopoidea detected in any of the samples, and is thought to be the only xylem-feeding Auchenorrhyncha collected.

Distribution

Specimen based records of *Bathyllus albicinctus* from the Atlas of Living Australia (ALA) and the Australian Plant Pest Database (APPD) were mined for *Bathyllus albicinctus* to supplement the Victorian Agricultural Insect Collection (VAIC) specimens for distribution and apparent hosts (Figures 2, 3 & 4). Close-up shots of each state are provided in the Appendices section below. Selleck et al. 2024 expand upon this with data from reference collections around Australia.



Figure 2. *Bathyllus albicinctus* specimen records in Australia taken from the ALA. Records are Australia-wide, excluding NT. There are 147 records identified as *B. albicinctus*, though only 29 records have a specimen associated with them. The remaining 118 records are observation records. Specimen records are known only from NSW, SA, Tas and WA. For state-specific maps refer to Figure A.1.

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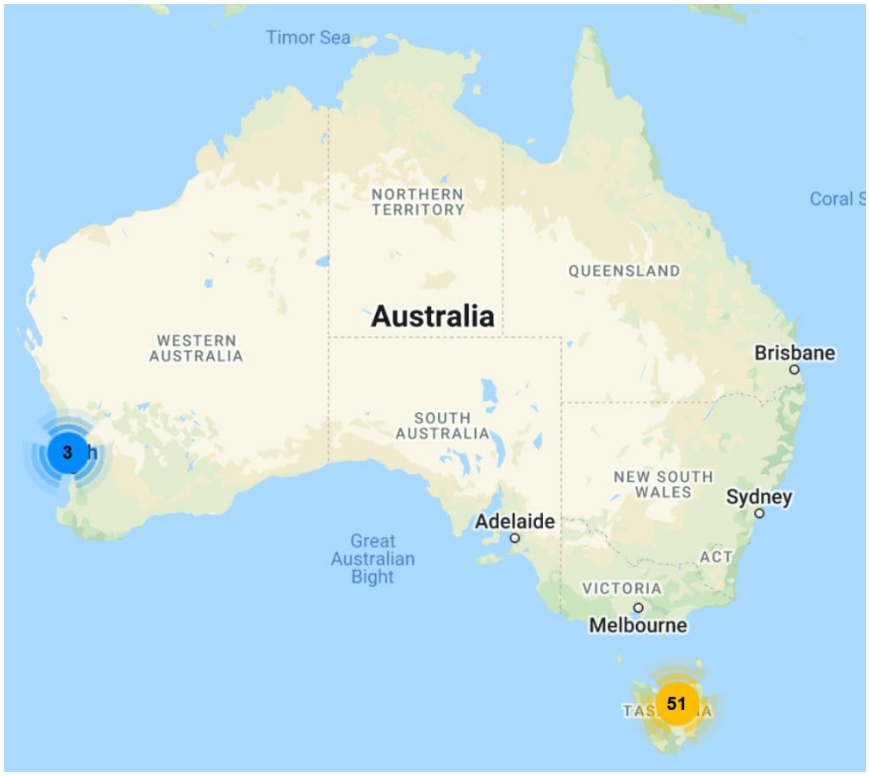


Figure 3. *Bathyllus albicinctus* specimen records in Australia taken from the APPD. Records are only from Tas and WA, but all 54 records are based on a specimen. Specimens from Victoria are in the VAIC but have not yet been uploaded to the APPD (that data is shown in Figure 4). For state-specific maps refer to Figure A.2.



Figure 4. *Bathyllus albicinctus* specimen records in Australia taken from the VAIC. Records are all based on specimens located at AgriBio, Bundoora. The 69 specimens are from QLD, Vic, and SA. For state-specific maps refer to Figure A.3.

Potential host species

Table 2. *Bathyllus albicinctus* has been collected on a variety of different native, ornamental, and weedy plants from different families. Whether the insect was feeding on every plant is unknown. Below is a list of plants associated with the recorded specimens, separated by family.

Common name	**Scientific name**	**Family**	**Location observed**
Lucerne/alfalfa	*Medicago sativa*	Fabaceae	Vic., Newstead
Coastal wattle	*Acacia sophorae*	Fabaceae	Vic., Bundoora NSW, Wooli
Shasta daisy	*Leucanthemum* x *superbum*	Asteraceae	SA, Adelaide
Daisy	-	Asteraceae	Vic., Bundoora
Skeleton weed	*Chondrilla juncea*	Asteraceae	QLD, Brisbane
Margeurite daisy	*Argyranthemum frutescens*	Asteraceae	Vic., St. Arnaud
Tea tree	*Leptospermum* sp.	Myrtaceae	Vic., Melbourne
Feijoa	*Feijoa sellowiana*	Myrtaceae	WA, Busselton
Yellow gum	*Eucalyptus leucoxylon*	Myrtaceae	SA, Carew
Rosemary	*Salvia rosmarinus*	Lamiaceae	Vic., Melbourne
Sage	*Salvia* sp.	Lamiaceae	-
Lavender	*Lavandula* sp.	Lamiaceae	Vic., Highton
Barley	*Hordeum vulgare*	Poaceae	SA, Port Rickaby
Grass	-	Poaceae	Vic, Woodlands
bayhops, beach morning glory, goat's foot	*Ipomoea pes-caprae*	Convulvulaceae	Vic, Greenvale QLD, Urangan
Aloe vera?	*Aloe* sp?	Asphodelaceae	SA, Carrickalinga
Geranium, cranesbill	*Geranium* sp.	Geraniaceae	Vic., Melbourne
Hop goodenia*	*Goodenia ovata*	Goodeniaceae	Vic., Melbourne
Avocado	*Persea americana*	Lauraceae	WA, Busselton
Cinnamon bells, common potato orchid	*Gastrodia sesamoides*	Orchidaceae	Tas, Latrobe
Beardtongue	*Penstemon* sp.	Plantaginaceae	Vic., Lal Lal
Grevillia, spider flower	*Grevillia* sp.	Proteaceae	WA, Glenfield

*Unknown if *Bathyllus albicinctus* specimen was recorded on *Goodenia*, or if the survey was centered around *Goodenia* in general.

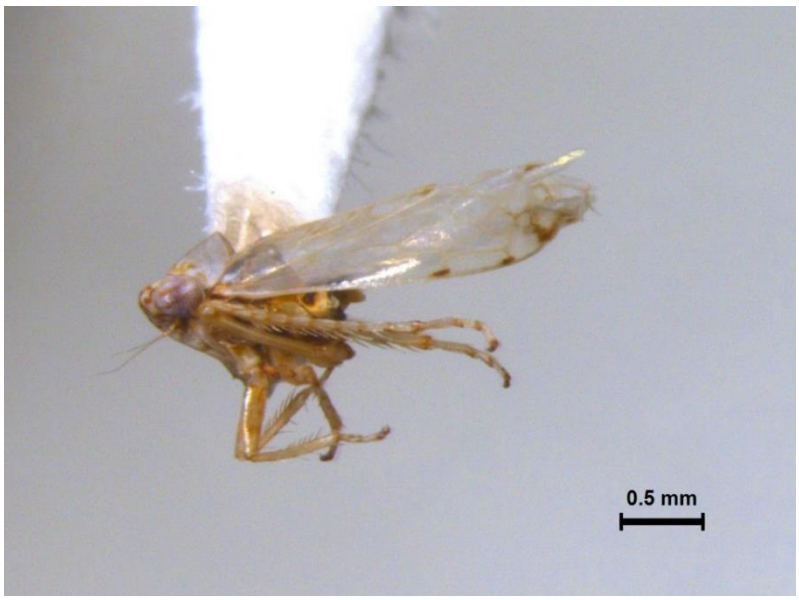
Images of Species Examined

Images were taken by Cait Selleck using Leica Application Suite (LAS version 4.5.0) software running a Leica DCF450 camera attached to a M205C microscope with MEB110 ring light. Multifocal image stacking was achieved using LAS software. Scale bars were also produced within LAS.

Thirteen specimens with successful DNA sequences are included below, representing nine different taxa.



VAITC 11054 - *Orosius argentatus* male, dorso-lateral view



VAITC 11054 – *Orosius argentatus* male, lateral view (abdomen removed for genitalia dissections)



VAITC 11063 – *Orosius argentatus* male, lateral view



VAITC 11063 – *Orosius argentatus* male, ventral view



VAITC 11064 – *Orosius argentatus* female, lateral view



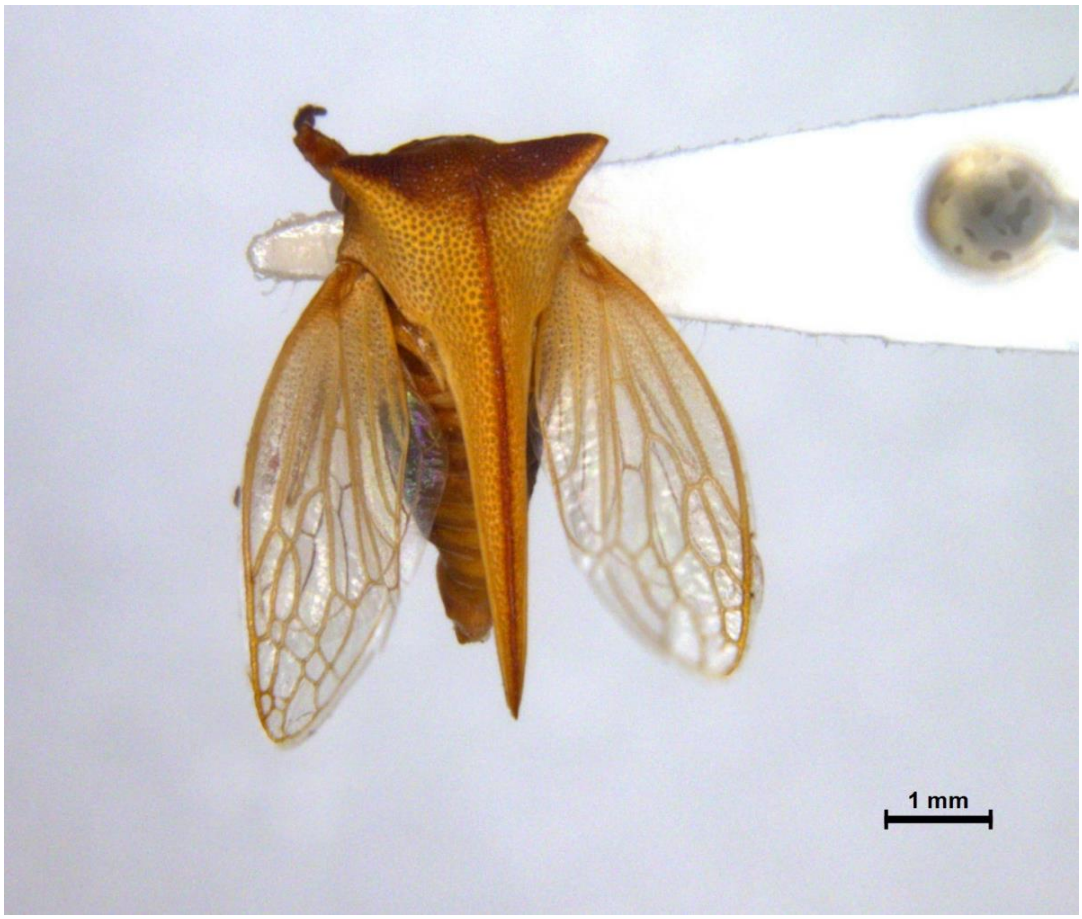
VAITC 11064 – *Orosius argentatus* female, antero-dorsal view



VAITC 11075 – *Orosius argentatus* female, dorsal view



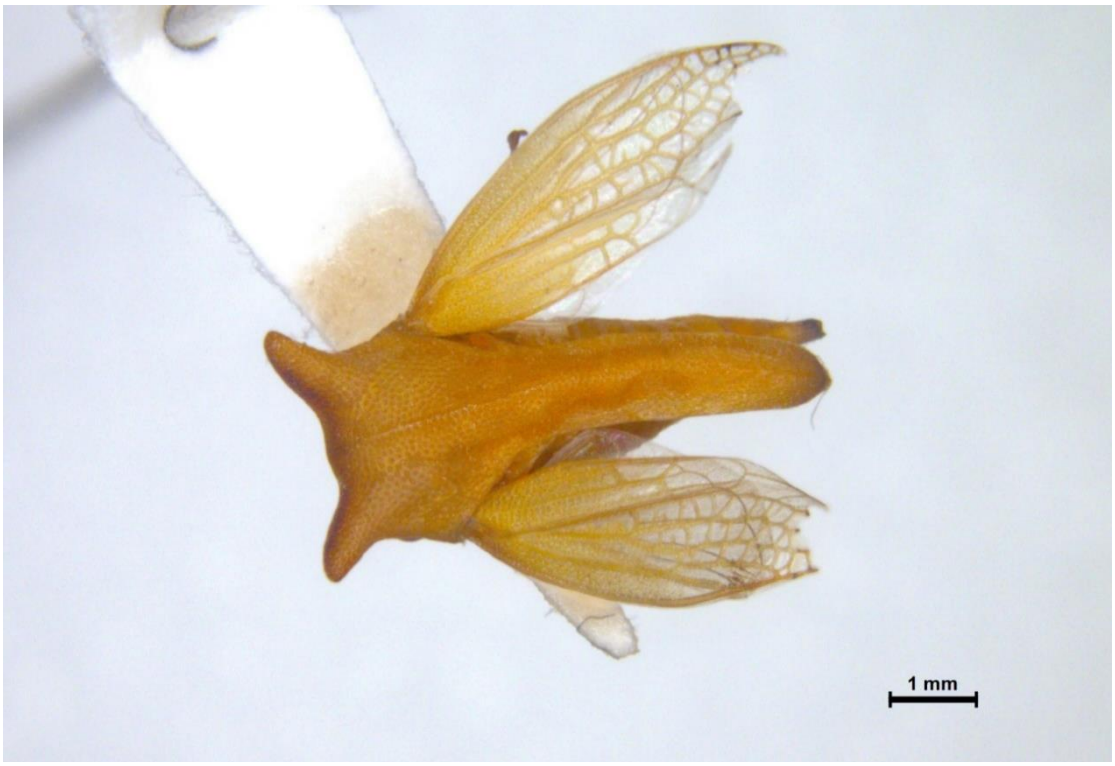
VAITC 11075 – *Orosius argentatus* female, lateral view



VAITC 11055 – *Sextius virescens* male, dorsal view



VAITC 11055 – *Sextius virescens* male, lateral view



VAITC 11078 – Membracidae female, dorsal view



VAITC 11078 – Membracidae female, lateral view



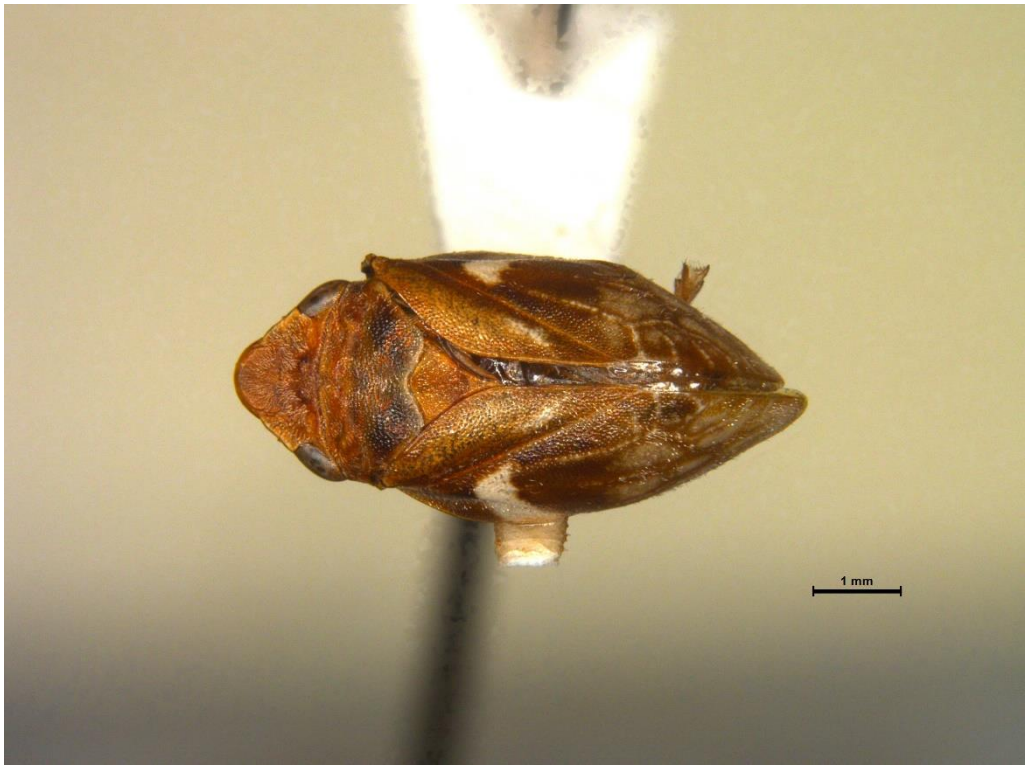
VAITC 11056 – *Bathyllus albicinctus* female, lateral view



VAITC 11056 – *Bathyllus albicinctus* female, anterior view



VAITC 11057 – *Bathyllus albicinctus* female, lateral view



VAITC 11057 – *Bathyllus albicinctus* female, dorsal view



VAITC 11058 – *Bathyllus albicinctus* male, dorsal view



VAITC 11058 – *Bathyllus albicinctus* male, lateral view



VAITC 11070 – *Siphanta* sp. male, lateral view



VAITC 11070 – *Siphanta* sp. male, dorsal view



VAITC 11072 – *Goniagnathus osmelaki* male, dorso-lateral view



VAITC 11072 – *Goniagnathus osmelaki* male, lateral view



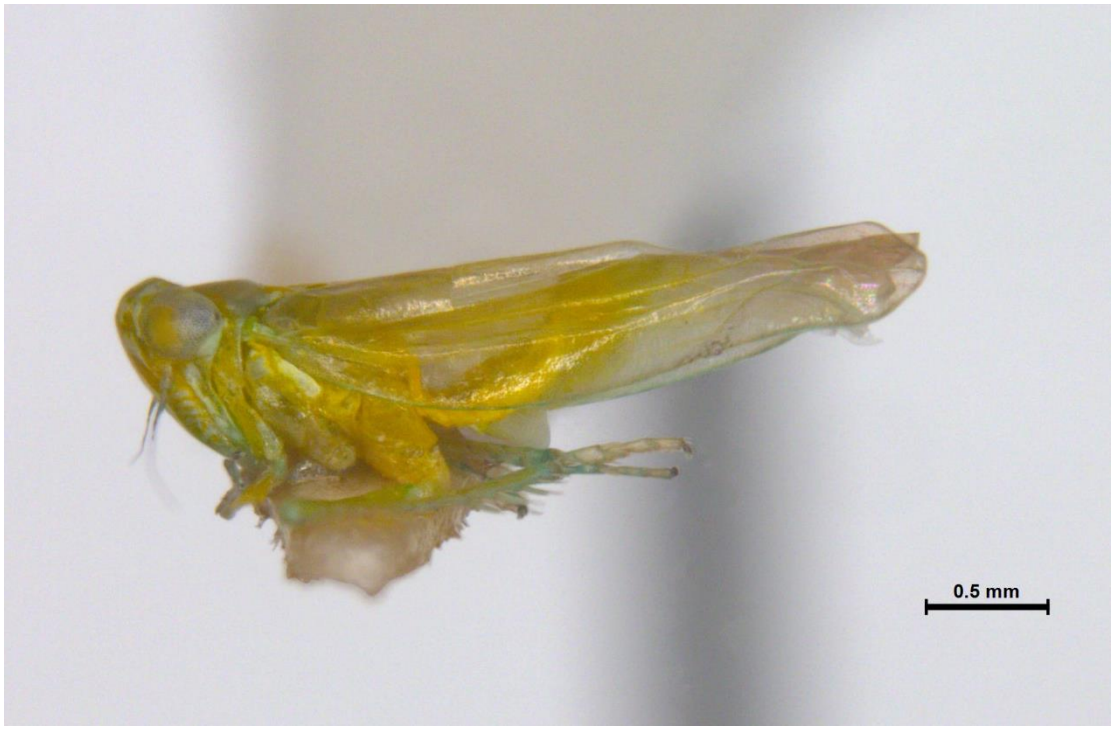
VAITC 11077 – *Paradorydium viridis* male, dorso-lateral view



VAITC 11077 – *Paradorydium viridis* male, ventral view



VAITC 11079 – *Austroasca* sp. female, dorsal view



VAITC 11079 – *Austroasca* sp. female, lateral view

CONCLUSION

Based on the data to date, there is only one species that is recognized as a xylem-feeder, collected from the Grampians Olive Orchard. As a result, *Bathyllus albicinctus* will be the focus of future host and distributional studies.

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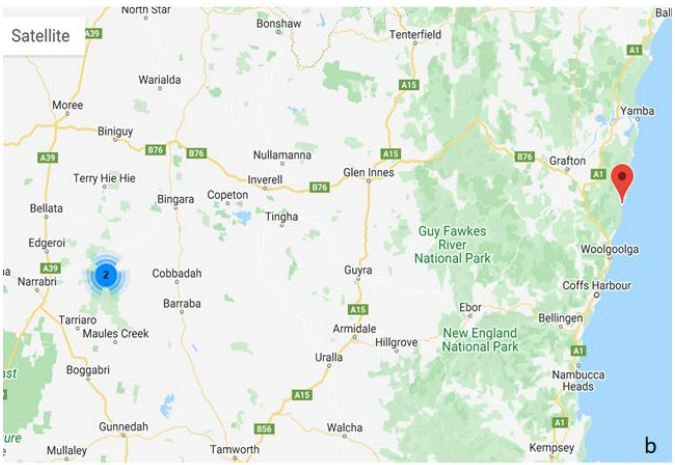
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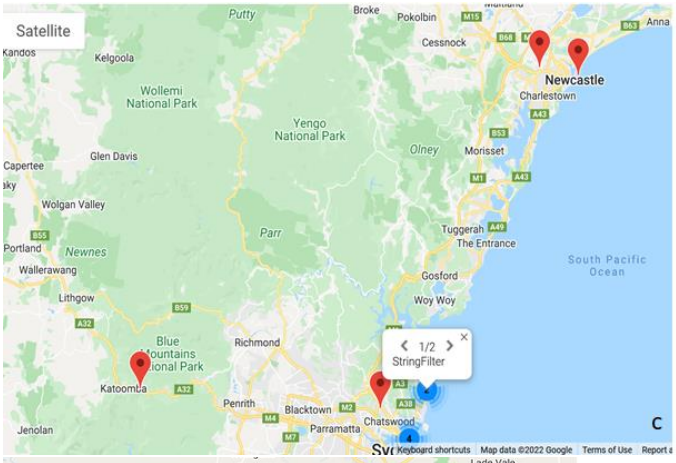
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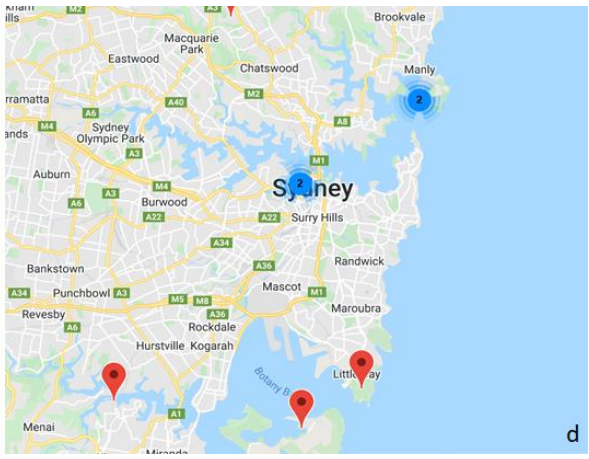
APPENDICES

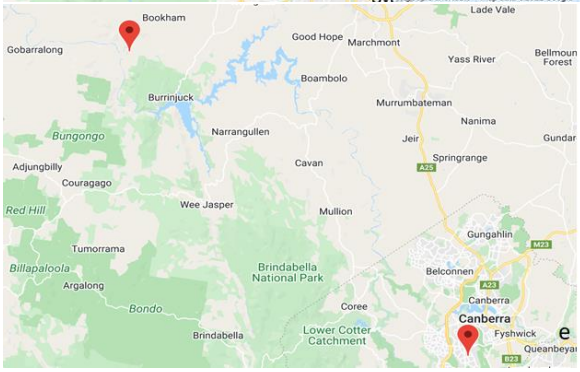
Detailed Collection Locations for Reference Collection Specimens

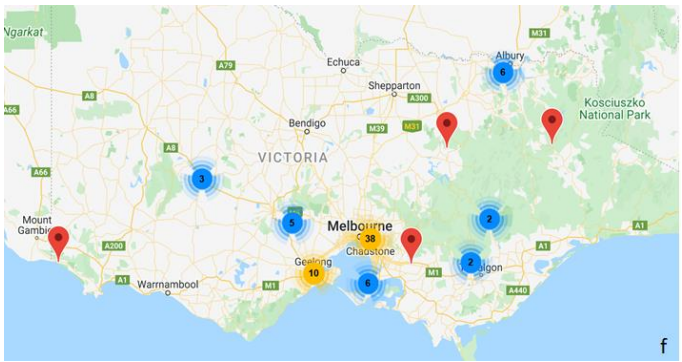












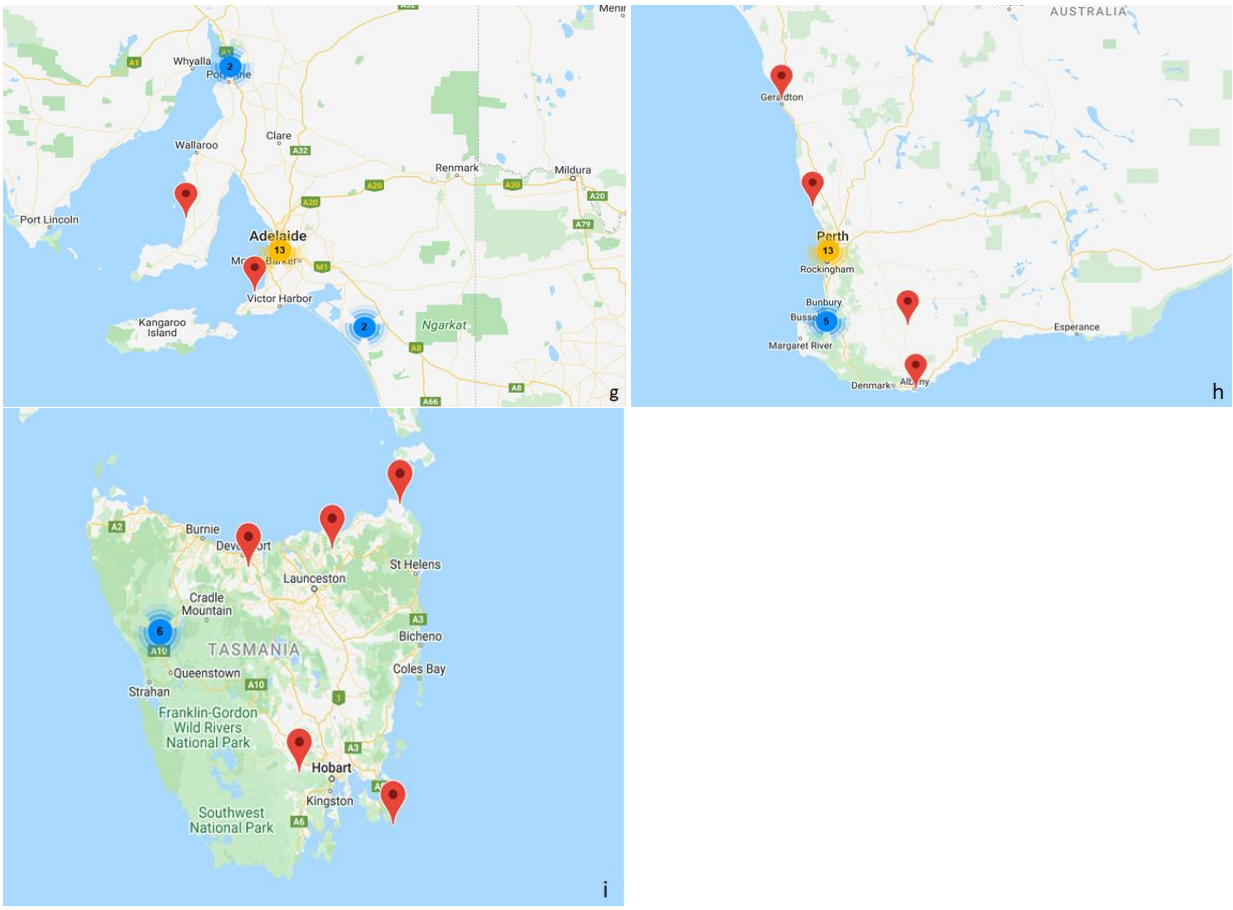


Figure A.1. Detail of *Bathyllus albicinctus* specimen records in each state, from ALA (Atlas of Living Australia). a - QLD; b – northern NSW; c – mid NSW, Newcastle; d – mid NSW, Sydney; e – ACT; f – Vic.; g – SA; h – WA; i – Tas.

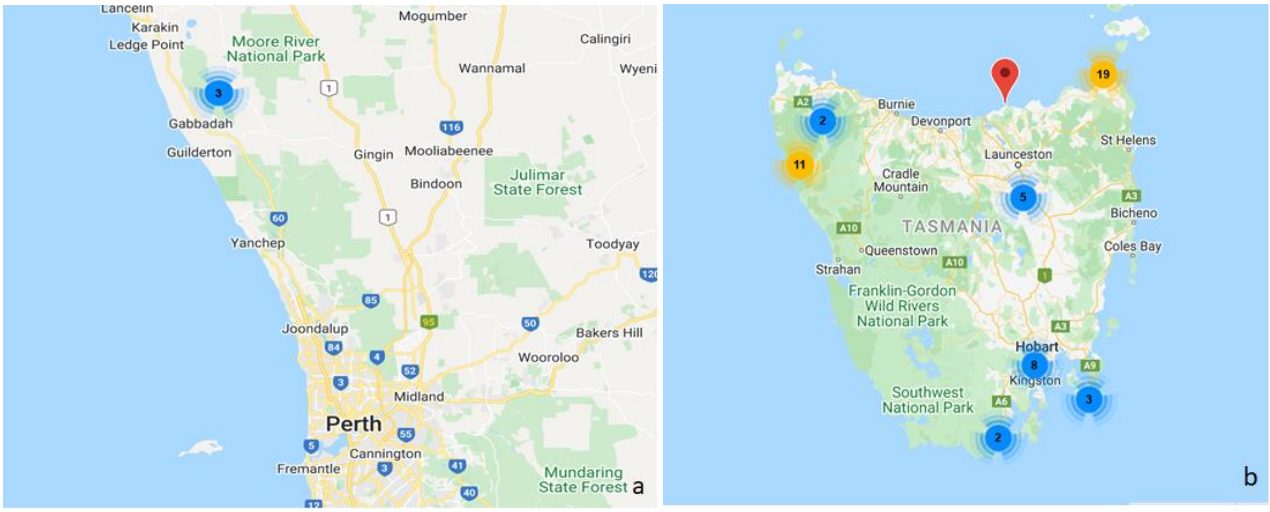


Figure A.2. Detail of *Bathyllus albicinctus* specimen records in each state, from APPD (Australian Plant Pest Database). a – WA; b – Tas.

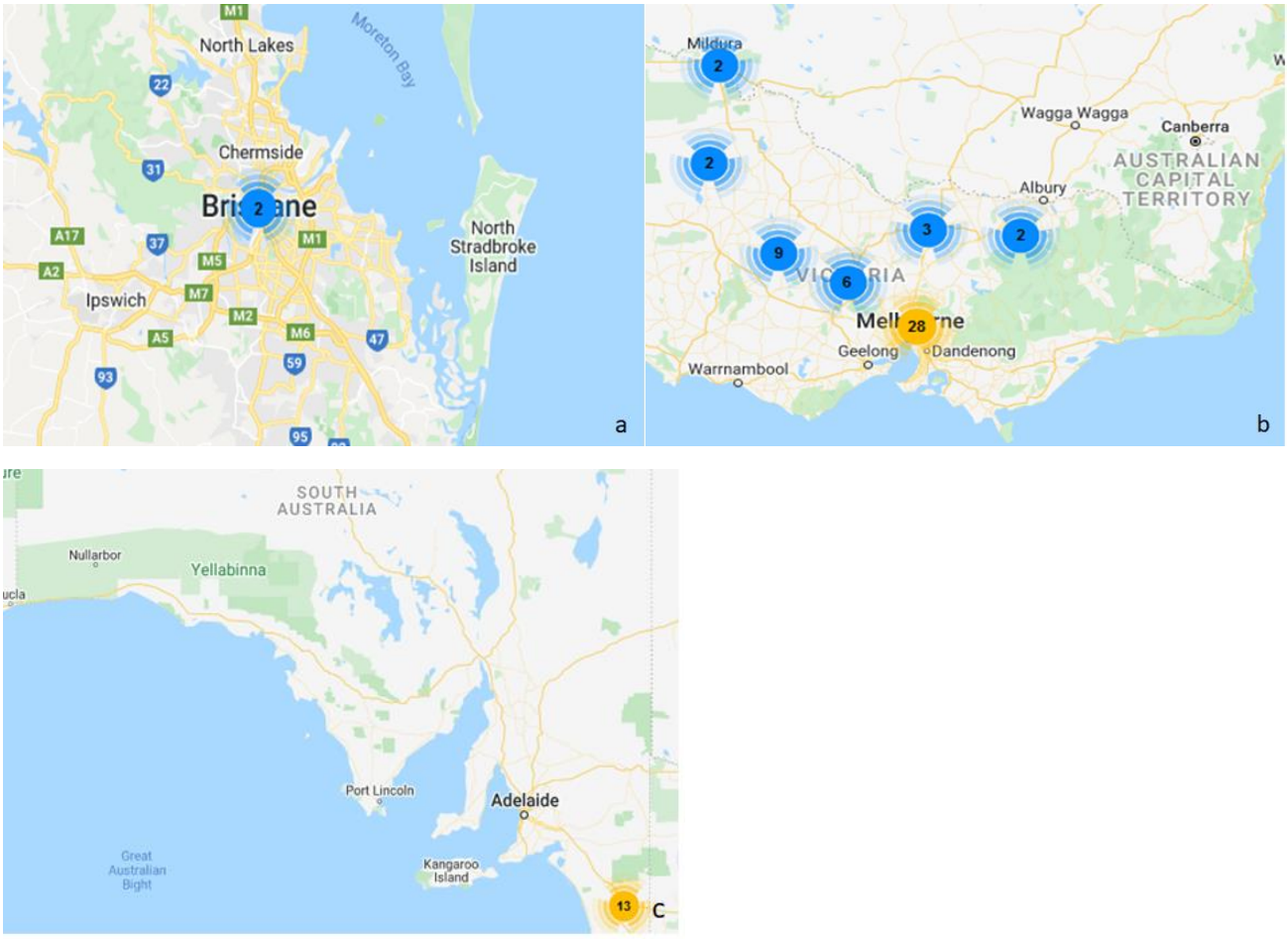


Figure A.3. Detail of *Bathyllus albicinctus* specimen records in each state, lodged in the VAIC (Victorian Agricultural Insect Collection). a – QLD; b – Vic.; c – SA.