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An assessment of the status of psylloid species (Hemiptera: Psylloidea) as potential pests of commercial citrus in southern Africa: Implications for Pest Management

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

In

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CENTRE FOR BIOLOGICAL CONTROL

DEPARTMENT OF ZOOLOGY AND ENTOMOLOGY

By

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DECLARATION

By electronically submitting this thesis, I, **Raynold Moagi** (**22M6852**), declare that the entire content presented herein is entirely my own, originating from my intellectual efforts. To the best of my knowledge and belief, I attest that this work has not been previously submitted in any form for the conferral of any academic qualification, such as a diploma or degree, either at this university or any other higher educational institution, nor has it been submitted to any organisation beyond academic realms. Therefore, I affirm that this thesis represents my original contribution and initial submission for the degree of Master of Science in Entomology at Rhodes University. Whenever external information (i.e. data, graphs, images, texts, tables, etc.) has been incorporated within this work, due acknowledgement has been provided. The sources have been cited throughout the thesis and further detailed in the references section.

Furthermore, the research documented in this thesis was conducted under the generous supervision of Prof M. Hill, Centre for Biological Control (CBC), Department of Zoology and Entomology, Rhodes University (RU), South Africa; Dr E. Mauda, Citrus Research International (CRI), South Africa; and Mr L. Mukwevho, School of Biology and Natural Sciences, University of Mpumalanga (UMP), South Africa.

Signature (Student):  Date: **30 June 2024**

DEDICATION

This Masters degree is dedicated with heartfelt gratitude and love. Foremost, to my only cherished mother, Thandi Leria Mamiane, whose unwavering support and sacrifices have been my guiding light throughout this academic journey. Furthermore, I extend my heartfelt appreciation to my first two elder brothers, Sean and Solly Moagi, and my two elder sisters, Cecilia and Tinyiko Moagi, for their continuous encouragement and belief in my potential. I extend this dedication to Thatego C. Moagi (a special, beautiful gift from God), my niece Nhlamulo V. Moagi, representing all my nieces, and my nephew Ndzalama A. Moagi, representing all my nephews; your presence in my life has been a source of joy and inspiration. I also extend my deepest appreciation to all those who have believed in my academic path and walked alongside me thus far. This includes my brother and sister-in-law, some of my relatives and friends, as well as church members (FBC-Faith Bible Church), whose constant love, advice, prayers and patience have been a source of strength. Their unwavering support in every aspect has been instrumental in achieving this great milestone. Lastly, this research work is also dedicated to the hardworking commercial citrus farmers in South Africa and beyond, whose dedication and efforts contribute significantly to our agricultural and economic landscape. Your resilience and commitment inspire me to strive for excellence in my field.

ABSTRACT

Psylloids (Hemiptera: Psylloidea), constitute a group of plant sap-sucking insects, some of which are economically significant pests in different ecosystems due to their potential to transmit Gram-negative bacteria, such as the *Candidatus* Liberibacter species. The African citrus triozid (ACT), *Trioza erytreae* (Del Guercio), which transmits African citrus greening and the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, which transmits Asian citrus greening are significant threats to citrus. Asian citrus psyllid poses a global economic threat due to its ability to vector "*Candidatus* Liberibacter asiaticus" (CLas), which can rapidly kill citrus trees. However, both ACP and CLas are currently not present in southern Africa but are present in East and West Africa.

In the Afrotropical region, 71 triozid species are known to occur and approximately 41 described *Diaphorina* species in southern Africa. Currently, two indigenous *Diaphorina* species, *Diaphorina punctulata* and *Diaphorina zebrana* have been documented to feed on citrus. There is a significant knowledge gap regarding the ecological roles of other indigenous psylloid species occurring within the citrus environments. Therefore, this study aimed to: (i) determine the diversity and community structure of psylloid species in citrus environments, and (ii) their host ranges through DNA analysis of gut contents to determine if they fed on citrus.

Field surveys were carried out across 12 distinct commercial citrus environments across Limpopo and Mpumalanga provinces between 2022 and 2023. Psylloids were collected using yellow sticky traps and an insect sweep-net. Collected psylloid specimens were preserved in 70% ethanol vials and identified to the lowest possible taxonomic level (i.e. genus or species) using both published and unpublished dichotomous identification keys. Furthermore, citrus leaf samples were collected from the same plants on which psylloids were found in the orchards. Genomic DNA (gDNA) was extracted from both leaf and psylloid samples using two different DNA extraction methods. To confirm if citrus DNA could be detected in the psylloid guts, all leaf gDNA samples were initially amplified using the rbcLaF/R primer pair, targeting a 530-bp region of the chloroplast *rbcL* gene through the polymerase chain reaction (PCR). Lastly, gut content analysis was performed on 11 psylloid species using the same primer pair through PCR to detect citrus DNA.

A total of 4,900 psylloids belonging to five families (i.e. Aphalaridae, Carsidaridae, Liviidae, Psyllidae and Triozidae), 19 genera and 47 species, were collected in citrus environments. More

psylloids were recorded in Limpopo (3,754) than in Mpumalanga (1,146). The most abundant species were *Pauropsylla trichaeta* (1,680), followed by *Diaphorina punctulata* (466), *Trioza erytreae* (426), *Diaphorina virgata* (371), *Euryconus* sp. (358), *Cacopsylla* sp. (311), *Retroacizzia mopanei* (263), *Acizzia russellae*-group (240), *Acizzia* sp.3 (216) and *Acizzia* sp.2 (140). Yellow sticky traps captured 3,265 psylloids in citrus orchards, while an insect sweep-net collected 1,635 psylloids (477 from citrus orchards and 1,158 from adjacent natural vegetation). Data from the insect sweep-net revealed that 22 psylloid species were recorded on citrus. In comparison, nine psylloid species were found on *Vachellia* spp. and unidentified plant species separately, whereas six, three and two psylloid species were recorded on marula, *Ficus* sp. and mopane, respectively. The abundance, richness and community structure of psylloids differed significantly between the collection methods, provinces and among plant species.

The rbcLaF/R primer pair amplified all citrus leaf gDNA samples, producing amplicons of the targeted 530-bp size. The PCR analysis of 11 psylloid species showed that the rbcLaF/R primer pair amplified plant DNA, with PCR-amplified plant DNA samples producing amplicons between 500-bp and 750-bp in the gut contents of five psyllid species: *Diaphorina punctulata*, *Diaphorina virgata*, *Diaphorina zebrana*, *Euryconus* sp. and *Trioza erytreae*. However, the targeted 530-bp plant DNA region was only amplified from the gut contents of *Euryconus* sp. and *Diaphorina punctulata*. This study documented psylloid diversity and community structure within commercial citrus environments. The findings indicate that the community of psylloids was diverse in citrus environments, with yellow sticky traps being more effective in monitoring different psyllid species within these environments. Furthermore, the PCR analysis detected citrus DNA in the gut contents of *Euryconus* sp. and *Diaphorina punctulata*, suggesting that they could be nibbling on citrus when their specific or main host-plants adjacent to citrus orchards are depleted. However, these insects do not lay their eggs or complete their life cycle on citrus, further confirming that citrus is not their host-plant. Thus, further studies, including Sanger sequencing of PCR-amplified plant DNA, are recommended to confirm the ingested plant species, and host-specific testing including infection trials needs to be conducted.

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LIST OF ABBREVIATIONS, UNITS AND SYMBOLS

Abbreviations:

A.D	Anno Domini
ABS	Alternaria brown spot
ACG	African citrus greening
ACP	Asian citrus psyllid
ACT	African citrus triozid
AGE	Agarose gel electrophoresis
AP	Apple proliferation
B.C	Before Christ
bp	Base pairs
CaLsol	*Candidatus* Liberibacter solanacearum
CBC	Centre for Biological Control
CBS	Citrus black spot
CG	Citrus greening
CGA	Citrus Growers Association
CL	Citrus leprosis
CLaf	*Candidatus* Liberibacter africanus
CLam	*Candidatus* Liberibacter americanus
CLas	*Candidatus* Liberibacter asiaticas
CRI	Citrus Research International
CTAB	Cetyltrimethylammonium bromide
CTV	Citrus tristeza virus
DAFF	Department of Agriculture, Forestry and Fisheries
ddH_2O	Double distilled water
DF	Degrees of freedom
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EC	Eastern Cape
EDTA	Ethylenediaminetetraacetic acid

ESFY	European stone fruit yellows
gDNA	Genomic deoxyribonucleic acid
GLM's	Generalised linear models
GPS	Geographic positioning system
HLB	Huanglongbing
H_2O	Water
IPM	Integrated pest management
ITS	Internal transcribed spacer
LD	Loading dye
LP	Limpopo
LRT	Likelihood-ratio tests
MP	Mpumalanga
mtCOI	Mitochondrial cytochrome c oxidase subunit I
MvGLM	Multivariate generalised linear models
NaCl	Sodium Chloride
nMDS	Non-metric multidimensional scaling
No.	Number
NSB	Non-specific binding
NTC	No template control
P	P-value or calculated probability
PC	Positive control
PCR	Polymerase chain reaction
PD	Pear decline
rbcL	Ribulose bisphosphate carboxylase gene large subunit
rcf	Relative centrifugal force
RT	Room temperature
RU	Rhodes University
SACs	Species accumulation curves
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SPK	Spike

UMP	University of Mpumalanga
USA	United States of America
USD	United States dollar
vs	Versus
ZAR	South African Rand
ZC	Zebra Chip

Units:

×g	Time gravity
µg/ML	Micrograms per millilitre
µL	Microliter
cm	Centimeters
DF	Degrees of freedom
g	Gram
ha	Hectares
kg	kilogram
M	Molar
m	Meters
Mb	Megabase pairs
mg	Milligrams
mg/mL	Milligrams per millilitre
mL	Milliliter
µM	Micromolar
mM	Millimolar
mm	Millimeters
MT	Metric tons
nm	Nanometers
ng/µL	Nanograms per microliter
pH	Potential of hydrogen
rcf	Relative centrifugal force
V	Volts

v/v	Volume per volume
w/v	Weight per volume
x^2	Chi-squared

Symbols:

%	Percentage
°C	Degrees Celsius

RESEARCH OUTPUTS

Publication co-authored during the study duration:

1. Burckhardt D., **Moagi R**. & Mauda E. (2023). *Anoeconeossa exsul* Burckhardt, a new eucalypt psyllid from Australia and South Africa (Hemiptera: Psylloidea). *Entomologische Zeitschrift · Schwanfeld*, 133(1), 11-15.

Poster Presentations and Conference Proceedings:

1. **Moagi R**., Mauda E., Mukwevho L. & Hill M. (2022). An assessment of the status of psylloid species (Hemiptera: Psylloidea), as possible vectors of *Candidatus* Liberibacter species, causative of citrus greening in South Africa. *CRI 11th Citrus Research Symposium*, Drakensberg, KZN (**21-24 August 2022**). *Poster presentation*

2. **Moagi R**., Mauda E., Mukwevho L. & Hill M. (2022). An assessment of the status of psylloid species (Hemiptera: Psylloidea), as possible vectors of *Candidatus* Liberibacter species, causative of citrus greening in South Africa. *Rhodes University's Centre for Biological Control Research Day*, Grahamstown, Eastern Cape (**25 November 2022**). *Oral presentation*

3. **Moagi R**., Mauda E., Mukwevho L. & Hill M. (2023). Psylloid community structure and diversity in and adjacent to citrus orchards. *23rd Congress of the Entomological Society of Southern Africa* (*ESSA*), Stellenbosch University, Western Cape (**11-14 July 2023**). *Oral presentation*

4. **Moagi R**., Mauda E., Mukwevho L. & Hill M. (2023). Psylloid community structure and diversity in and adjacent to citrus orchards. *Annual Postgraduate Conference*, Rhodes University, Grahamstown, Eastern Cape (**8-9 September 2023**). *Oral presentation*

5. **Moagi R**., Mauda E., Mukwevho L. & Hill M. (2023). An assessment of the status of psylloid species (Hemiptera: Psylloidea), as possible vectors of *Candidatus* Liberibacter species, causative of citrus greening in South Africa. *Rhodes University's Centre for Biological Control Research Day*, Grahamstown, Eastern Cape (**24 November 2023**). *Oral presentation*

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Background to the study

Citrus spp. (Rutaceae: Sapindales) are the most economically important perennial agricultural fruit crop, extensively cultivated and consumed in more than 140 countries across tropical and sub-tropical regions of the world (Liu *et al.*, 2012; Zhong & Nicolosi, 2020; Lombardo *et al.*, 2023). Citrus cultivation extends beyond mere economic importance; it plays a significant role in ensuring food security, nutritional well-being and creating substantial employment opportunities (Gmitter & Hu, 1990; Liu *et al.*, 2012; Wu *et al.*, 2018; Khan *et al.*, 2021). The nutritional benefits of citrus fruits have been extensively documented, including but not limited to the rich provision of essential compounds such as carotenoids, polyphenols and vitamin C, all contributing to human health (Liu *et al.*, 2012; Genovese *et al.*, 2014; Nagano *et al.*, 2018; Sun *et al.*, 2019; Duru *et al.*, 2022). Currently, numerous citrus species of commercial importance are cultivated across various regions globally, and the most well-known ones include sweet oranges, lemons, limes, grapefruits and mandarins (Zhong & Nicolosi, 2020; Duru *et al.*, 2022; Aidoo *et al.*, 2023; Volk *et al.*, 2023). Within this diverse group of citrus species, sweet oranges are the most popular ones, contributing to over half of the world's total citrus production (Khamis *et al.*, 2017; Richard *et al.*, 2018; Jaouad *et al.*, 2020; Aidoo *et al.*, 2023; Lombardo *et al.*, 2023).

The highest citrus-producing countries across the globe include China with an annual production exceeding 32.7 million metric tons (MT), followed closely by Brazil with 16.6 million MT and India contributing 9.7 million MT. The United States of America (USA) and Spain also contribute significantly by producing 7.8 million MT and 6.8 million MT, respectively (Mendonça *et al.*, 2017; González-González *et al.*, 2020; Lombardo *et al.*, 2023). Within the African continent, citrus has been produced among several countries, with Egypt and South Africa currently ranked as the highest citrus producers, and the majority of citrus fruits produced within these two countries are mainly export-orientated to international markets (Khamis *et al.*, 2017). In South Africa, citrus production has recently experienced exponential growth, elevating the country to the 14th position among the world's top citrus producers and further making it the second-largest exporter of fresh

commercial citrus fruits globally (Inglese & Sortino, 2019; CGA, 2020), with an annual average of 1.6 million MT of citrus fruits recently produced in the country (Idamokoro *et al.*, 2022).

The citrus production in South Africa, as in many other citrus-growing regions around the world, has, however constantly been constrained by numerous factors, irrespective of its high agricultural and economic importance (Jaouad *et al.*, 2020). Constraints attributed to a gradual decline in citrus production, include biological (i.e. pests and diseases), socio-economic and environmental factors (Khamis *et al.*, 2017; Aidoo *et al.*, 2020). Of particular concern, include the arthropod pests and diseases, which have become significant contributors to the decline of citrus production (Kilalo *et al.*, 2009; Khamis *et al.*, 2017). These constraints have a significant impact on citrus trees, affecting various plant components, including but not limited to roots, leaves and fruits (Haile *et al.*, 2022). As such, they not only compromise the quality and quantity of citrus production but further pose a significant threat to the overall sustainability of the citrus industry globally. Among the arthropod pests recorded, psylloids are currently recognised as the most destructive insect pests in the citrus industry (Kilalo *et al.*, 2009; Khamis *et al.*, 2017; Rwomushana *et al.*, 2017; Aidoo *et al.*, 2021).

Psylloids (Hemiptera: Psylloidea), commonly referred to as jumping-plant lice, are phytophagous, phloem-feeding insects that are generally host-specific (Burckhardt *et al.*, 2021; Zhao *et al.*, 2023). This group includes species of significant economic concern, and some are vectors of devastating disease-causing pathogens (Burckhardt, 1994; Hodkinson, 2009; Burckhardt *et al.*, 2020). Among these phloem-feeding hemipteran pests, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) and *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), are undoubtedly the most significant threats or constraints to commercial citrus production, and are thought to be responsible for substantial global economic losses (Aubert, 1987; Cocuzza *et al.*, 2017; Rwomushana *et al.*, 2017). Although the direct feeding damage by these psyllid pests on citrus trees is considerable (Hodkinson, 1984, 2009; Burckhardt, 1994), the economically important damage is caused by the vectored phloem-limited bacteria species: "*Candidatus* Liberibacter africanus" (CLaf), "*Candidatus* Liberibacter asiaticas" (CLas) and "*Candidatus* Liberibacter americanus" (CLam) (da Graça, 1991; Halbert & Manjunath, 2004; Bové, 2006; Gottwald, 2010; Ruíz-Rivero *et al.*, 2021). These phloem-limited bacterial species are causative agents of citrus greening (CG), by far the most current devastating and widespread disease affecting citrus species for which there is still no cure available for infected trees globally (Bové, 2006; Gottwald, 2010; Zhang *et al.*, 2010; Wang *et al.*, 2017a).

Although *T. erytreae* and *D. citri* are considered the most economically important psyllid vectors of disease-causing pathogens (Alves *et al.*, 2014; Moreno *et al.*, 2021), the distinction between these pests lies within their primary roles. *Trioza erytreae* serves as the primary vector responsible for transmitting CLaf, which is associated mainly with the African citrus greening disease (ACG) in southern Africa (McClean & Oberholzer, 1965a, 1965b). However, in other parts of the world, *D. citri* is a primary vector, responsible for transmitting both CLas and CLam (Capoor *et al.*, 1967; Martinez & Wallace, 1967; Shimwela *et al.*, 2016). These pathogenic bacteria have consistently been associated with citrus greening disease, also known as Huanglongbing (HLB) (Jagoueix *et al.*, 1996; Teixeira *et al.*, 2005; Hall *et al.*, 2012). Among these pathogens, CLas has however been reported as the most widespread and destructive pathogen, responsible for rapid tree decline and ultimately death (Gottwald *et al.*, 2007; Pietersen *et al.*, 2010; Bové, 2014), thus, making *D. citri* the most critical pest of global economic concern (Bové, 2006; Gottwald, 2010).

Recently, there have been documented occurrences of *D. citri* and its associated CLas in different regions of the African continent and also some Indian Ocean islands (Catling, 1970a; Shimwela *et al.*, 2016; Rwomushana *et al.*, 2017; Ajene *et al.*, 2020a; Oke *et al.*, 2020; Aidoo *et al.*, 2023; Sétamou *et al.*, 2023). However, both *D. citri* and CLas are currently not present in southern Africa, as confirmed by previous research (Teixeira *et al.*, 2005; Pretorius & van Vuuren, 2006; Pietersen *et al.*, 2010; Bové, 2014), and the imminent arrival of these potentially harmful agents in the region is a cause for concern. Within southern Africa, the only known psyllid pest is *T. erytreae*, which poses a significant risk as a potential vector of CLaf, the bacterium responsible for ACG disease, affecting all commercial citrus orchards in the region (McClean & Oberholzer, 1965a, 1965b).

There is currently limited knowledge regarding the role of indigenous psylloid species occurring in South African commercial citrus environments (i.e. citrus orchards and their surrounding natural vegetation). Understanding the diet-breadths of these psylloids in citrus environments is important for determining their potential to feed on citrus plants and their capacity to act as alternative insect vectors of the phloem-limited plant-pathogens associated with citrus greening disease. This current study, therefore, aimed to investigate the diet-breadth of indigenous psylloid species occurring in commercial citrus environments and further provide confirmation regarding their potential role in vectoring any *Candidatus* Liberibacter species, the bacteria associated with citrus greening disease in South Africa.

1.2. The citrus plant

1.2.1. Taxonomic classification and morphological characteristics of citrus.

Citrus Linnaeus, is a distinctive genus belonging to the angiosperm sub-family Aurantioideae of the Rutaceae family (Nicolosi, 2007; Penjor *et al.*, 2013; Lv *et al.*, 2015; Nagano *et al.*, 2018; Wu *et al.*, 2018; Aslam *et al.*, 2021; Haile *et al.*, 2022). The Rutaceae family comprises seven sub-families, with Aurantioideae being one of them, which is divided into two tribes, namely the *Clauseneae* and *Citreae* (Morton, 2009; Penjor *et al.*, 2013; Lv *et al.*, 2015). Among these, the tribe *Citreae* is divided into three sub-tribes (Penjor *et al.*, 2013; Nagano *et al.*, 2018), including the sub-tribe *Citrinae*, the most important group within the Rutaceae family (Swingle & Reece, 1967). The genus *Citrus* and its closely related genera belong to the sub-tribal group ‘‘true citrus trees’’ in the sub-tribe *Citrinae* (Nicolosi, 2007; Penjor *et al.*, 2013). Globally, there are currently eight commercially cultivated species of citrus, among which five are considered major economic importance (Xu *et al.*, 2013; Hynniewta *et al.*, 2014; Jaouad *et al.*, 2020). These species include grapefruits (*Citrus paradisi*), lemons (*Citrus limon*), limes (*Citrus aurantifolia*), sweet oranges (*Citrus sinensis*) and mandarins (*Citrus reticulata*) (Penjor *et al.*, 2013; Xu *et al.*, 2013; Hynniewta *et al.*, 2014; Richard *et al.*, 2018; Jaouad *et al.*, 2020). Furthermore, in each citrus species, different cultivars have been developed showing variation in their distinctive morphological characteristics, including canopy shape and size, fruit colour, size, quality, maturity season and seed count per fruit (Novelli *et al.*, 2006).

All cultivated species of citrus are characterised by their evergreen nature, growing either as trees or shrubs, generally achieving a height ranging from 5 to 15 meters (m) upon reaching full maturity (Hussain *et al.*, 2021; Haile *et al.*, 2022). These citrus plants or trees display distinctive evergreen leaves that are alternately arranged and simple in structure, generally with winged stalks (Miguel *et al.*, 2008; Hussain *et al.*, 2021; Haile *et al.*, 2022). Furthermore, these leaves have a shallowly cup-shaped structure and generally measure between 4 to 6 centimeters (cm) in length, with a width ranging from 2 to 3 cm (Miguel *et al.*, 2008; Hussain *et al.*, 2021). In addition to their foliage, citrus plants produce highly fragrant white flowers or blossoms, comprised of 5 petals and numerous stamens, creating a visually appealing spectacle, especially during the flowering seasons (Miguel *et al.*, 2008; Morton, 2009; Liu *et al.*, 2012; Hussain *et al.*, 2021). The produced citrus

fruits, known as hesperidiums, are modified berries, and their sizes and shapes vary according to specific species and variety (Miguel *et al.*, 2008) (Figure 1. 1).

Figure 1. 1: Morphology of citrus flowers and ripe fruits (**Photo credit**: Peter Stephen).

1.2.2. Origin, history and distribution of citrus.

The native origin of citrus remains uncertain; however, it has been generally accepted that citrus originates from the tropical and sub-tropical regions of south-eastern Asia, primarily China and Indonesia (Scora, 1975; Ramana *et al.*, 1981; Liu *et al.*, 2012; Haile *et al.*, 2022). From these origins, citrus trees were domesticated and subsequently introduced to other regions of the world (i.e. Europe and the southern parts of Africa around 310 B.C. and 1650 A.D., respectively) primarily through migration, trade and cross-continental exchange (Scora, 1975; Liu *et al.*, 2012; Haile *et al.*, 2022). During the beginning of the 20th century, the production of citrus experienced exponential growth, driven by an increase in market demands and advancements in marketing strategies aimed at enhancing the quality of citrus fruit products (Ramana *et al.*, 1981). Currently, citrus production is practised across more than 140 countries, including South Africa, primarily situated in the tropical and sub-tropical regions of the world (Liu *et al.*, 2012; Zhong & Nicolosi,

2020; Aslam *et al.*, 2021; Mvondo-She *et al.*, 2021; Haile *et al.*, 2022). This widespread practice or cultivation of citrus is mainly attributed to the fertile soil and suitable climatic conditions found within these global growing regions (Khan *et al.*, 2021; Volk *et al.*, 2023).

1.2.3. The South African citrus industry.

South Africa has a leading position in the global citrus fruit industry as the 14th largest producer of fresh commercial citrus fruits, with a remarkable annual average production exceeding 1.6 million metric tons (MT) (Inglese & Sortino, 2019; Idamokoro *et al.*, 2022). This achievement is further complemented by its position as the second-largest exporter of citrus fruits (by volume) on a global scale after Spain (Inglese & Sortino, 2019). The export figures for 2019, as detailed by CGA (2020), revealed that more than 89 million cartons (where one carton equates to 15kg) of citrus fruits were exported from South Africa to international destinations. The majority of South African commercial citrus fruits produced are, however, destined mainly for international export markets with this attributed primarily to the high fruit quality produced and the high revenue generated from international or foreign markets (Sinngu & Antwi, 2014; Joseph *et al.*, 2020, 2021; Manenzhe, 2021). Among these noteworthy exports, citrus cultivars including the Valencia and Navel oranges are the most frequently produced fruits for international markets, followed closely by mandarins, lemons and grapefruits (Sinngu & Antwi, 2014). Although the majority, accounting for 76 percent (%) of the South African citrus fruits are exported, the remaining portion, which accounts for 24%, serves domestic purposes, with 18% being allocated for processing and 6% for local consumption or supplied directly to the local markets (Sinngu & Antwi, 2014; CGA, 2018, 2020; Inglese & Sortino, 2019). The reach of South African citrus extends across diverse continents, with Europe emerging as the leading recipient of fresh citrus fruit exports, followed by the Middle East, accompanied by several other regions that import smaller citrus volumes (Edmonds, 2021).

Citrus has become one of the most important driving forces in the agricultural industry, making substantial contributions to the economy in terms of both gross value and remarkable production volume in South Africa (Sinngu & Antwi, 2014). Currently, it holds the esteemed position of being the third-largest horticultural industry in the country, following closely behind the deciduous fruits and vegetables industries (Sinngu & Antwi, 2014; Inglese & Sortino, 2019). During the production

season of 2016/17, the citrus fruit industry made a substantial contribution of R19.1 billion (USD 1 = ZAR 18.53) to the overall gross value of agricultural production within South Africa. This impressive figure accounted for 25 % of the total gross value, which amounted to R57.3 billion, in the horticulture industry during the corresponding period, as outlined by DAFF (2019), cited by Joseph *et al.* (2021) and Manenzhe (2021). In addition to its significant contribution to the county's economy, the citrus industry remains a major source of employment, particularly during peak periods such as harvest time when labour demand is at its highest (Sinngu & Antwi, 2014). This industry is characterised by its labour-intensive nature, and it currently provides job opportunities to approximately 100,000 permanent farm workers, with the majority of these workers engaged in activities such as picking and packing in the citrus orchards (Sinngu & Antwi, 2014; Vahrmeijer *et al.*, 2015; Joseph *et al.*, 2021). However, Mather (1999) previously reported that determining the exact number of seasonal farm workers remains challenging with this attributed primarily to the complex challenges in organising and tracking such workers in South Africa. In addition to the on-farm workforce, an unspecified number of individuals are employed across the entire supply chain services. This includes roles in transportation, port handling and related logistical support (Sinngu & Antwi, 2014; Genis, 2018). Therefore, it is estimated that the well-being or livelihoods of over a million households is/are dependent on the citrus fruit industry (Genis, 2018).

The citrus fruit production in South Africa comprises five broad categories, namely oranges (i.e. Valencia, Navels, Deltas and Midknight), soft citrus (i.e. Clementine, Satsuma, Mandarins and Naartjies), grapefruit (i.e. Star Ruby and Pummelos/Pomelos), as well as lemons and limes (i.e. Eureka and Genoa) (Inglese & Sortino, 2019; Manenzhe, 2021). More than 81, 638 hectares (ha) of land in South Africa is currently dedicated primarily to citrus production and occurs throughout all the provinces, except Gauteng province (CGA, 2019) (Figure 1. 2). It is evident that the highest citrus fruit production occurs in the Limpopo province, as it has the largest area of land dedicated to citrus production, followed by Eastern Cape and Western Cape provinces (CGA, 2019) (Figure 1. 2). The other remaining provinces, according to their respective hectare sizes, are Mpumalanga, KwaZulu-Natal, Northern Cape, North West and Free State (CGA, 2019) (Figure 1. 2).

The diverse climatic conditions across these different regions (as listed above) have significantly contributed towards the success of the production of citrus fruits in South Africa. The regions such as Mpumalanga, Limpopo and KwaZulu-Natal, are characterised by their warmer climates and

have become the key contributors to the industry, with production primarily focused on grapefruits and Valencia oranges (Sinngu & Antwi, 2014). However, the cooler climates prevalent in the Western Cape and Eastern Cape provinces are better suited mainly for the mandarins, lemons and Navel oranges production (Sinngu & Antwi, 2014). Although these distinct climatic regions have been advantageous for citrus fruit cultivation, it is essential to acknowledge that each distinct citrus-producing region across the country harbours its group of significant pests due to the suitability of these climates and also providing a conducive environment for the establishment of numerous insects (Urquhart, 1999; Smith & Peña, 2002). Consequently, the citrus-production regions across the country face significant challenges resulting from the direct damages inflicted by insect pests and the transmission of diseases, a concern that remains conspicuously high.

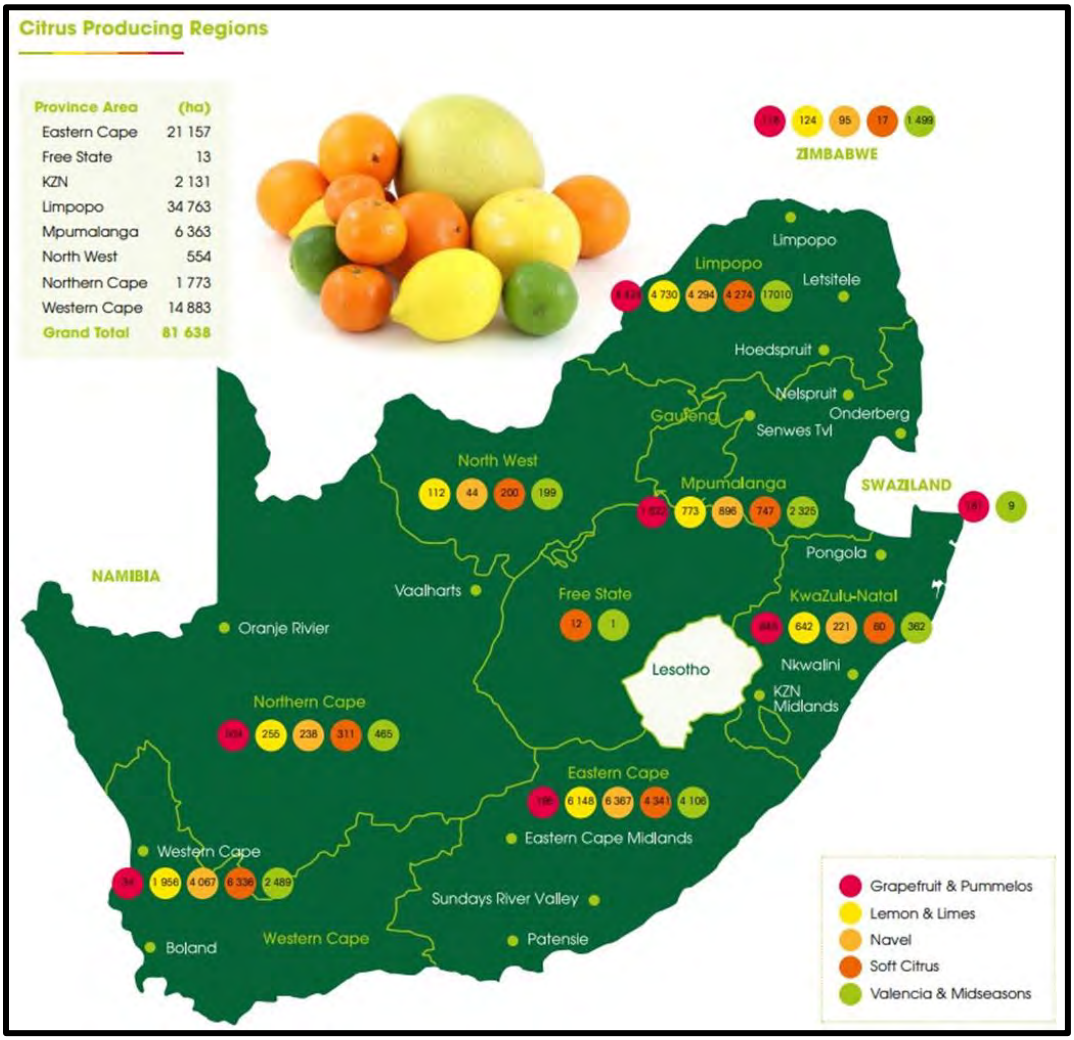

Figure 1. 2: Commercial citrus-producing regions in South Africa, along with the corresponding cultivars within each region (CGA, 2019).

1.2.4. Constraints of the South African citrus production.

Citrus is known to be constrained by numerous factors that are attributed to the significant decline in both the quantity and quality of commercial citrus fruits globally (Smith & Peña, 2002). Among the major constraints extensively documented within citrus production, include inadequate or poor orchard management practices, environmental challenges, insufficient planting materials and the susceptibility of citrus species and cultivars to various pests and diseases (Oerke, 2006; Kilalo *et al.*, 2009; Khamis *et al.*, 2017). Of these constraints, pests and diseases have been considered the most significant constraints of citrus production in many regions of the world (Kilalo *et al.*, 2009). The detrimental impact inflicted by these biological threats leads to substantial reductions in fruit yield, elevated production costs and diminished income due to the degradation of market-worthy quality (Oerke, 2006). A group of highly detrimental diseases has been identified as key factors that are responsible for the substantial decline in citrus production. These include, among others, the Alternaria brown spot (ABS), Citrus black spot (CBS), Citrus greening (CG), Citrus leprosis (CL), Phytophthora and Citrus tristeza virus (CTV) (Smith & Peña, 2002; Jaouad *et al.*, 2020).

In the South African context, more than 100 different pest species of economic importance have been documented on citrus (Jaouad *et al.*, 2020). The documented pests are insects, nematodes and mites (Talhouk, 1975; Smith *et al.*, 1997), with insects comprising the largest and most significant group. These insect pests, which belong to various orders and families, are of major concern in the agricultural industry since others have the ability to vector and transmit numerous plant-pathogens (Smith & Peña, 2002). The key problematic insect pests of citrus have been documented, among them, include the African citrus triozid, *Trioza erytreae* (Del Guercio); citrus thrips, *Scirtothrips aurantia* Faure; citrus mealybug, *Planococcus citri* (Risso); false codling moth, *Thaumatotibia leucotreta* (Meyrick); Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann); Natal fruit fly, *Ceratitis rosa* Karsch; and red scale, *Aonidiella aurantii* (Maskell) (Smith & Peña, 2002; Jaouad *et al.*, 2020). Other additional major insect pests, though less economically important, have also been documented, including marula fruit fly, *Ceratitis cosyra* (Walker), among them (Smith & Peña, 2002). Of these significant pests, the psyllid *Trioza erytreae* is currently regarded as one of the most destructive citrus pests of economic concern due to its potential to act as a vector, that is, the ability to transmit plant-pathogenic bacteria that cause CG disease, a major concern for citrus production (Bové, 2006; Gottwald *et al.*, 2007; Khamis *et al.*, 2017; Rwomushana *et al.*, 2017).

1.2.5. The psylloid species (Hemiptera: Psylloidea).

The superfamily Psylloidea, commonly known as "psyllids" or "jumping plant-lice", constitute a diverse group of small insects closely related to aphids (Aphidoidea), adelgids, phylloxerans (both from Phylloxeroidea), whiteflies (Aleyrodoidea) and scale insects (Coccoidea), belonging to the hemipteran sub-order Sternorrhyncha (Hodkinson, 1974, 2009; Ouvrard *et al.*, 2015b; Horton *et al.*, 2021; Civolani *et al.*, 2023). Psylloids are distinctive plant-feeding insects (phytophagous) that are generally highly specific when selecting the host-plants on which to develop, and their feeding occurs primarily through piercing the plant tissue and sucking the phloem sap from the vascular plants (Hodkinson, 2009; Burckhardt *et al.*, 2014; Ouvrard *et al.*, 2015a). Globally, the taxonomic classification of psylloids currently encompasses over 4,000 species that are classified under more than 200 genera (Burckhardt & Ouvrard, 2012). This diverse assemblage is further grouped into eight recognised families: i.e. Aphalaridae, Calophyidae, Carsidaridae, Homotomidae, Liviidae, Phacopteronidae, Psyllidae and Triozidae (Burckhardt & Ouvrard, 2012; Burckhardt *et al.*, 2021). All psylloids are predominantly found in terrestrial environments, with the highest species diversity encountered in the tropical and southern temperate regions throughout the world (Burckhardt *et al.*, 2021; Moreno *et al.*, 2021).

As with other hemipteran insects, psylloids have transcended their biological niches to become key pests, causing significant damage to different agricultural crops, as well as forest and horticultural trees (Burckhardt, 1994; Munyaneza, 2010a; Munyaneza *et al.*, 2010b; Ouvrard *et al.*, 2015a, 2015b). This includes species of Homotomidae, Liviidae, Psyllidae and Triozidae families (Moreno *et al.*, 2021). The widespread infestations of these pests have resulted in substantial economic losses globally (Burckhardt, 1994). The adverse effects of these insect pests on their associated host-plants have been reported as occurring both directly and indirectly (Burckhardt, 1994; Hodkinson, 2009; Munyaneza *et al.*, 2010; Moreno *et al.*, 2021). These pests inflict considerable damage by feeding directly on plant sap and injecting toxic saliva, leading to visible symptoms like leaf notching and curling (Burckhardt, 1994; Aidoo, 2023). Furthermore, their high population on the associated host-plants can lead to copious amounts of honeydew secretion and leaf deformation, directly harming the plants (Aidoo, 2023). The honeydew secretion on plant leaves and shoots facilitates the growth of sooty mould, which inhibits photosynthesis in the leaves, ultimately compromising the overall health and vitality of the plants (Burckhardt, 1994; Cocuzza *et al.*, 2017; Hailu & Wakgari, 2019).

Although the detrimental impact of their direct feeding is significant on their associated host-pants, the most destructive aspect of these pests arises indirectly from their potential role as vectors of different plant-pathogens, such as the economically significant bacteria, phytoplasmas and viruses (Munyaneza, 2010a; Munyaneza *et al.*, 2010b; Burckhardt *et al.*, 2014; Antolinez *et al.*, 2017; Cho *et al.*, 2017; Morrow *et al.*, 2020). Consequently, these pests are currently considered major threats of economic concern, particularly in the agricultural industry (Burckhardt, 1994; Munyaneza *et al.*, 2010b).

1.2.5.1. Psylloid species of economic importance:

Among the multitude of psylloid species considered agricultural pests across the globe, only a few have been known to potentially vector and transmit the plant-pathogenic bacteria (Burckhardt, 1994; Ouvrard *et al.*, 2015a, 2015b). These species are primarily classified under the genera of *Arytainilla*, *Bactericera*, *Cacospylla*, *Diaphorina* and *Trioza* (Tamborindeguy *et al.*, 2017), all classified under the Psyllidae and Triozidae families (Moreno *et al.*, 2021). Significantly, these psylloid species are known to consistently maintain an association with phloem-limited bacteria, including the *Candidatus* Liberibacter species (Rhizobiaceae: Alphaproteobacteria: Rhizobiales) and *Candidatus* Phytoplasma species (Bacilli: Acholeplasmatales), both recognised as significant groups of plant-pathogens (Burckhardt, 1994; Halbert & Manjunath, 2004; Tedeschi *et al.*, 2006; Munyaneza *et al.*, 2010b; Nissinen *et al.*, 2014; Antolinez *et al.*, 2017; Cooper *et al.*, 2019; Jarausch *et al.*, 2019). These plant-pathogens are the causative agents of several emerging diseases that are responsible for significant economic losses, particularly in the global agricultural industry (Burckhardt, 1994; Munyaneza, 2010a; Munyaneza *et al.*, 2010b; Ouvrard *et al.*, 2015a, 2015b).

The economically important and devastating disease-vectoring psyllids in the agricultural industry, include those in the genus of *Cacopsylla* (all from Hemiptera: Psyllidae), all of which have the ability to vector and transmit a range of *Candidatus* Phytoplasma species, causing the emergence of critical diseases [i.e. Apple proliferation disease (AP); Pear decline disease (PD); and European stone fruit yellows (ESFY)] that cause severe damage respectively in the commercial apple, pear and stone fruit orchards (Burckhardt & Hodkinson, 1986; Cho *et al.*, 2017; Bertaccini *et al.*, 2019a; Morrow *et al.*, 2020). For example, the *Cacopsylla melanoneura* and *Cacopsylla picta* in Europe are vectors of "*Candidatus* Phytoplasma mali", causing AP disease in apple orchards (Tedeschi &

Alma, 2004; Bertaccini *et al.*, 2019a). Similarly, in the United Kingdom, *Cacopsylla pyricola*, and in France and Italy, *Cacopsylla pyri* transmit "*Candidatus* Phytoplasma pyri", resulting in PD disease that impacts pear orchards (Davies *et al.*, 1992; Carraro *et al.*, 1998; Moreno *et al.*, 2021). Some psyllid species have recently been reported as vectors of bacteria species in the *Candidatus* Liberibacter genus (Moreno *et al.*, 2021). *Bactericera trigonica* and *Trioza apicalis* (= *Dyspersa apicalis*) (both from Hemiptera: Triozidae) have emerged as the primary vectors for "*Candidatus* Liberibacter solanacearum" (CaLsol), responsible for Zebra chip disease (ZC), which adversely affects carrot plants in the regions of Europe and North Africa (Munyaneza *et al.*, 2014; Antolinez *et al.*, 2017), whereas *B. cockerelli* (Hemiptera: Triozidae) transmits CaLsol, causing significant damage to the cultivated potato and tomato crops within the Americas and New Zealand (Liefting *et al.*, 2009). However, none of the above-mentioned psyllid vectors, together with their associated plant-pathogens, have been reported in commercial citrus production globally.

In the context of the citrus industry, there are currently two well-known phloem-sap feeding psyllid vectors, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) and *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Rwomushana *et al.*, 2017; Aidoo *et al.*, 2021, 2023). These two psyllids are widely considered major pests of considerable economic significance globally (Cocuzza *et al.*, 2017). Both *T. erytreae* and *D. citri* have greatly affected commercial citrus production, the related industries and economies in regions where they have been established (Kilalo *et al.*, 2009; Ekesi, 2015; Lewis-Rosenblum *et al.*, 2015; Cocuzza *et al.*, 2017). Their global status as major pests of economic importance has been attributed not to their direct-feeding damages (Khamis *et al.*, 2017; Rwomushana *et al.*, 2017; Ajene *et al.*, 2020b), but mainly to their potential to act as vectors and transmitters for the three distinct isolated plant-pathogenic bacterial species in the "*Candidatus* Liberibacter" genus: the "*Candidatus* Liberibacter asiaticus" (CLas), "*Candidatus* Liberibacter americanus" (CLam) and "*Candidatus* Liberibacter africanus" (CLaf) (McClean & Oberholzer, 1965a, 1965b; Capoor *et al.*, 1967; Halbert & Manjunath, 2004; Bové, 2006; Hall *et al.*, 2012; Shimwela *et al.*, 2016). These plant-pathogenic bacterial species are the primary causative agents responsible for establishing citrus greening (CG), a disease that significantly affects all commercial citrus species and cultivars across the globe (da Graça, 1991; Jagoueix *et al.*, 1996; Teixeira *et al.*, 2005; Bové, 2006; Ruíz-Rivero *et al.*, 2021).

Trioza erytreae, commonly known as the African citrus triozid (ACT), is locally associated with a phloem-limited bacterium referred to as "*Candidatus* Liberibacter africanus" (CLaf), causing the African citrus greening (ACG) disease (McClean & Oberholzer, 1965a, 1965b; Catling, 1970a; van den Berg, 1990; da Graça, 1991; Bové, 2006). In other parts of the world, the citrus greening disease is also called Huanglongbing (HLB) and is caused by another phloem-limited bacterium known as "*Candidatus* Liberibacter asiaticus" (CLas) (Jagoueix *et al.*, 1996; Teixeira *et al.*, 2005). *Diaphorina citri*, a small insect psyllid called the Asian citrus psyllid (ACP), serves as the primary vector of CLas, and has successfully established its presence in nearly all major citrus-producing regions throughout the world, significantly impacting the viability of commercial citrus cultivation (Capoor *et al.*, 1967; Halbert & Manjunath, 2004; Teixeira *et al.*, 2005; Shimwela *et al.*, 2016). Globally, the widespread distribution of these two psyllid vectors, together with their associated CG-Liberibacter species, is currently driving the rapid spread of citrus greening disease throughout major citrus-producing regions (Duran-Vila & Bové, 2015). This expansion poses a significant and growing threat to local citrus industries within these regions (Cocuzza *et al.*, 2017).

1.2.5.2. The plant-pathogen Candidatus *Liberibacter species and their associated citrus greening disease:*

The genus "*Candidatus* Liberibacter", is a phloem-limited, Gram-negative bacterium categorised under alpha-subdivision of proteobacteria (α-Proteobacteria) in the Rhizobiaceae family (Garnier & Bové, 1983; Jagoueix *et al.*, 1994; da Graça & Korsten, 2004). "*Candidatus* Liberibacter" genus consists of three distinct and well-known plant-pathogenic bacteria species that significantly impact all commercial citrus species across the globe. These species are identified as follows: "*Candidatus* Liberibacter asiaticus" (CLas) across Asia, "*Candidatus* Liberibacter americanus" (CLam) across the Americas and "*Candidatus* Liberibacter africanus" (CLaf) across Africa (Jagoueix *et al.*, 1994; Teixeira *et al.*, 2005; da Graça *et al.*, 2016). Among these bacteria, CLas is the most predominant pathogen in most citrus-producing regions and has certain degrees of tolerance to heat, whereas CLaf and CLam are heat sensitive (Jagoueix *et al.*, 1994; da Graça & Korsten, 2004; Jantasorn *et al.*, 2012; Dala-Paula *et al.*, 2019). Despite these thermal preferences, their natural host range in the terrestrial environments includes primarily two types of plants; those that support the psylloid vectors and those that allow the bacterial pathogens to multiply (Thakuria *et al.*, 2023). Specifically, these host-plants are mainly commercially cultivated citrus trees and some related plant genera, all

belonging to the Rutaceae family (Aubert, 1987; van den Berg, 1990; Halbert & Manjunath, 2004). Among the related plant species include *Calodendrum capense*, *Murraya paniculata* and *Severinia buxifolia* (Manjunath *et al.*, 2008; Halbert *et al.*, 2012; Shimwela *et al.*, 2016).

Although the *Candidatus* Liberibacter species are associated with a wide host range, all belonging to the Rutaceae family, the three bacteria are economically important because of their devastating impact on citrus trees (da Graça, 1991; Gottwald, 2010). The three *Candidatus* Liberibacter species have been documented to primarily inhibit the sieve tubes of the phloem of host-plants upon which their respective insect vectors feed; hence their transmission occurs through the acquisition process when these vectors feed on the phloem sap of the infected plants. This initial encounter is followed by inoculation of susceptible citrus plants with the bacteria during subsequent feedings (Liu & Tsai, 2000; Bové, 2006; Tatineni *et al.*, 2008; Pelz-Stelinski & Killiny, 2016; Morán *et al.*, 2023). As these bacteria spread through the vessels inside leaves and roots, the phloem tissues in these specific areas become significantly blocked, hindering the transport of essential plant nutrients and sugars (Bové, 2006; Tatineni *et al.*, 2008; Bendix & Lewis, 2018; Tipu *et al.*, 2021; Thakuria *et al.*, 2023). As such, this process leads to the establishment of the disease commonly known as citrus greening (CG) (Bové, 2006; Gottwald, 2010; Farnsworth *et al.*, 2014; da Graça *et al.*, 2015). However, pathogens are acquired most effectively when immatures feed on the phloem of the infected plants. Inoue *et al.* (2009) found that the adult *D. citri* failed to transmit *Candidatus* Liberibacter asiaticus to citrus seedlings after being exposed to an infected plant for 24 hours, and Pelz-Stelinski *et al.* (2010) found that adult *D. citri* acquired low rates of *Candidatus* Liberibacter asiaticus from the infected plants compared to immatures which showed a high rate of acquisition.

Citrus greening is currently considered the most aggressive, devastating and widespread bacterial disease that affects most commercially important citrus species, posing severe pathogenic threats to all citrus-production regions of the world (Bové, 2006; Gottwald, 2010; Tipu *et al.*, 2021; Aidoo, 2023). The disease has been associated with substantial economic losses and the collapse of several commercial citrus industries in regions where it has been established (i.e. Asia, the Americas and Africa) (Bové, 2006; Farnsworth *et al.*, 2014; da Graça *et al.*, 2015). Within the African continent, particularly in South Africa, the commercial citrus industry has experienced a significant decline, as approximately 100,000 citrus trees were commercially rendered unproductive and economically unsustainable (Oberholzer *et al.*, 1965). Therefore, the prevalence of this disease led to the virtual

elimination of the majority of CG-affected citrus trees in commercial citrus orchards (Buitendag & von Broembsen, 1993; Pretorius & van Vuuren, 2006). Subsequently, the economic impact of citrus greening disease on the South African commercial citrus industry became evident, resulting in an estimated annual financial loss of R35 million (van den Berg *et al.*, 1987).

Globally, the citrus greening disease affects practically all citrus species and cultivars, irrespective of the rootstock used (Manjunath *et al.*, 2008; Halbert *et al.*, 2012; Ghosh *et al.*, 2023; Thakuria *et al.*, 2023). Symptoms of citrus greening disease are highly variable and differ with the age of the infection, the environment and especially the cultivar (McClean & Oberholzer, 1965b; Oberholzer *et al.*, 1965; Mcclean & Schwarz, 1970; da Graça, 1991). Primary symptoms usually include some sort of asymmetric leaf mottle (Hu *et al.*, 2021; da Graça *et al.*, 2022) (Figure 1. 3). In limes, it is a panel motle, whereas, in sweet orange, it is irregular, usually in more than two colours (Oberholzer *et al.*, 1965; Gottwald *et al.*, 2007). Pummelos can have a fine mosaic and sometimes a panel mottle (Gottwald *et al.*, 2007). Tangerines have more fruit symptoms compared to leaf symptoms because the symptomatic leaves quickly drop or fall off. Lemons can have eye-popping yellow and green stripes (Oberholzer *et al.*, 1965). Infected grapefruit leaves tend to curl (Bové, 2006; Gottwald, 2010). Secondary symptoms (chronic symptoms) look like nutrition deficiency (Oberholzer *et al.*, 1965; Halbert & Manjunath, 2004; Tipu *et al.*, 2021). According to studies by Gottwald (2010) and Tipu *et al.* (2021), the severely CG-infected citrus trees generally become stunted, sparsely foliated, experience leaf drops followed by out-of-season flushing and blossoming, and twig die-back, thus losing their economic viability.

Other symptoms have also been discovered in the infected citrus fruits, including but not limited to reduced fruit size, lopsided, and an extremely bitter taste, probably due to high acid and low sugar content in the fruit, thus rendering them commercially valueless (McClean & Oberholzer, 1965b; Halbert & Manjunath, 2004; Bové, 2006; da Graça *et al.*, 2015). Furthermore, the infected citrus trees have been reported to experience an excessive and premature fruit drop, while those remaining on the tree become unevenly coloured with greening at the stylar end (Bové, 2006; Kokane *et al.*, 2020; Ghosh *et al.*, 2021) (Figure 1. 3). Despite the extensive scientific investigations, finding an effective method to cure citrus greening disease has remained elusive. The only effective approach to date involves the removal of all CG-infected trees from the healthy ones to prevent the spread of

the bacteria in commercial citrus orchards (da Graça, 1991; Bové, 2006; Gottwald, 2010; Zhang *et al.*, 2010; Inoue *et al.*, 2020).

Figure 1. 3: The characteristics of citrus greening disease (CG) symptoms on infected citrus trees: Yellowing leaves (**A**) with irregular blotchy mottle patterns on both sides (**C**), excessive fruit drop (**B**) and underdeveloped, poorly coloured fruits at the stylar end (**D**). Healthy citrus leaf shown for comparison (**C**, top-left corner) (**Photo credit**: J.M. Bové and Peter Stephen).

1.2.5.3. Transmission of Candidatus *Liberibacter species by psyllid vectors:*

The spread of plant-pathogenic bacteria *Candidatus* Liberibacter species (CLas, CLam and CLaf) has been associated with the insect vectors and plant experiments (graft and dodder) (Garnier & Bové, 1983; van Vuuren, 1993; Shimwela *et al.*, 2016). As for seed transmission, several studies have been conducted to determine and confirm whether the pathogenic bacteria can be transmitted through the plant seed; however, they had contradicting results. For example, the experimental seed transmission studies conducted by Capoor *et al.* (1974) where seeds from the infected citrus

trees were planted, confirmed that after a high percentage of seed germination, all seedlings were healthy, and no disease symptoms were discovered or found. Furthermore, Hartung *et al.* (2010), Hilf (2011) and van Vuuren *et al.* (2011) conducted seed transmission tests using seeds collected from the symptomatic fruits, and the results also confirmed that neither CLaf nor CLas are transmitted through the plant seeds. However, Albrecht & Bowman (2009), and Hilf (2011) found results suggestive of seed transmission, because a low percentage of tested seedlings grown from the seeds of symptomatic fruits were CLas-positive. Therefore, due to these contradictory reports, no conclusive studies exist yet regarding the possibility of plant-seeds transmitting the *Candidatus* Liberibacter species. Since *Candidatus* Liberibacter species are phloem-limited, however, several studies have documented that only phloem-feeding insects can potentially acquire and effectively transmit these bacteria to the host-plants on which their respective insect vectors feed (Weintraub & Beanland, 2006; Carmo-Sousa *et al.*, 2020).

In citrus environments, the primary mode of the effective occurrence and the natural spread of both pathogenic bacteria and the resulting citrus greening disease has been discovered to be facilitated naturally by the feeding and flight behaviour of two adult psyllid vectors: namely *T. erytreae* and *D. citri* (McClean & Oberholzer, 1965a; Capoor *et al.*, 1967; Teixeira *et al.*, 2005) (Figure 1. 4). Adult citrus psyllids utilise their mandibular and maxillary stylets to feed on plant stems, as well as new and mature leaves, with a preference for young foliage (Green & Catling, 1971; Hall *et al.*, 2012) (Figure 1. 4). When an adult psyllid that is uninfected lands on an already infected citrus tree and feeds on new leaves, it ingests and incubates the Liberibacter bacterium inside its gut, therefore, becoming a pathogen vector (Moll & Martin, 1973; Hodkinson, 2009; Aidoo, 2023). However, at this stage, the vector lacks the potential to transmit the bacterium that it has ingested to other host-plants, establishing a persistent infection in the psyllid (Aidoo, 2023). This initial infection inside the psyllid vector becomes primed for transmission, occurring in a stochastic or random manner, to a new, susceptible citrus tree when the psyllid vector resumes feeding (Xu *et al.*, 1988; Hung *et al.*, 2004; Aidoo, 2023).

Direct adult acquisition and transmission of the pathogen are rare, according to Inoue *et al.* (2009) and Pelz-Stelinski *et al.* (2010). The multiplication of the plant-pathogens inside the psyllid vector is very important for effective transmission into new host-plants (Inoue *et al.*, 2009; Pelz-Stelinski *et al.*, 2010). Subsequently, when the psyllid vector migrates to feed on another healthy citrus tree,

then, the transmission of the Liberibacter bacterium species takes place (Aidoo, 2023). This occurs through the transfer of the pathogen from the salivary glands of the psyllid vector, released through the stylets, and introduced into the plant's tissues (Aidoo, 2023). Studies by van Vuuren & van der Merwe (1992), and Ammar *et al.* (2016) have documented that the extended or prolonged feeding periods of the psyllid vector on the hosts can lead to higher transmission rates of the bacterium. Once established inside the plant, the pathogen spreads systematically, often causing initially subtle or imperceptible symptoms (Aidoo, 2023). As the infections progress, more pronounced symptoms begin to manifest, accompanied by a gradual decline in the health of the affected plant (Aidoo, 2023). Furthermore, both citrus psyllid pests, *T. erytreae* and *D. citri*, show a stronger attraction to infected citrus trees because the vectored pathogen induces the infected plants to release or produce a chemical called methyl salicylate which makes infected trees very attractive to these pests (Mann *et al.*, 2012). As a result, the infected citrus trees become the most preferable targets over the healthy trees for *T. erytreae* and *D. citri* (Volpe *et al.*, 2024). However, after feeding and due to the low nutritional value of the diseased trees, these psyllid pests eventually shift their preference towards uninfected citrus trees in citrus orchards where they continue feeding (Mann *et al.*, 2012; Volpe *et al.*, 2024). This shift promotes the widespread of the greening-associated Liberibacter bacteria in citrus orchards (Mann *et al.*, 2012), and further increases the likelihood of infecting other psylloid species that were previously unexposed to these greening-associated bacteria.

Figure 1. 4: The psyllid vectors of greening-associated *Candidatus* Liberibacter species, preferably feeding on newly emerging citrus shoots: Adult *Trioza erytreae* (**A**), and adult *Diaphorina citri* with immatures excreting honeydew (**B**) (**Photo credit**: Peter Stephen and David Hall).

1.2.6. Justification of the study.

Insect pests and the plant-pathogens they carry pose significant threats to citrus orchards through infesting the citrus plants/trees and thus causing extensive damage to commercial citrus production (Kanakala & Ghanim, 2016; Dalio *et al.*, 2017; Sun *et al.*, 2019). The presence of phloem-feeding psyllid pests, *Trioza erytreae* and *Diaphorina citri*, have seriously/greatly threatened commercial citrus production in nearly all major global citrus-producing regions where they have established (Aubert, 1987; Tolba & Soliman, 2015; Cocuzza *et al.*, 2017; Rwomushana *et al.*, 2017). Insect interaction with commercial citrus trees has contributed significantly towards the near-collapse of several agricultural citrus fruit industries globally (Bové, 2006; Gottwald, 2010). The substantial economic devastation caused by these pests is primarily attributed to their role as potential vectors and transmitters of plant-pathogenic bacteria responsible for causing devastating citrus greening diseases (da Graça, 1991; Bové, 2006; Gottwald, 2010). Although the citrus greening is attributed to three primary vectored-Liberibacter species globally, the greening disease associated with the pathogen-vector combination of *Candidatus* Liberibacter asiaticas and *D. citri* has been considered the deadliest global citrus disease, responsible for the death of millions of citrus trees (Bové & Ayres, 2007; Luo *et al.*, 2017; Yuan *et al.*, 2021). Furthermore, CLas and *D. citri* manifest as heat-tolerant species and have widespread distribution, with recent detections or reports in other African regions, and this expansion of their range poses a significant threat to southern African commercial citrus growers, should these pests migrate beyond their established East African regions (Shimwela *et al.*, 2016; Rwomushana *et al.*, 2017; Rasowo *et al.*, 2019; Ajene *et al.*, 2020a; Oke *et al.*, 2020; Sétamou *et al.*, 2023). To complicate things further, *D. citri* and the local vector, *T. erytreae* have been documented to potentially transmit *Candidatus* Liberibacter africanus and CLas, respectively, but only under experimental conditions (Massonie *et al.*, 1976; Cocuzza *et al.*, 2017), and there is a possibility of similar transmissions occurring within the natural field conditions if both pathogen and vector species coexist (Bové, 2006; Rasowo *et al.*, 2019), and could worsen the present losses associated with citrus pests.

In southern Africa, the citrus greening disease is associated with the Liberibacter bacterium CLaf and is vectored by the indigenous psyllid, *Trioza erytreae*, African citrus triozid (ACT) (McClean & Oberholzer, 1965a, 1965b). *Trioza erytreae* has been recorded as the only psyllid species that feeds and breeds on citrus trees in the southern African commercial citrus industry (Hodkinson,

1984; van den Berg, 1990; Cocuzza *et al.*, 2017). However, other indigenous psylloid species have been recorded in commercial citrus orchards during early detection surveys of *Diaphorina citri*, Asian citrus psyllid (ACP). Considering the significant role that some species of psylloids play as phloem-feeders and vectors of economically important plant-pathogens in agricultural ecosystems (Burckhardt, 1994), it is therefore important to gather comprehensive insights into the group of psylloid species occurring within the commercial citrus environments. In the Afrotorpical region, *Diaphorina aurberti* has been recorded in Comoro Island with immatures and adults completing their life cycles on citrus (Hollis, 1987). Furthermore, other *Diaphorina* spp., such as *Diaphorina amoena, D. communis, D. murrayi, D. punctulata* and *D. zebrana*, have also been reported from citrus (Capener, 1970a, 1970b; Halbert & Manjunath, 2004). Understanding the psylloid diversity in and around the citrus orchards as either potential feeders or potential vectors for any *Candidatus* Liberibacter species that might be associated with the citrus greening disease is vital in long-term IPM planning against pests of economic importance. Consequently, this knowledge will contribute significantly towards addressing the potential challenges that might arise from the native psylloid species within the citrus industry in South Africa, and further provide a major contribution to the efficient management of the industry, ultimately establishing a concrete foundation for proactive and effective solutions aimed at mitigating the potential threats that might arise from these species.

1.3. General research purpose of the study

The primary purpose of this research was to conduct a comprehensive and systematic investigation with the aim of identifying and further documenting the native psylloid species occurring across diverse commercial citrus environments (i.e. citrus orchards and their adjacent natural vegetation). Furthermore, this research study seeks to uncover the ecological significance of these indigenous psylloids in commercial citrus orchards, with a specific focus on their potential role as feeders on citrus plants.

1.3.1. The following specific objectives have been set to achieve the purpose of this study:

- To determine the diversity and community structure of indigenous psylloid species in citrus environments.
- To determine the presence of citrus DNA in the guts of indigenous psylloid species residing in citrus environments.

1.4. Thesis Outline

This thesis consists of four chapters. The general introduction is followed by two research or data chapters and a general discussion.

Chapter 1: General introduction.

Background of the study, literature review (an overview of literature about the citrus taxonomic classification; its origin, history and distribution; its economic importance; and abiotic and biotic constraints), justification, aims and objectives, and chapter layouts of the thesis, are provided.

Chapter 2: The diversity and composition of psylloids associated with citrus orchards in the Afrotropical region, South Africa.

Within this chapter, field surveys were carried out across diverse commercial citrus environments in Limpopo and Mpumalanga provinces, South Africa, to determine the diversity and community structure of psylliod species present within these environments. The host-plants associated with some of the psylloid species and the best method for monitoring psyllid pests in commercial citrus orchards are also investigated.

Chapter 3: Do native and invasive psylloid species feed on different varieties of citrus plants in citrus orchards? A molecular gut content analysis.

Within this chapter, gut contents of the filed-collected psylloid species were analysed to determine their potential feeding on citrus plants. It further describes some citrus plants that are likely to be threatened by psylloid infestations in orchards.

Chapter 4: General discussion.

Gives an overview of the study, and summarises key findings from **Chapters 2** and **3**, highlighting their significance within the context of citrus orchard management and plant health, followed by conclusions and recommendations for future work.

CHAPTER 2

THE DIVERSITY AND COMPOSITION OF PSYLLOIDS ASSOCIATED WITH CITRUS ORCHARDS IN THE AFROTROPICAL REGION, SOUTH AFRICA

2.1. Introduction

Insects represent the most diverse and abundant group of living organisms established in different environments across the globe, with the majority of them associated with plants (Bahrndorff *et al.*, 2016; Nayak *et al.*, 2021; Yana *et al.*, 2022). Among these, psylloids (Hemiptera: Sternorrhyncha: Psylloidea), commonly referred to as "jumping plant-lice" or "psyllids", constitute a highly diverse group of plant sap-sucking insects that inhibit nearly all terrestrial environments and are distributed throughout all biogeographic regions except Antarctica (Hodkinson, 2009; Ouvrard *et al.*, 2015a, 2015b; Maryanska-Nadachowska *et al.*, 2018; Burckhardt *et al.*, 2021). Characterised by their specialised feeding habits, typically restricted to a narrow range of hosts, these sap-sucking insects are generally associated with either a single or a few host-plant species, particularly those within the same taxonomic groups (Hodkinson, 2009; Burckhardt *et al.*, 2014). The majority of psylloids are associated with widely distributed groups of plants from diverse families, including Fabaceae, Myrtaceae, Asteraceae, and those in the order Sapindales (Hodkinson, 1974, 2009; Ouvrard *et al.*, 2015b); hence their widespread presence across different ecosystems (i.e. agricultural, forest and natural environments). Importantly, some psylloid species have become significant pests, posing serious threats to the growth and productivity of their associated host-plants in the aforementioned environments across different regions globally, including Africa, Asia and North America (Halbert & Manjunath, 2004; Grafton-Cardwell *et al.*, 2013; Antolinez *et al.*, 2017; Cocuzza *et al.*, 2017; Benhadi-Marín *et al.*, 2021).

Globally, the known diversity of the Psylloidea currently exceeds 4,000 described species, with several species, including *Bactericera cockerelli* (Šulc), *Bactericera trigonica* (Hodkinson) both from Triozidae, *Cacopsylla* species from Psyllidae and *Glycaspis brimblecombei* (Moore) from Aphalaridae, considered pests of economic importance known to affect a wide range of cultivated plants, including apples, avocados, carrots, eucalyptus, potatoes, pears, stone fruits and tomatoes (Burckhardt, 1994; Hollis & Martin, 1997; Hodkinson, 2009; Martínez *et al.*, 2018; Burckhardt *et al.*, 2021). Although the considerable impact is induced during sap-sucking, the plant-pathogens

vectored by some psylloid pests were documented as a major threat to the overall plant health and productivity of their hosts, particularly in global agroecosystems (Burckhardt, 1994; Munyaneza *et al.*, 2010b; Moreno *et al.*, 2021). Among the most concerning plant-pathogens are *Candidatus* Liberibacter and Phytoplasmas, which have been documented as significant threats to some of the aforementioned cultivated fruits and vegetables, including apples, carrots, citrus, potato, tomato, pear and stone fruits (Halbert & Manjunath, 2004; Tedeschi *et al.*, 2006; Munyaneza *et al.*, 2010b; Nissinen *et al.*, 2014; Antolinez *et al.*, 2017; Cho *et al.*, 2017; Morrow *et al.*, 2020).

In citrus production, two main psyllid pests are of particular concern: *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) and *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), along with their associated bacterial species (Halbert & Manjunath, 2004; Bové, 2006; Gottwald, 2010; Chapter 1). These psyllid pests serve as vectors and transmit phloem-limited bacterial agents responsible for establishing citrus greening (CG) disease, also known as Huanglongbing (HLB), primarily on citrus plants globally, including *Candidatus* Liberibacter species [i.e. *Ca*. Liberibacter africanus (CLaf), *Ca*. Liberibacter asiaticus (CLas) and *Ca*. Liberibacter americanus (CLam)] (Bové, 2006; Cocuzza *et al.*, 2017; Siverio *et al.*, 2017; Ruíz-Rivero *et al.*, 2021). Citrus greening is a global devastating and incurable disease that manifests as continuous fruit drop, stunted growth, die-back, production of poor-quality fruits and asymmetrical yellow mottles or severe chlorosis on the plant foliage, among others, leading to significant economic losses within the citrus production globally (Bové, 2006; Gottwald *et al.*, 2007; Manjunath *et al.*, 2008; Paula *et al.*, 2018). As an example of the destructive potential of these combined agents, around 100 million infected commercial citrus trees were lost across Asia with an additional one million trees in Brazil since the initial report of the disease in São Paulo in 2004 (Gottwald *et al.*, 2007; Zhang *et al.*, 2010). In Africa, the reported yield losses associated with CG disease have ranged from 25% to 100% (Rasowo *et al.*, 2019).

Given the significant role of psylloids in citrus production and their potential impact on plant health and productivity, the current study aimed to document their morphospecies in citrus environments. With the coexistence of diverse plant species within these environments and the close interactions between plants and herbivorous insects, we hypothesised that the citrus environments harbour a considerable diversity of psylloids. Thus, the current chapter focused on assessing the diversity and community structure of psylloids in citrus orchards and their surrounding natural vegetation. The knowledge on the diversity of indigenous psylloids, their association with the citrus plants and

their potential role in transmitting plant-pathogen-causing citrus greening disease will inform the citrus communities of the need to prioritise the management of native psylloid species due to their integral role in these environments.

2.2. Materials and Methods

2.2.1. Study sites.

Field surveys for psylloids collection were conducted in commercial citrus environments located in the summer rainfall regions of the north-eastern part of South Africa (Figure 2. 1). The surveys were conducted at 12 randomly distributed commercial citrus orchards along the selected gradient. A total of eight citrus environments were situated in the Limpopo province (i.e. four in Burgersfort region; one in Ba-Phalaborwa region; one in Giyani region; and the last two in Hoedspruit region). Furthermore, four citrus environments were selected in Mpumalanga province, with two located in Mbombela region and the remaining citrus environments in Komati-Malelane region (Figure 2. 1). Lastly, the sampling sites were recorded using a GPS (Geographic Positioning System) device, the Garmin GPSMAP® 65/65S (GARMIN, USA) and mapped.

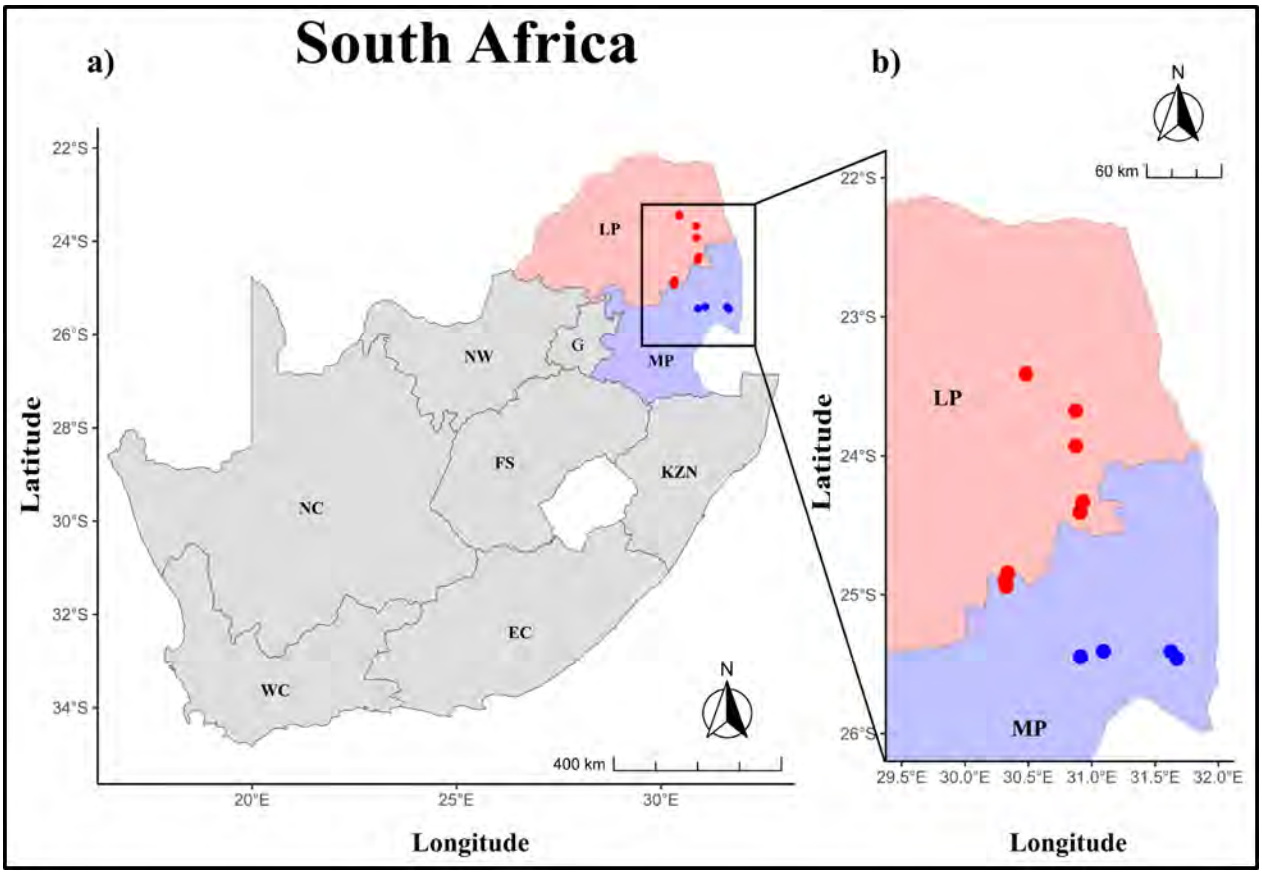

Figure 2. 1: A South African map of the distinct commercial citrus environments surveyed within Limpopo [(**LP**) (red dots)] and Mpumalanga [(**MP**) (blue dots)] provinces, South Africa.

2.2.2. Surveys of psylloid species in different citrus environments.

Selected sites were visited monthly to collect psylloids infesting citrus trees and those adjacent to citrus orchards. This was achieved by using a combination of passive and active psylloid sampling techniques/methods which involved the use of double-sided yellow sticky traps and insect sweep-net, respectively. Double-sided yellow sticky traps [e.g. measuring 200 × 140 millimeters (mm)] sourced from Insect Science (Pty) Ltd were hung on randomly distributed citrus trees throughout the orchards to sample psylloids (Figure 2. 2). A minimum of five to ten yellow sticky traps were deployed at the edges of each citrus orchard, and the numbers were determined by the size of each plot. All sticky traps, with each labelled with the site name, trap number and date of deployment, were consistently hung at the outer canopy of each selected citrus tree at a height ranging between 1.5-2 meters (m) in orchards (Figure 2. 2). To minimise any potential dust contamination, yellow sticky traps were also placed in the innermost rows of the orchards. Furthermore, these sticky traps

were spaced at a distance of at least 500 m apart in the citrus orchards to ensure adequate spacing and avoid the possibility of capturing or collecting the same psylloids multiple times.

Figure 2. 2: A double-sided yellow sticky trap hung on a single branch of each selected citrus tree in a commercial citrus orchard (**A**), at a height of approximately 1.5-2 m (**B**) (**Photo credit**: Leani Serfontein and Tshepang Makitla).

To complement the passive collection method, psylloids were also collected actively by using an entomological/insect sweep-net with an approximate diameter of 65 centimeters (cm). The active collection method was conducted both within the citrus orchards, particularly along the rows where yellow sticky traps were deployed, and along the adjacent natural vegetation located approximately 10-50 m away from these orchards (Figure 2. 3). During active collection, psylloids were collected

directly from the plant canopy for 20 minutes per sampling plot. Furthermore, a minimum of five plants were randomly selected for sampling in each plot, and five sweeps using a sweep-net were performed at each plant. Following the collection process, all collected yellow sticky traps were sealed with plastic cling-wrap and placed in a container box to prevent any potential contamination or damage to the trapped psylloids during transportation. Actively collected psylloid specimens were preserved in the glass vials half-filled with 70% ethanol until the sorting process. Lastly, all psylloids collected using both yellow sticky traps and insect sweep-net were sampled during citrus-flushing seasons, commencing from August 2022 to April 2023.

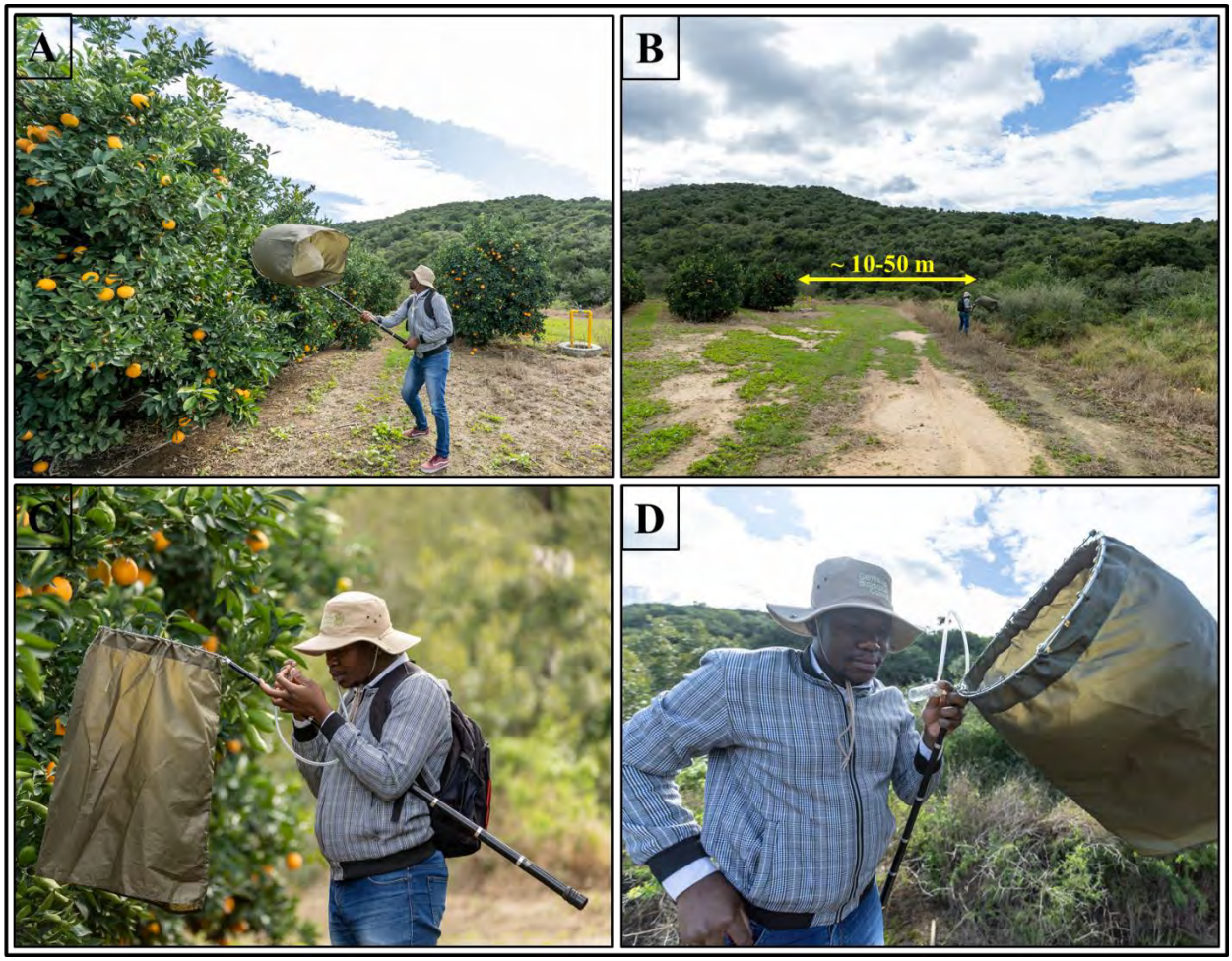

Figure 2. 3: Active collection of psylloids conducted using an insect sweep-net in a citrus orchard along the rows where the sticky traps were deployed (**A**), and along the adjacent natural vegetation (**B**). All psylloid specimens from both habitats were aspirated into glass vials half-filled with 70% ethanol (**C** and **D**) (**Photo credit**: David Taylor).

2.2.3. Preparation, identification and storage of collected psylloid specimens.

Collected sticky traps were removed from the storage container box and placed inside a refrigerator until required for isolation and identification. Psylloid specimens collected using sticky traps were isolated from these sticky traps using HistoChoice under a stereo-microscope. The morphological characteristics of adult and immature psylloids collected using sticky traps and sweep-net were used during the identification of these species. Psylloid specimens were sexed and identified to the genus level and, where possible, to the morphospecies level using both published and unpublished dichotomous identification keys developed by Daniel Burckhardt for the identification of psylloids in the Afrotropical region (Capener, 1973; Hollis, 1984; Burckhardt, 2022, *unpublished key*). However, representative psylloid specimens that could not be confidently identified were then submitted for identification to a specialist taxonomist of psylloids at Citrus Research International (CRI), situated in Mbombela, Mpumalanga Province. For this study, the term "morphospecies" was used to refer to all psylloids that were identified and those that have not yet been identified to the either genus or species level. Lastly, all psylloid specimens collected using the above-two-described collection methods were housed at CRI Entomology Laboratory, where they were then preserved in 1.5-milliliter (mL) Eppendorf tubes, each filled with 70% ethanol and tagged with the labelled information such as collection site and trap number, sampled plant, collection method, deployment or collection date and species name. All the preserved psylloid specimens were then stored at room temperature (RT) in the laboratory, awaiting further analysis or future research purposes.

2.2.5. Association between plant and psylloid species.

The association between trees and psylloid species was investigated by quantifying the abundance of each psyllid species on specific plant species in the surveyed citrus environments. For each plant species sampled, the total abundance of each psyllid species was determined by dividing the total count of individuals of that species across all sampled plants by the total number of sampled plants of the same species. To gather additional information, detailed information about the sampled plant species was recorded during the field surveys. In instances where the plants could not be identified, herbarium specimens were collected and pressed for further identification in the laboratory. These identifications were verified by a botanist. Any signs of feeding damage on plant leaves attributed

to the psylloid infestations were recorded and photographed for further analysis and documentation (Figure S 2. 1).

2.2.6. Statistical analysis.

Multivariate generalised linear models (MvGLM) (Wang *et al.*, 2012) were performed to measure statistical differences in the community structure of psylloids sampled on different plant species using different collection methods in the selected commercial citrus environments across different study regions in Limpopo and Mpumalanga provinces. To achieve this, the '*manyglm*' R function from the '*mvabund*' R package (Wang *et al.*, 2012) was used to model the multivariate species abundances as the response variable, with plant species and collection methods (e.g. trapping versus sweep-netting) used as categorical fixed effects. Likelihood-ratio tests (hereafter 'LRT') and pit-strap bootstrapping were used to compute *P*-values, using 999 bootstrap replicates, to assess the statistical significance of fixed effects. To visualise variation in the assemblages of psylloid species between plant species and collection methods, model-based unconstrained ordination was employed using the '*boral*' R package (Hui, 2015). A Bayesian hierarchical correlated response model was fit to the species-abundance matrix, using latent variables to account for residual correlations between psylloid species.

Univariate Generalized Linear Models (Univariate GLM's) were performed to determine the species of psylloids that contributed to the variation within the community structure between the plant species sampled and collection methods. Pit-strap bootstrapping was used to compute adjusted p-values corrected for multiple testing and correlations between species (Wang *et al.*, 2012). Univariate GLM's were specified using a negative binomial distribution to account for overdispersion in preliminary models specified with a Poisson distribution.

Lastly, to estimate the adequacy of collection efforts when psylloids were collected using traps or an insect sweep-net at different citrus environments, the species accumulation curves (SACs) were computed using a series of custom wrapper R functions around the 'poolaccum' function from the 'vegan' R package (Oksanen *et al.*, 2022). Species accumulation curves plotted the observed species richness (hereafter 'Observed') against the number of surveys performed. Three nonparametric species richness estimators, namely: Chao 2 ('chao2'), second-order jackknife ('jack2') and the bootstrap estimator ('boot') were used to measure the adequacy of sampling

efforts at the citrus orchards and adjacent plots (Bell *et al.*, 2014). Sampling effort was considered sufficient if observed species richness was comparable to estimated species richness (approached an asymptote). All data was analysed using the R version 4.3.1 (R Core Team, 2023).

2.3. Results

2.3.1. Abundance of psylloids in commercial citrus environments across provinces.

A total of 4,900 psylloids assigned to five families, 19 genera and 47 morphospecies were collected in commercial citrus environments in Limpopo and Mpumalanga provinces. Among the collected psylloids, 3,754 individuals were recorded in Limpopo, while 1,146 psylloid counts were recorded in Mpumalanga (Figure 2. 4). A high proportion of sampled psylloids was *Pauropsylla trichaeta* with 1,680 individuals recorded in this species. Among the abundant species were *Diaphorina punctulata*, *Trioza erytreae*, *Diaphorina virgata*, *Euryconus* sp., *Cacopsylla* sp., *Retroacizzia mopanei*, *Acizzia russellae*-group, *Acizzia* sp.3 and *Acizzia* sp.2 with 466, 426, 371, 358, 311, 263, 240, 216 and 140 individuals recorded, respectively. The abundance of between 1 and 98 was recorded for the remaining 37 morphospecies. Among the collected individual psylloids, a total of 3,265 psylloids were collected in citrus orchards using yellow sticky traps. Lastly, 477 individual psylloids were collected in the same citrus orchards, while up to 1,158 were collected in adjacent natural vegetation using a sweep-net in both microhabitats, with the highest numbers recorded on marula [*Sclerocarya birrea* (Anacardiaceae)] (545), followed by mopane [*Colophospermum mopane* (Fabaceae)] (239) and *Ficus* sp. (Moraceae) (229), the least abundant psylloids recorded on *Vachellia* spp. [formally known as *Acacia* (Fabaceae)] (79) and unidentified plant species (66) (Figure 2. 4).

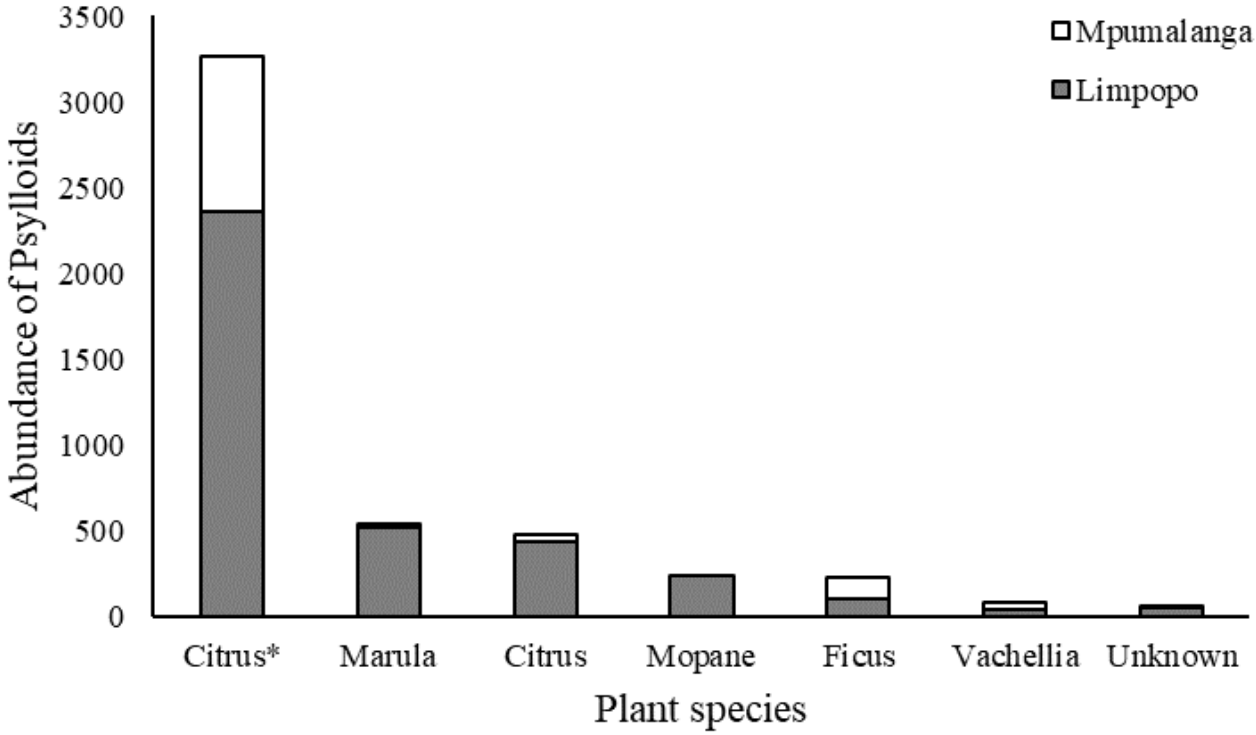

Figure 2. 4: The abundance of psylloids sampled using sticky traps (*) and a sweep-net on Citrus and other plant species naturalised adjacent to orchards in Limpopo and Mpumalanga provinces.

Yellow sticky traps used in citrus orchards had a higher abundance of psylloids compared to insect sweep-net in the surrounding natural vegetation. Furthermore, when comparing the abundance of psylloids collected using a sweep-net on citrus and other plant species constituting the adjacent natural vegetation, the numbers differed among plant species. The interaction between provinces and plant species influenced the abundance of psylloids recorded with sweep-net in the surveyed citrus environments (Table 2. 1).

Table 2. 1: Univariate Generalized Linear Models (GLM's) results indicate significant differences in psylloid abundances collected through two distinct sampling methods across diverse citrus environments between the provinces and among the different sampled plant species.

Sampling methods	**Province**			**Plant species**			**Province vs Plant species**		
	DF	x^2	***P- value***	**DF**	x^2	***P- value***	**DF**	x^2	***P- value***
Sticky traps	1	283,238	< **0,001***	-	-	-	-	-	-
Sweep-net	1	107,46	**< 0,001***	5	950,94	< **0,001***	4	26,254	< **0,001***

Statistically significant *P-values* are highlighted in bold and marked with (*).

2.3.2. Species richness of psylloids in commercial citrus environments across provinces.

The family Psyllidae had the highest represented genera with 12 morphospecies, followed by the Triozidae with three morphospecies. At least two morphospecies were recorded for Aphalaridae and Liviidae families separately. The remaining family Carsidaridae had one morphospecies. Lastly, 14 morphospecies were represented by singletons, with only two morphospecies that could not be assigned to either family or genera. The species richness of psylloids recorded using both the yellow sticky traps and sweep-net did not significantly differ between the provinces (Table 2. 2), despite the noticeably higher abundances of psylloids sampled in Limpopo compared to those sampled in Mpumalanga. Data collected using sweep-net showed that species richness of psylloids significantly differed among plant species with the highest richness (22 morphospecies) recorded on citrus (Table 2. 2). A total of nine psylloid morphospecies were recorded on *Vachellia* spp. and unidentified plant species separately, whereas six, three and two morphospecies were recorded on marula, *Ficus* sp. and mopane, respectively. Among psylloid morphospecies collected using insect sweep-net, 11 morphospecies were unique to citrus, while eight were found on the adjacent plant species. Lastly, 11 morphospecies were shared among the different plant species (Table S 2. 1).

Table 2. 2: Univariate Generalized Linear Models (GLM's) results indicate significant differences in psylloid species richness between provinces and among sampled plant species in different citrus environments, as assessed using two distinct sampling or collection methods.

Sampling methods	**Province**			**Plant species**			**Province vs Plant species**		
	DF	***x²***	***P- value***	**DF**	***x²***	***P- value***	**DF**	***x²***	***P- value***
Sticky traps	1	1,172	0,279	-	-	-	-	-	-
Sweep-net	1	1,676	0,195	5	32,41	**< 0,001***	4	4,486	0,344

Statistically significant *P-values* are highlighted in bold and marked with (*).

2.3.2. Community structure of psylloids among the plant species and between collection methods.

Diaphorina punctulata and *Diaphorina virgata* were abundant and frequent on marula compared to other plant species adjacent to citrus orchards. For example, the abundance of *D. punctulata* on marula (14.6 ± 20.5 SD) surpassed that of unidentified plants (0.857 ± 1.57 SD), citrus (0.380 ± 2.10 SD) and *Ficus* (0.167 ± 0.408 SD) by 17-fold, 38-fold and 87-fold, respectively. *Diaphorina*

virgata had a 108-fold increase in abundance on marula (18.1 ± 48.8 SD) than on *Vachellia* spp. (0.167 ± 0.408 SD), and a 132-fold increase in abundance compared to citrus (0.137 ± 0.731 SD). However, *A. russellae*-group and *R. mopanei* showed higher abundance and frequency, but when sampled primarily on *Vachellia* spp. and mopane, respectively. This surpassed their occurrence and abundance on other plant species in citrus environments. *Acizzia russellae*-group, for example, had an eight-fold and 197-fold greater abundance when sampled on *Vachellia* spp. (1 ± 2.45 SD) than on mopane (0.125 ± 0.354 SD) and citrus (0.00507 ± 0.0711 SD), respectively. A similar trend was further observed with *R. mopanei*, which showed a greater abundance of 706-fold on mopane trees (29.8 ± 30.4 SD) than on citrus (0.0422 ± 0.394 SD). The community structure of psylloid species showed statistical significance among the different plant species sampled (x^2 = **11.54**, df = **5**, *P* = **0.002**) in commercial citrus environments across the study regions (Table 2. 3). Univariate GLM's indicated that *Acizzia russellae*-group, *Diaphorina punctulata*, *Diaphorina virgata* and *Retroacizzia mopanei* were the primary drivers of the observed differences in psylloid community structure between plant species (Table 2. 3). The non-metric multidimensional scaling (nMDS) ordination plot below shows a significant overlap in the community structure of psylloids sampled across diverse plant species or genera in different citrus environments (Figure 2. 5).

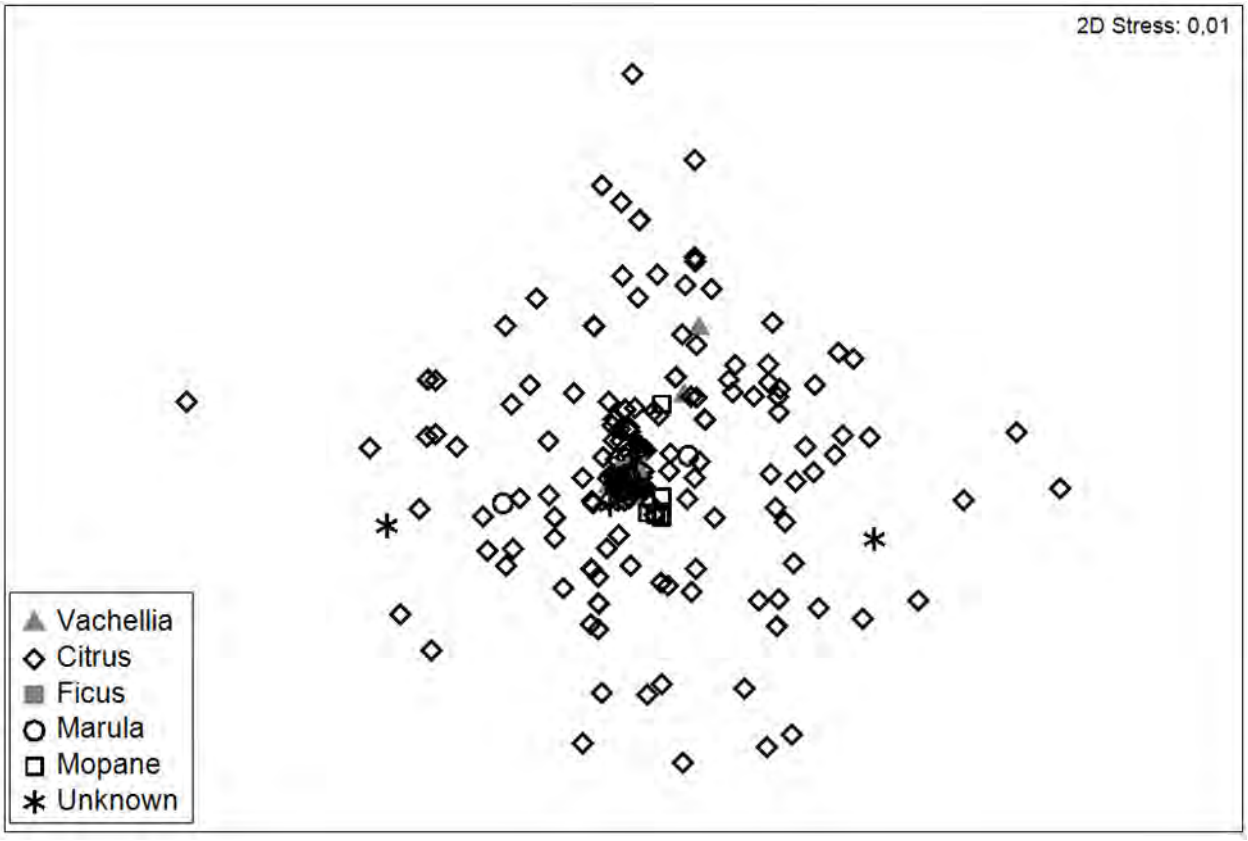

Figure 2. 5: Non-metric Multidimensional Scaling (nMDS) ordination plot showing the difference in the community structure of psylloids collected on diverse plant species or genera in commercial citrus environments across the Limpopo and Mpumalanga provinces.

Similarly, there was a significant difference in the psylloid community structure, depending on the specific sampling method used, either the trapping or sweep-netting (x^2 = **13.71**, df = **1**, *P* = **0.001**) (Table 2. 3). *Acizzia* sp.3 and *Cacopsylla* sp. were identified as the key drivers responsible for the observed differences in psylloid community structure between these two different methods (Table 2. 3). The findings showed a significant difference in the abundance of *Acizzia* sp.3 and *Cacopsylla* sp. when comparing the trapping method to the sweep-netting method. Specifically, *Acizzia* sp.3 showed a 53-fold higher abundance with the trapping method (0.504 ± 5.80 SD) in comparison to the sweep-netting method (0.0095 ± 0.0974 SD). Similarly, the abundance of *Cacopsylla* sp. was six times higher when sampled using the trapping method (0.675 ± 4.10 SD) in comparison to the sweep-netting method (0.114 ± 0.900 SD). The nMDS ordination plot presented below shows overlaps between the two collection/sampling methods used for sampling psylloids among diverse plant species or genera in different citrus environments (Figure 2. 6).

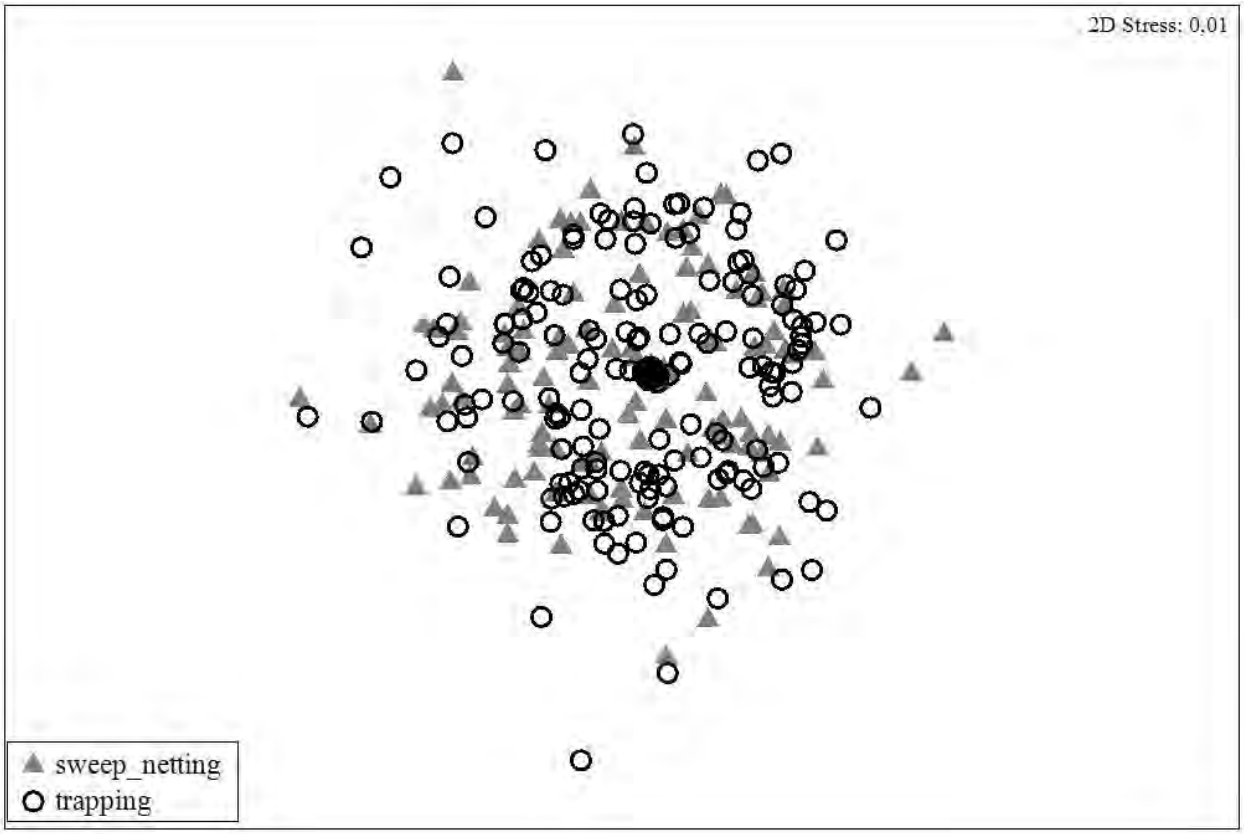

Figure 2. 6: Non-metric Multidimensional Scaling (nMDS) ordination plot showing a relationship between collection methods (trapping versus sweep-netting) and the psylloid community sampled from multiple host-plant genera in commercial citrus environments. Each dot represents a psylloid community, and samples plotted nearby (clustered) indicate similar species composition obtained using the two different collection methods.

Although many psylloid species were responsible for driving the statistical significances within the community structure either between the plant species sampled or collection methods used, two specific species contributed to these statistical significances between both plant species sampled and the collection methods used. *Euryconus* sp. and *Pauropsylla trichaeta* were the only species responsible for the observed statistical significance in the community structure between both plant species sampled and collection methods used (Table 2. 3). These two significant results indicate that the abundances of *Euryconus* sp. and *P. trichaeta* differed depending on which plant species they were sampled. This variability in their abundances was further pronounced when considering the collection methods used (trapping versus sweep-netting). Specifically, the results highlighted that *Euryconus* sp. was more abundant on marula (1.12 ± 4.24 SD), demonstrating a two-fold and

three-fold increase in abundance compared to when sampled respectively on the citrus (0.569 ± 3.19 SD) and unidentified plants (0.429 ± 1.13 SD). Furthermore, within the context of collection methods, trapping (0.753 ± 3.74 SD) yielded a four-fold greater abundance of *Euryconus* sp. as opposed to sweep-net (0.181 ± 1.27 SD). In the case of *P. trichaeta*, its abundance on *Ficus* (37.7 ± 33.4 SD) greatly surpassed that of citrus (2.44 ± 13.2 SD), unidentified plants (0.714 ± 1.89 SD) and marula (0.125 ± 0.342 SD) respectively by 16-fold, 53-fold and 302-fold factors. Furthermore, in terms of collection methods, the abundance of *P. trichaeta* was observed to be three-fold higher when sampled through the trapping method (3.35 ± 15.5 SD) rather than the sweep-netting method (1.22 ± 8.16 SD).

Table 2. 3: The response of each psyllid species to the plant species sampled and collection methods used during the field surveys in different commercial citrus environments of Limpopo and Mpumalanga provinces.

Family	Morphospecies	Abundance	No. of plant species associated	Plant species		Collection methods	
				x^2	*P-value*	x^2	*P-value*
Aphalaridae	*Agonoscena crotalariae*	98	2	0.728	1.000	0.172	1.000
Aphalaridae	*Glycaspis brimblecombei*	1	1	0.055	1.000	0.113	1.000
Carsidaridae	*Mesohomotoma libesei*	4	1	0.064	1.000	0.131	1.000
Liviidae	*Euphyllura speciosa*	29	2	1.144	0.993	0.155	1.000
Liviidae	*Euphyllura* cf. *speciosa*	9	1	0.07	1.000	0.142	1.000
Liviidae	*Peripsyllopsis* sp.	4	1	0.361	1.000	0	1.000
Psyllidae	*Acizzia* sp.1	11	1	0.071	1.000	0.806	0.997
Psyllidae	*Acizzia* sp.2	10	2	1.506	0.952	2.408	0.388
Psyllidae	*Acizzia* sp.3	216	2	0.268	1.000	3.572	**0.049***
Psyllidae	*Acizzia* sp.4	10	1	0.07	1.000	0.143	1.000
Psyllidae	*Acizzia karrooensis*	28	2	2.854	0.544	0.14	1.000
Psyllidae	*Acizzia russellae*	67	1	0.083	1.000	2.272	0.465
Psyllidae	*Acizzia russellae*-group	240	3	4.134	**0.047***	0.127	1.000
Psyllidae	*Cacopsylla* sp.	311	3	1.534	0.952	4.123	**0.016***
Psyllidae	*Ciriacremum capense*	54	1	0.081	1.000	2.98	0.165
Psyllidae	*Diaphorina albomaculata*-group	1	1	0.055	1.000	0.113	1.000
Psyllidae	*Diaphorina* cf. *acokantherae*	1	1	0.055	1.000	0.2	1.000

(*) reflects the statistical significance between the plant species sampled and the collection methods used.

Table 2. 3 (Continued): The response of each psyllid species to the plant species sampled and collection methods used during the field surveys in different commercial citrus environments of Limpopo and Mpumalanga provinces.

Family	Morphospecies	Abundance	No. of plant species associated	Plant species		Collection methods	
				x^2	*P-value*	x^2	*P-value*
Psyllidae	*Diaphorina* cf. *bicolor*	2	1	0.06	1.000	0.122	1.000
Psyllidae	*Diaphorina* cf. *loranthi*	29	4	3.056	0.386	1.637	0.722
Psyllidae	*Diaphorina* cf. *petteyi*	13	1	0.072	1.000	0.087	1.000
Psyllidae	*Diaphorina fabulosa*	1	1	0.055	1.000	0.2	1.000
Psyllidae	*Diaphorina loranthi*	1	1	0.055	1.000	0.2	1.000
Psyllidae	*Diaphorina tenebrosa*	3	1	0.062	1.000	0.127	1.000
Psyllidae	*Diaphorina punctulata*	466	4	7.315	**0.007***	3.123	0.126
Psyllidae	*Diaphorina virgata*	371	3	4.549	**0.022***	1.75	0.662
Psyllidae	*Diaphorina zebrana*	42	1	0.08	1.000	1.224	0.961
Psyllidae	*Diaphorina* sp.1	15	1	3.507	0.152	0.131	1.000
Psyllidae	*Diaphorina* sp.2	41	2	0.073	1.000	0.148	1.000
Psyllidae	*Euryconus* sp.	358	3	6.365	**0.010***	4.3	**0.012***
Psyllidae	*Epiacizzia* sp.	2	1	0.06	1.000	0.214	1.000
Psyllidae	*Parapsylla capensis*	1	1	0.055	1.000	0.2	1.000
Psyllidae	*Palaeolindbergiella* sp.	25	2	0.434	1.000	1.863	0.571
Psyllidae	*Prunus psyllid*	60	1	0.082	1.000	0.166	1.000
Psyllidae	*Psylla loranthi*-group	36	1	0.079	1.000	0.598	1.000

(*) reflects the statistical significance between the plant species sampled and the collection methods used.

Table 2. 3 (Continued): The response of each psyllid species to the plant species sampled and collection methods used during the field surveys in different commercial citrus environments of Limpopo and Mpumalanga provinces.

Family	Morphospecies	Abundance	No. of plant species associated	Plant species		Collection methods	
				x^2	*P-value*	x^2	*P-value*
Psyllidae	*Psylla* cf. *loranthi*-group	7	1	0.068	1.000	0.138	1.000
Psyllidae	*Psyllidae* sp.	16	2	3.468	0.152	0.143	1.000
Psyllidae	*Retroacizzia mopanei*	263	2	4.416	**0.035***	0.316	1.000
Triozidae	*Baeoalitriozus afrobsoletus*	30	2	1.36	0.980	2.112	1.465
Triozidae	*Pauropsylla trichaeta*	1680	4	7.572	**0.007***	9.452	**0.001***
Triozidae	*Trioza carvalhoi*	1	1	0.055	1.000	0.113	1.000
Triozidae	*Trioza erytreae*	326	1	0.095	1.000	2.008	0.528
Triozidae	*Trioza* cf. *erytreae*	3	1	0.062	1.000	0.127	1.000
Triozidae	*Trioza* cf. *xylopia*	2	1	0.06	1.000	0.122	1.000
Triozidae	*Trioza* sp.1	2	1	0.06	1.000	0.122	1.000
Triozidae	*Trioza* sp.2	1	1	0.055	1.000	0.133	1.000
Triozidae	*Trioza* sp.3	2	1	0.344	1.000	0	1.000
Unknown	*Psylloid* sp.1	7	1	0.068	1.000	0.138	1.000

(*) reflects the statistical significance between the plant species sampled and the collection methods used.

2.3.3. Estimating the sampling efforts of collection methods (trapping versus sweep-netting) for psylloid species in commercial citrus environments.

Species accumulation curves (SACs) showed significant variability in their shapes across all the graphs, both the bottom (representing the trapping method) and top graphs (representing the sweep-netting method) (Figure 2. 7). Specifically, the SACs represented by the bottom graphs, which correspond to the trapping method, tended to become more stable or reached an asymptotic state (plateau). This observation strongly suggests that our sampling efforts were effective and sufficient, as evidenced by the data in Figure 2. 7 (bottom graphs). These results provide a strong confirmation that the trapping method had effectively captured an adequate number of individual species within citrus environments (i.e. citrus orchards and their surrounding natural vegetation). Therefore, further sampling efforts using this method are anticipated to yield only a few increases in the count of previously undiscovered species within these specific environments. However, the SACs illustrated in the top graphs, representing the sweep-netting method, showed a continuation rise, forming an almost straight-line pattern irrespective of the number of field surveys conducted to sample psylloid species within the selected commercial citrus environments (Figure 2. 7, top graphs). Consequently, the SACs in these top graphs did not reach an asymptote, thus, indicating an ongoing accumulation. The persistent absence of plateauing or an asymptotic state across all the growth line-curves in the top graphs strongly indicates that the conducted surveys were not adequate to capture all psylloid species present in the surveyed commercial citrus environments (Figure 2. 7, top graphs). Therefore, this observation suggests that further or additional sampling efforts using the sweep-netting method could potentially lead to the discovery of more or a greater number of psylloid species within these specific environments.

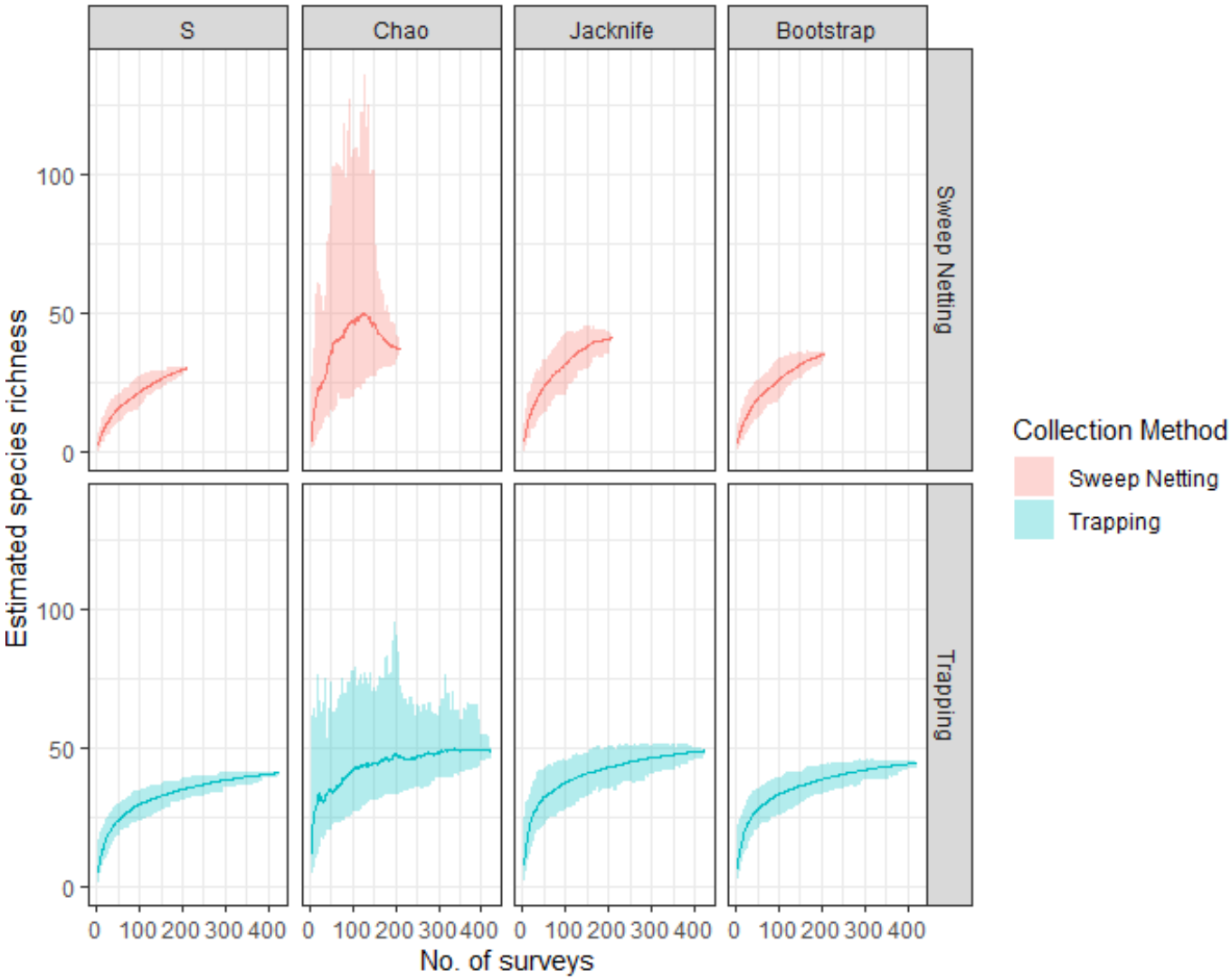

Figure 2. 7: The graphs represent the results of the species accumulation curves (SACs) analysis, comparing the species richness between two collection methods used for sampling psylloid species in the selected commercial citrus environments: Sweep-netting method is shown on the top graphs, whereas the trapping method is presented on the bottom graphs.

2.4. Discussion

Different morphospecies of psylloids were collected in both citrus orchards and adjacent natural vegetation, referred to as citrus environments, across sites selected in Limpopo and Mpumalanga provinces. The observed establishment patterns within these environments corroborate with those presented by Watanasit & Nhu-Eard, 2011, who outlined the establishment of ant species not only

in homogeneous (monoculture) rubber plantations but also in heterogeneous (mixed vegetation) rubber plantations. A similar trend was observed by Kasseney *et al.* (2019) in the botanical garden of the University of Lomé, where they documented the establishment of ant and termite fauna in different habitat types, including both open grassland areas and those covered with mixed exotic and native plants. These studies collectively suggest that the adaptability of insects across diverse habitat types is generally attributed to their ability to exploit a broad range of plant species within a particular environment. However, the current study presents a different scenario for psylloids, as none of the species collected 0in citrus orchards, except for *Trioza erytreae*, have been previously reported to be associated with citrus. This raises the possibility that the presence of these psylloids in the citrus orchards could be influenced by factors other than the host-plant availability, possibly such as favourable climatic conditions that are suitable for their survival and activity, particularly during the day. Supporting this hypothesis, previous studies by Afreh-Nuamah (1985, 2007) have shown that citrus orchards provide a habitat capable of supporting a diverse entomofauna complex, including saprophagous, phytophagous, pollinators, dispersers and decomposers, which depend on different or several environmental factors for their daily activities (Aidoo *et al.*, 2014).

Despite the ecological differences between the citrus orchards and adjacent natural vegetation, this study documented a total of 47 psylloid morphospecies across these environments. This diversity complements the recent findings of Mazzardo *et al.* (2017), who documented 34 psylloid species in wooded seasonal vegetation, correlating this diversity to the diversity of plant species present. Similarly, Percy (2011) found that psylloid diversity on Macaronesian Islands was significantly influenced by the diversity of associated host-plants. Studies conducted by Lin *et al.* (2015) and Naman & Abdullah (2019) further corroborate our current findings. Therefore, the higher diversity of psylloid species documented in the surveyed citrus environments in this study can be primarily attributed to the abundance and diversity of plant resources available, which provide both essential food and oviposition sites that support the overall life cycles and behaviours of psylloid species.

The abundance and diversity of psylloids collected using yellow sticky traps were higher compared to those collected using insect sweep-net. Using multiple collection methods is a common practice to enhance arthropod sampling efficiency (Minor *et al.*, 2021; Majeed *et al.*, 2022; Mukwevho *et al.*, 2023, 2024). Nicholaus *et al.* (2016), for example, used a combination of pitfall traps, sweep-nets, beating sheets and hand collection to assess the community structure and diversity of beetles

in the grassland and wooded habitats in Mbeya, Tanzania. They found that pitfall traps were most effective in capturing both the highest number of individuals and the greatest diversity of beetle species. Therefore, this emphasises the utility of using multiple methods for thorough biodiversity studies. Similar trends have been reported in ant studies, where pitfall traps, monoliths and baits were compared, with the former method often yielding higher capture rates across diverse habitats (Kasseney *et al.*, 2019; Hacala *et al.*, 2021). Adams *et al.* (2017) found that sweep-nets were more effective than insect vacuums for sampling arthropods in both oiled and non-oiled salt marshes in southern Louisiana, sampling more individuals and a greater diversity of arthropod taxa. However, the findings of the current study diverge from those of Adams *et al.* (2017), as yellow sticky traps proved to be more effective compared to sweep-net in capturing psylloids. According to Yi *et al.*, (2012), the choice of sampling method often relies on the active behaviour of the targeted insect group. In agricultural ecosystems, psylloids, similar to other Hemipteran insects such as whiteflies and leafhoppers, are effectively monitored using yellow sticky traps due to their attraction to the sticky surface (Berlinger, 1980; Gerling & Horowitz, 1984; Aldini *et al.*, 2003; Döring & Chittka, 2007; Matsukura *et al.*, 2011; Sétamou *et al.*, 2014, 2019; Miranda *et al.*, 2017). Therefore, the success of yellow sticky traps in this study can be primarily attributed to their ability to attract and capture psylloids, making them particularly suitable for monitoring in this context.

When determining the habitat-specific differences, the abundance of psylloids collected using only sweep-net was higher in the adjacent natural vegetation compared to citrus orchards, whereas the species richness was greater in the citrus orchards. The variations in arthropod abundance, species richness and composition between agroecosystems and natural undisturbed vegetation are well-documented (Sanabria *et al.*, 2014; Aidoo *et al.*, 2016). The differences observed in our study may result from the influence of the habitat type and vegetation structure, which are well-documented factors impacting arthropod populations (Gardner *et al.*, 1995; Landsman & Thiel, 2021; Minor *et al.*, 2021). For example, the higher psylloid abundance in natural vegetation may be attributed to the more complex and diverse habitat structure, which provides a variety of niches and resources that support larger populations. However, the increased species richness in citrus orchards suggests that these managed environments might offer a wider range of microhabitats or plant resources suitable for a greater diversity of psylloid species. Our findings are further supported by Chiawo *et al.* (2017), who studied bee diversity and floral resources across diverse habitats and observed an increased bee abundance in farmlands with abundant floral resources compared to forested

habitats, emphasising the significant role of resource availability in influencing insect abundance or populations. Similar patterns have been observed in studies on fruit flies by Bhusal *et al.* (2020) and Karki *et al.* (2023), who found that differences in insect abundance across locations were largely driven by the availability of food sources. These parallels suggest that the availability of resources, whether floral or otherwise, is a significant factor influencing insect abundance across different habitats. Ojija (2016) also assessed the abundance, diversity and species richness of some arthropods in grassland and woodland habitats, and found significant differences in the arthropod abundance between grassland and woodland habitats, attributing these differences to variations in food resource availability.

In contrast, all citrus orchards in our study were sometimes treated with insecticides targeting citrus pests, resulting in fewer encounters with psylloids. This decline in their abundance was particularly evident in orchards treated with broad-spectrum insecticides a few days before sampling. Several studies have consistently shown that the application of pesticides in agroecosystems leads to a significant decline in the abundance and diversity of arthropod communities, such as both targeted pests and non-targeted species (Desneux *et al.*, 2007; Dutcher, 2007; Pisa *et al.*, 2014; Simon-Delso *et al.*, 2015). These chemicals, including insecticides, herbicides and fungicides, act directly on insects, either killing them upon contact or ingestion, thereby diminishing their population sizes (Krauss *et al.*, 2011; Michalko & Pekár, 2017; Jacobsen *et al.*, 2019). Therefore, the application of insecticides in citrus orchards in the current study likely contributed to the significant decline in psylloid abundance. This aligns with the findings of Park *et al.* (2015), who studied the impact of pesticides on wild bee communities visiting apple orchards and observed a linear decline in the abundance and species richness of wild bees with increasing pesticide use. A similar pattern was recently reported by Paudel & Tiwari (2022), who investigated insect diversity across a multitude of habitats (i.e. mango orchards, litchi orchards, vegetable fields, organic fields and uncultivated land) using pitfall traps for collection, and found a significantly lower abundance in the cultivated fields subjected to frequent insecticide applications, thus, emphasising the impact of insecticide use on arthropod populations. Similarly, previous research by Thomson & Hoffmann (2006) in Australian citrus orchards indicated that pesticide-treated orchards had lower arthropod species richness than untreated ones. Their study also noted that untreated orchards had higher populations of beneficial predators like lacewings and lady beetles, which naturally reduced pest populations. However, in the current study, psylloids were not found in insecticide or pesticide-treated citrus

orchards. Fernández *et al*. (2005) also noted a significant decline in the diversity and abundance of predatory mites in pesticide-treated citrus orchards in Spain.

Despite the similar species richness and composition across Limpopo and Mpumalanga provinces, significant differences were observed in the abundance of psylloids between the two regions. Both provinces are characterised by rich natural vegetation and extensive agricultural activities (Mucina & Rutherford, 2006). It has been observed in an ecological study that regions with abundant food resources generally support larger and more diverse populations of organisms (Hanya & Chapman, 2013), as the availability of such resources is crucial for the survival and reproduction of species. Therefore, the combination of rich natural vegetation and agricultural activities in both provinces creates an environment with plentiful food resources, supporting higher populations of herbivorous insects and their predators. However, despite these similarities in their ecological characteristics, a consistently higher abundance of psylloids was recorded in the citrus environments of Limpopo compared to Mpumalanga. This difference may be attributed primarily to the number of field sites surveyed within each province. Specifically, eight diverse citrus environments were surveyed in Limpopo, whereas only four were surveyed in Mpumalanga. Each surveyed field site in Limpopo hosted a diverse range of plant species, which is significant as psylloids often exhibit associations with specific plant species in ecosystems (Hodkinson, 2009; Burckhardt *et al.*, 2014). Therefore, the increased number of field sites surveyed in Limpopo might have allowed for sampling a wider variety of plants, thereby resulting in a higher recorded psylloid abundance compared to relatively limited sampling plants in Mpumalanga province, which led to a lower psylloid count. However, suppose the number of sites selected for surveys was equal between Limpopo and Mpumalanga; in that case, we believe that high psylloid abundance would have also been recorded in the latter province as most plants would have also been sampled. In addition, the adjacent natural vegetation in Limpopo harboured a greater diversity of host-plants (with six recorded) compared to those in Mpumalanga (five host-plants recorded). This richer diversity in host-plants could have provided additional resources and habitats for psylloids, thus, contributing to their higher abundance in the citrus environments in this province. The increased availability of host-plants in the surrounding vegetation potentially offered more feeding and breeding opportunities for these insects, further enhancing their population in Limpopo province.

Some psylloid genera that include global pests of diverse plant species have been documented with varying impacts across agricultural and forest ecosystems, such as *Cacopsylla*, *Diaphorina* (both from Psyllidae), *Glycaspis* (Aphalaridae) and *Trioza* (Triozidae) (Halbert & Manjunath, 2004; Steinbauer *et al.*, 2015; Cho *et al.*, 2017; Jarausch *et al.*, 2019; Morrow *et al.*, 2020). Psyllid pests like those in the *Cacopsylla*, for example, vector and transmit the *Candidatus* Phytoplasma species, which leads to severe diseases and significant declines in orchards of apple, pear and stone fruits (Cho *et al.*, 2017; Bertaccini *et al.*, 2019b; Morrow *et al.*, 2020). In the context of citrus, species such as *Diaphorina citri* and *Trioza erytreae* respectively from the *Diaphorina* and *Trioza* genera, remain significant concerns for the citrus industry globally, as they act as vectors for *Candidatus* Liberibacter species, the bacteria that cause citrus greening disease (Bové, 2006; Rwomushana *et al.*, 2017). Furthermore, some *Trioza* species are economically significant vectors of *Candidatus* Liberibacter species, causing Zebra Chip disease in Solanaceous crops globally (Munyaneza *et al.*, 2010b; Antolinez *et al.*, 2017).

In contrast, while *Glycaspis* is not economically significant in agriculture, it poses a threat to forest ecosystems, particularly eucalyptus species globally (Brennan *et al.*, 1999; Steinbauer *et al.*, 2015). During field surveys in citrus environments, the only psyllid species observed from this genus was *Glycaspis brimblecombei*, commonly known as the red gum lerp psyllid, which primarily infests eucalyptus plant species like *E. camaldulensis* Dehnh and *E. tereticornis* Smith (Brennan *et al.*, 2001; de Queiroz *et al.*, 2013; Ben Attia & Rapisarda, 2014; Lucia *et al.*, 2016; Bush *et al.*, 2020). In a previous study by Frasconi *et al.* (2013), it was found that infestations by this psyllid increase the susceptibility of hosts to other pests and diseases, potentially leading to the death of susceptible varieties. However, during field surveys, we did not encounter any eucalyptus plant species in the adjacent natural vegetation. Therefore, the consistent absence of eucalyptus plants in this habitat across all the study sites, suggests that other plant species nearby might have harboured this psyllid. This further implies that *Glycaspis brimblecombei* might have migrated (possibly by wind-assisted migration) from those plants in the vicinity to citrus orchards. This hypothesis could also apply to other psylloid species that were collected in citrus orchards, where we could not link most of these psylloid species with their primary hosts, as confirmed by the absence of immatures, following the concept of "psylloid host-plant" as defined by Burckhardt *et al.* (2021).

The community structure of arthropods, including insects, spiders and mites, in agroecosystems is influenced or often shaped by a multitude of ecological and environmental factors. These factors include the type and diversity of natural vegetation surrounding the agricultural areas, agricultural practices and land use patterns, population levels of insects and their natural enemies, and climatic conditions, among others (Stone, 1993; Branquart & Hemptinne, 2000; Potts *et al.*, 2003; Luke *et al.*, 2014). For example, Janda *et al.* (2019) assessed the diversity and community structure of ant species across five different habitats and discovered that annual precipitation and minimum annual temperature significantly influenced the diversity and composition of ant communities, especially in semi-desert habitats. A similar pattern was also observed in other ant studies, where habitat type and vegetation structure strongly influenced community composition (Costa-Milanez *et al.*, 2014; Luke *et al.*, 2014; Sanabria *et al.*, 2014). In the current study, the community structure of psylloids was significantly influenced by the plant species available, thereby affecting the abundance and distribution of other psyllid species in the surveyed citrus environments. For example, *Diaphorina punctulata* and *Diaphorina virgata*, were sampled across different plant species, including citrus, *Ficus* (Fig) and marula; however, their significant abundance was sampled on marula compared to other plant species. Furthermore, the consistent presence of these *Diaphorina* species within the surveyed citrus environments across all study regions correlated with the prevalence of the marula, therefore, indicating that marula is the main host-plant for these psyllid species. This host-plant confirmation was further supported by the presence of immatures collected from the same plant species as the adults. These findings are supported by the previous research by Capener (1970a, 1970b) who also identified marula as a host-plant for both *Diaphorina punctulata* and *Diaphorina virgata*.

Globally, psyllid species in the genus of *Acizzia* are associated with *Vachellia* spp., and to a lesser extent, some *Albizia* species (see Webb & Moran, 1974; Yen, 2002; Taylor & Moir, 2009). In this study, *Acizzia russellae*-group was sampled from three different plant species, including *Vachellia* spp., citrus and mopane. However, the abundance was significantly higher when collected only on *Vachellia* spp., and its distribution in the surveyed environments further correlated primarily with the establishment of this plant species. Furthermore, the immatures of this psyllid were observed and collected from the same plant species as adults, thus confirming *Vachellia* spp. as a host. These findings are consistent with those from Powell *et al.* (2012), who also reported a high abundance of other *Acizzia* species associated with specific *Vachellia* spp. However, our study provides more

detailed and accurate information concerning the specific host-plant of *Acizzia russellae*-group by including the immatures. Webb & Moran (1974) previously discovered immatures and adults of *Acizzia russellae* on *Vachellia karroo* Hayne in southern Africa. Although our study could not classify *Vachellia* spp. to species level rather to the genus level, the presence of immatures on this sampled plant species suggests the possibility of this plant being *Vachellia karroo*. Future studies should consider botanical classification materials to enhance accuracy in identifying specific host-plants at the species level.

A previous study by Oppong *et al.* (2010, cited Ernst & Sekhwela, 1987) documented the mopane plants as the primary hosts for several endemic insect species, of which *Retroacizzia mopanei* is among them. Our current study aligns with these findings, as immatures of *Retroacizzia mopanei* were collected on mopane plants in the adjacent during the surveys, thus, indicating an association with this specific host. Furthermore, during the field surveys in citrus environments, the adults of *Retroacizzia mopanei* were collected on mopane and some citrus plants. Despite occurring on two distinct plant species, the study has found that the abundance of *Retroacizzia mopanei* on mopane plants was significantly higher compared to citrus, and its distribution was further correlated with the presence of the mopane plants. According to Mashabane *et al.* (2001) and Fakazi *et al.* (2021), mopane is a valuable indigenous plant in southern Africa that serves different purposes in rural communities, including hosting the larvae of the mopane worm (*Imbrasia belina*), contributing to local food security and commercial activities. Despite the association with the mopane, the current study identified *Retroacizzia mopanei* as a pest due to its feeding activity which caused damage to the mopane plant leaves. The observed specific damage attributed to *Retroacizzia mopanei* on the surveyed mopane plants includes the construction of lerps that covered large areas of the plant leaf surfaces. The lerp formation by *Retroacizzia mopanei* on leaves, as documented by Oppong *et al.* (2010), affects the photosynthetic rates, possibly impacting food security in these communities. Furthermore, Oppong *et al.* (2010) documented that lerps not only significantly impact plant health but further attract other organisms, including birds, baboons and humans that utilise them as a food supplement. However, removing lerps by these organisms can cause secondary damage to mopane leaves during the process (Oppong *et al.*, 2010).

The consistent dominance of some psylloid species in the surveyed environments was primarily attributed to the establishment of specific plant species, which were also confirmed as their host-

plants (as previously discussed), supporting their populations in these environments. However, the effectiveness of the collection methods used, particularly yellow sticky traps and sweep-net, also played a significant role in assessing the abundance and diversity of other psyllid species across these environments. Several studies have documented that yellow sticky traps are widely employed across different fields, including agriculture and forestry, for monitoring hemipteran insects such as aphids, leafhoppers, psylloids and whiteflies, which are known as pests of different crops and vectors of severe plant-pathogens. The literature consistently emphasised that yellow sticky traps are exceptionally effective, often surpassing alternative collection methods in capturing the species richness and abundance (Berlinger, 1980; Gerling & Horowitz, 1984; Krysan & Horton, 1991; Aldini *et al.*, 2003; Matsukura *et al.*, 2011; Sétamou *et al.*, 2014; Bodino *et al.*, 2020; Czarnobai De Jorge *et al.*, 2023). The findings of this study corroborate the existing literature, emphasising the effectiveness of yellow sticky traps over the alternative sweep-netting method in monitoring the occurrence and abundance of hemipteran insects in the agricultural sector, particularly in citrus orchards. When comparing these two methods, yellow sticky traps consistently captured higher abundances of psyllids compared to sweep-netting, particularly for species such as *Acizzia* sp.3 and *Cacopsylla* sp., which showed a 53-fold and six-fold increase in abundance, respectively. A similar trend was further observed for *Euryconus* sp. and *Pauropsylla trichaeta*, where yellow sticky traps yielded a four-fold and three-fold greater abundance, respectively. These findings are consistent with the previous work of Hall & Hentz (2010), who, in their study on the abundance of adult *Diaphorina citri* in citrus orchards, also discovered that yellow sticky traps captured a greater abundance of adult *Diaphorina citri* compared to an alternative stem-tap sampling method. Although our study differed from Hall & Hentz (2010) in methodology by employing a sweep-net (conducted in both citrus orchards and the adjacent natural vegetation) rather than the stem-tapping method, which was confined primarily to citrus orchards in their study, the consistent effectiveness of sticky traps in capturing a significantly higher abundance of some species of psylliods remained evident across the sampling period in the surveyed citrus environments.

The edges of habitats, particularly the first two rows of crops which are commonly defined as edge effects, often support or harbour a higher abundance of insects migrating from surrounding natural vegetation compared to the interior of these habitats, particularly within the agricultural landscapes (Olson & Andow, 2008; Sétamou & Bartels, 2015; Mola *et al.*, 2021). This edge effect is well-documented, with studies such as Tscharntke *et al.* (2005) and Romero *et al.* (2008) discovering

that field edges and interiors support different insect communities, with edges frequently hosting greater species abundance, diversity and ecosystem services. In our study, among the fundamental factors that might have contributed to the effectiveness of yellow sticky traps in capturing a higher abundance of psylloid species in citrus orchards could be attributed to their strategic placement. Specifically, these sticky traps were consistently deployed on citrus trees along the orchard edges, particularly focusing only on trees in the second row adjacent to indigenous vegetation surrounding these orchards. This trapping approach is consistent with the findings of Boina *et al.* (2009), who reported higher captures of adult *Diaphorina citri* on yellow sticky traps positioned along the orchard block edges adjacent to the fallow ground compared to those deployed in the interior area.

Our results also support the work of Sétamou & Bartels (2015), who monitored *D. citri* populations across different orchard sites (i.e. edge, adjacent and interior) every two weeks by using lime-green sticky traps and visual observations. Despite methodological differences (i.e. sampling methods and study sites), Sétamou & Bartels (2015) consistently found higher psyllid densities and flush-shoot infestation levels on edge trees and a gradual decline towards the interior trees in both mature grapefruit and sweet orange orchards across their study. Interestingly, our findings contrast with those of Krauss *et al.* (2011), who found higher cereal aphid abundances at field edges compared to centers in both organic and conventional fields using sweep-nets rather than sticky traps. Despite using both yellow sticky traps and sweep-net sampling along the citrus orchard edges in our study, the former consistently outperformed the latter in capturing psylloids. This suggests that although sweep-nets are useful for some insects, yellow sticky traps may offer a more reliable method for monitoring psylloid populations in citrus orchards. However, this may also suggest that different sampling methods may yield varying results depending on the target insect species.

The effectiveness of yellow sticky traps is, however, not limited to psylloids, as evidenced by other studies on diverse insect populations. In a recent study by Dimitrova *et al.* (2020), for example, the authors assessed canopy arthropod diversity in an olive orchard by comparing the effectiveness of yellow and transparent sticky traps. Their findings revealed that although the diversity between the two methods was similar, the total abundance of arthropods captured on yellow sticky traps was significantly higher in comparison to that of an alternative sampling method. Furthermore, our observations are supported by another recent study by Dongiovanni *et al.* (2023) conducted in the region of Apulia, southern Italy, which focused on assessing the effectiveness of yellow sticky

traps against sweep-netting for monitoring the spittlebug species, such as *Philaenus spumarius* (Linnaeus) and *Neophilaenus campestris* (Fallen) (Hemiptera: Aphrophoridae), which vector a xylem-inhabiting, Gram-negative bacterium, *Xylella fastidiosa* Wells et al. (Xanthomonadales: Xanthomonadaceae), in olive orchards and other primary agricultural crops, such as cherry and almond, currently threatened by this bacterium. Their findings consistently confirmed that sticky traps captured a significantly higher abundance of spittlebug species compared to sweep-netting. These consistent findings across diverse studies further support the reliability and efficiency of yellow sticky traps as a preferred sampling method for monitoring insects, specifically pests, in agricultural environments. However, while the current study revealed the effectiveness of yellow sticky traps in capturing a significantly higher abundance of specific psyllid species, it is important to acknowledge that the sweep-netting method, which, even though less effective for some psylloid species, remains valuable, particularly in the context of collecting the abundance of other psylloids, therefore, indicating the importance of considering multiple collection methods for understanding the insect abundance in agricultural environments.

The psylloid abundance, species richness and community structure recorded in this study showed the effectiveness of employing a combination of different collection/sampling methods (i.e. yellow sticky traps and insect sweep-net) in accurately sampling species richness and relative abundance of psylloids in citrus environments. This observation strongly complements the findings of existing research, which, although focused on different insects, including ants, bees, butterflies and other arthropods, consistently showed the effectiveness of employing different sampling methods for accurately assessing their diversity and community structure established in a given environment (Watanasit & Nhu-Eard, 2011; Kasseney *et al.*, 2019; Naman & Abdullah, 2019; Dimitrova *et al.*, 2020; Hacala *et al.*, 2021). Despite the overall effectiveness of these combined sampling methods in revealing a higher abundance and diversity of psylloids, the analysis of species accumulation curves (SACs) showed an inadequate sampling effort in capturing the full extent of psylloid species present across all the surveyed citrus environments. Specifically, the sweep-netting graphs showed a continuous rise in growth line-curves lacking the characteristic asymptotic or plateauing lines that determine the efficient sampling efforts in a particular habitat, suggesting the possibility of an undiscovered psylloid species. This persistent absence of the plateauing lines across all the sweep-netting graphs strongly recommends that additional sampling efforts, using only the sweep-netting method, should be conducted again as this could lead to the discovery of more and new

psylloid species in addition to what has already been collected sampled across all the surveyed citrus environments. In contrast, the trapping method, displayed asymptotic states across all the growth line-curves, confirming the effectiveness of this method in capturing a sufficient number of psylloid species throughout all the surveyed citrus environments, particularly citrus orchards. Therefore, continuous sampling efforts using the trapping method may not be necessary, as it may yield previously recorded psylloid species or only a few additional psylloid species that might have been missed during the previous field surveys. Therefore, these findings confirm that, with the trapping method alone, many psylloid species were adequately sampled in the citrus environments. However, due to the combined use of trapping and sweep-netting methods, the overall sampling adequacy for psylloid species present in the selected citrus environments is called into question.

Although an adequate number of psylloid species present across all surveyed citrus environments could not sampled through the combined use of trapping and sweep-netting methods, the analysis of species richness revealed that both methods individually demonstrated efficiency in sampling the psylloid diversity and community structure, but with significant variations in their efficiency. The sweep-netting method, for example, recorded an estimated number of around 20 species of psylloids after the initial field surveys, eventually increasing to 30 after 200 surveys, as evidenced in the top graphs. However, the trapping method also captured a similar initial count, but with the number continuously increasing after each set of 100 surveys, eventually peaking at approximately 40 psylloid species after 400 surveys (see S estimator in Figure 2. 7, both top and bottom graphs). These variations in the sampling efficiency of both methods were evident across multiple species richness estimators, such as the Chao estimator, Jacknife estimator and Bootstrap estimator (Figure 2. 7, both top and bottom graphs). The analysis of species richness using Chao estimators indicated that, after 100 surveys, approximately 48 psylloid species were captured using the sweep-netting method. In contrast, the trapping method estimated approximately 45 psylloid species (Figure 2. 7, both top and bottom graphs). Jacknife estimators, however, suggested approximately 30 psylloid species were recorded after 100 surveys using the sweep-netting method, and around 40 psylloid species were captured using the trapping method after the same number of surveys. Furthermore, the Bootstrap estimators showed that, after 100 surveys, approximately 26 and 35 psylloid species were recorded using the sweep-netting and trapping methods, respectively. However, beyond the initial surveys, the S, Chao, Jacknife and Bootstrap estimators in the top graphs became unstable,

indicating a continuous rise in recorded species. However, the bottom graphs showed evidence of levelling-off (reaching an asymptote) between 300 and 400 surveys in all estimators.

The results of this study revealed that although both trapping and sweep-netting methods were individually effective in sampling psylloid species within citrus environments, the trapping method consistently outperformed sweep-netting method, particularly in citrus orchards, as evidenced by the higher species richness recorded (as discussed). The higher species richness recorded using the trapping method can likely be attributed to the prolonged exposure of yellow sticky traps in citrus orchards, as these traps were left in the orchards for a month during the sampling period, as detailed in the methodology section. This extended exposure time likely increased the chances of sampling more psylloid populations over time, capturing variations in species presence that might have been missed with the more sporadic or transient sweep-netting method. These findings complement recent research by Thompson *et al.* (2021), where they observed a higher abundance of wild bee species when using sticky traps. However, there is a significant difference, particularly in the methodology part, as yellow sticky traps in the current study were deployed for a month in citrus orchards. In contrast, Thompson *et al.* (2021) deployed the traps for only two-week intervals. The effectiveness of yellow sticky traps in this current study can be further explained by the visual cues they provide, which are critical for many phytophagous insects during orientation and host-plant selection. Studies have documented that visual traps that mimic the visual characteristics of host-plants are highly effective in monitoring insect pest populations on cultivated crops (Prokopy & Owens, 1978; Sétamou *et al.*, 2014; Czarnobai De Jorge *et al.*, 2023). Among hemipteran insects, such as aphids, whiteflies and psylloids, there is a well-documented attraction to yellow and green visual targets, with psylloids showing a strong preference for yellow visual targets, particularly within the 500 to 600 nanometers (nm) wavelength range (Döring & Chittka, 2007; Döring, 2014; Miranda *et al.*, 2017; Sétamou *et al.*, 2019). Although this study did not directly investigate visual preferences, it is plausible that the higher psylloid abundance collected using yellow sticky traps, as compared to sweep nets, can be attributed to their innate visual attraction to the colour yellow. This visual preference likely enhances the effectiveness of yellow sticky traps in capturing psylloid species in citrus orchards.

In contrast, the sweep-netting method was less effective in sampling a high abundance of psylloid species. One possible constraint that might have contributed to this sampling inefficiency could be

attributed to the specific timing of sampling. This observation is consistent with that from the study by Hacala *et al.* (2021, cited Tavares *et al.*, 2008), who reported the significant impact of sampling time on the efficiency of the bait-trapping method. In their study, focusing on ant species, the bait-trapping method was conducted only in the middle of the day and during sunny weather conditions, while the pitfall traps remained active for 12 weeks. As such, the pitfall traps collected a significant abundance of ant species than the bait-trapping method. In their discussion section, they mentioned that some ant species being active at different times, including night, early day or late afternoon, could therefore have been missed by the bait-trapping method. In our study, unlike the continuous exposure of the trapping method, the sweep-netting method was only conducted once each month, specifically during trap replacement as detailed under the methodology section, and therefore, this might have resulted in missing some psyllid species that were not actively foraging on that specific day of trap replacement, affecting the overall sampling efficacy of this method. Furthermore, other studies reported that smaller insect individuals are difficult to spot and capture by an observer with a sweep-net (Cane et al., 2000; Roulston et al., 2007). Therefore, since psylloids are minute insects (1-10 mm) (de Queiroz *et al.*, 2012), this could also be another factor that affected the efficacy of this method; the same inline context was found by Thompson *et al.* (2021).

In addition, the sampling efficacy of the sweep-netting method was affected by challenges related to variable weather conditions, including rain, which hindered the active collection of insects, and windy conditions that hindered the effective sampling. During the rainy days, no active collection occurred because psylloids were not actively foraging but instead hiding, consequently, rendering the active collection not effective. The wind conditions adversely affected the performance of the sweep-netting method, as it would sway or blow the net away from the targeted site of a particular plant, and during the aspiration process, psylloids that were caught could easily escape the net due to wind assistance. These weather-related challenges collectively resulted in a few to no psylloids recorded in many instances. Apart from weather constraints, the height restrictions in certain plants were also observed to contribute to the limitation of the effectiveness of the sweep-netting method. For example, sampling, particularly on tall plants, proved challenging because the canopy of these plants could not be reached (hence some plants were not sampled), thus, resulting in fewer or no collection of psylloids. The sweep-netting method, conducted in both citrus orchards and adjacent natural vegetation, faced additional constraints. Specifically, fence restrictions limited access to

some adjacent natural vegetation in other citrus orchard farms, therefore, affecting the complete sampling of psylloid species using the sweep-netting method throughout the entire study.

In conclusion, this study makes a significant contribution to our understanding of the diversity and community structure of psylloid species in citrus environments, addressing a significant gap in the existing literature. Through extensive field surveys, we found a higher diversity of psylloid species across diverse citrus environments. Furthermore, this study revealed that psylloid distribution and abundance in citrus environments were strongly influenced by the presence of a diverse group of plant species, with other plants identified as hosts for some psylloid species, aligning with findings from the previous research. Although some of the documented psylloid species belong to genera known globally for harbouring pests of cultivated plants, none of the psylloids identified in our study, except for *Trioza erytreae*, is currently classified as a pest in the global agricultural industry. However, their presence in citrus environments, particularly orchards, raises concerns about their potential to feed on citrus and threaten the industry. This approach will help establish a foundation for confirming their pest status and further provide a fundamental understanding of effective pest management strategies. Despite the success of the combined sampling methods (i.e. yellow sticky traps and insect sweep-net) in revealing a diverse psylloid community, additional sampling efforts, particularly using sweep-net, are needed to discover more psylloid species in citrus environments. Our assessment, supported by different species richness estimators, showed that trapping methods consistently captured a significantly higher abundance of psylloids, confirming the reliability of trapping as a preferred method for monitoring psylloids in commercial citrus orchards. However, challenges, such as the weather conditions, sampling time and plant height restrictions, affected the efficiency of the sweep-netting method. Given these findings, future research or studies should take these challenges into account when selecting and evaluating sampling or collection methods for psylloids in citrus environments. Therefore, addressing these knowledge gaps and improving the sampling approaches will greatly strengthen our ability to monitor and manage the potential threats posed by psylloids to the citrus industry, thereby contributing to the long-term sustainability of citrus crop cultivation and protection.

CHAPTER 3

DO NATIVE AND INVASIVE PSYLLOID SPECIES FEED ON DIFFERENT VARIETIES OF CITRUS PLANTS IN CITRUS ORCHARDS? A MOLECULAR GUT CONTENT ANALYSIS

3.1. Introduction

Psylloids are among the phytophagous phloem-feeding insects capable of vectoring economically important plant-pathogens associated with *Candidatus* Liberibacter species that pose major threats to economically important agricultural plants including citrus (Hodkinson, 1984, 2009; Syfert *et al.*, 2017; Burckhardt *et al.*, 2021; Horton *et al.*, 2021; Zhao *et al.*, 2023). There are approximately 4,000 described psylloid species with narrow host-plant range across the globe (Hodkinson, 1974, 2009; Ouvrard *et al.*, 2015b; Percy *et al.*, 2018; Burckhardt *et al.*, 2021; Panizzi *et al.*, 2021). The direct feeding associated with psylloids threatens plant health and can lead to significant declines in crop production (Hodkinson, 1984, 2009; Burckhardt, 1994). However, the devastating damage is inflicted by those psylloid species vectoring plant-pathogens, particularly in agricultural, forest and natural ecosystems (Burckhardt, 1994; Hodkinson, 2009; Munyaneza *et al.*, 2010b; de Queiroz *et al.*, 2012; Moreno *et al.*, 2021). Knowledge of the aspects of biology, ecology and behaviour of psylloids constantly caught within citrus environments (see Chapter 2) is important in gaining an understanding concerning the role these species might play in Integrated Pest Management (IPM) in commercial citrus orchards surrounded by indigenous vegetation with several native host-plants that could be closely related to citrus.

Several psylloid species have been previously documented feeding on citrus and its relatives, such as *Diaphorina communis* Mather, *Mesohomotoma lutheri* (Enderlein), *Psylla citricola* Yang & Li, *Psylla citrisuga* Yang & Li, *Psylla murrayi* Mathur and *Trioza citroimpura* Yang & Li (Halbert & Manjunath, 2004; Cen *et al.*, 2012). However, none of these citrus-feeding psyllids are known to vector plant-pathogenic bacteria causing devastating disease in citrus (Cen *et al.*, 2012). Currently, two known psyllid species are associated with citrus globally and are significant vectors of a Gram-negative phloem-restricted bacteria *Candidatus* Liberbacter species. African citrus triozid, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae) and the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), are the only two psyllids recorded to feed on citrus (preferably

on flush-shoots) and can transmit the plant-pathogens responsible for significant economic loss in global citrus industry (Rwomushana *et al.*, 2017; Bin *et al.*, 2019; Aidoo *et al.*, 2021, 2023; Chapter 1). *Trioza erytreae* and *Diaphorina citri* feeding may result in significant plant damage, leading to a loss of plant vigour; however, the economically significant impact of these psyllids is attributed primarily to their ability to act as vectors of three main species of phloem-restricted bacteria in the genus *Candidatus* Liberibacter (Rhizobiaceae: Alphaproteobacteria): the *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter americanus (CLam) and *Candidatus* Liberibacter africanus (CLaf) (Halbert & Manjunath, 2004; Bové, 2006; Ruíz-Rivero *et al.*, 2021; Morán *et al.*, 2023).

Candidatus Liberibacter africanus and *Candidatus* Liberbacter asiaticus are the primary causative agents for what is commonly known as citrus greening (CG) disease across the globe (Bové, 2006; Duran-Vila & Bové, 2015; Cocuzza *et al.*, 2017). The citrus greening disease is often referred to as Huanglongbing (HLB) in most parts of the world and is primarily vectored by *D. citri*, and is widely distributed in citrus-growing regions of Asia and the Americas (Bové, 2006; Chapter 1). In other parts of the world, *T. erytreae* is the main natural vector of *Candidatus* Liberibacter africanus (CLaf) which is distributed across Africa and other cooler regions of Asia (Halbert & Manjunath, 2004; Carmo-Sousa *et al.*, 2020). However, a recent laboratory study by Reynaud *et al.* (2022) has found that in ideal conditions, *T. erytreae* can transmit both CLaf and CLas. The establishment of the greening disease in commercial citrus orchards causes significant losses in both the production and quality of citrus fruits, with this, attributed primarily to its rapid spread, incurable nature, and the challenges associated with preventing new infections and the quick development of symptoms (Timmer *et al.*, 2003; Bové, 2006; Zhang *et al.*, 2010).

Several recent studies have documented occurrences of psylloid species infesting plants that have previously not been recorded as their hosts, or in geographical regions where their primary host-plants or host families do not occur (Hodkinson, 2009; Burckhardt & Ouvrard, 2012; Burckhardt *et al.*, 2014; Cooper *et al.*, 2019). In Chapter 2, several psylloid species were recorded within both citrus orchards and the adjacent natural vegetation, collectively referred to as citrus environments. The presence and constant occurrence of these psylloid species in these environments might pose a challenge when establishing an IPM programme to manage vectors of citrus greening diseases. There is currently no existing literature on different indigenous psylloid species feeding habits and

potential transmission of disease-causing pathogens on citrus, except for *T. erytreae* and *D. citri.* In South Africa, there are approximately 27 described *Diaphorina* species. Of these species, two (i.e. *Diaphorina punctulata* Pettey and *Diaphorina zebrana* Capener) have been previously found feeding on citrus (Catling & Atkinson, 1974). These two species and other *Diaphorina* species (including some undescribed ones) have been regularly recorded in yellow sticky traps for early detection of ACP in commercial citrus orchards (see Chapter 2). The triozid species mainly found in citrus environments is *T. erytreae* (Hollis, 1984). Several rutaceous plants including citrus are known to host *T. erytreae* (van den Berg, 1990; Halbert & Manjunath, 2004), but the host range of the triozids is generally broader than that of other psylloids families (Hollis, 1984). This study aimed to analyse the gut contents of psylloids actively collected in different citrus environments between Limpopo and Mpumalanga provinces, to confirm their potential feeding on citrus or alternative plants adjacent to the orchards using DNA sequencing techniques.

3.2. Materials and Methods

3.2.1. Study sites and sample collections.

3.2.1.1. Insects:

Psylloid species collected in Chapter 2 using insect sweep-net were used for gut content analysis. In summary, psylloid species were collected across 12 different commercial citrus environments (i.e. citrus orchards and their adjacent natural vegetation), of which eight were situated in Limpopo and the remaining four in Mpumalanga. Host-plants were determined by the collection of different life stages of each psyllid, such as eggs, immatures and adult psyllid presence on the plants. Field surveys were conducted monthly, covering both the citrus orchards and the indigenous vegetation surrounding orchards. All collected specimens were preserved in 1.5 millilitres (mL) tubes (vials), each filled with 70 percent (%) of ethanol (v/v). These vials were then stored at room temperature (RT) in the laboratory to ensure the psylloid specimens remained in good and stable condition for the following Deoxyribonucleic acid (DNA) extraction.

3.2.1.2. Plant materials:

Fifteen mature citrus varieties/cultivars were chosen for leaf sampling, with 13 selected from Farm A, one from Farm B and one from Farm C (Table 3. 1). A single leaf sample was randomly selected in each selected tree and inspected for disease symptoms before collection (Table 3. 1). All citrus varieties/cultivars selected for leaf sampling aligned with those from which psylloid species were collected in citrus orchards.

Table 3. 1: Sampling locations and the selected citrus species used in this study for leaf sampling.

Farm	GPS coordinates	Citrus variety or cultivar sampled
Farm A	S 33° 29' 17.18" E 25° 40' 26.72"	2PH Lemon; Delta; Delta Valencia; Lemon; Eureka Lemon; Lemon SDL RL; Mandarin; Midknight Valencia orange; Navel; Orri; Palmer navel; Turkey; and Valencia swingle.
Farm B	S 33° 31' 24.86" E 25° 41' 13.27"	Nadorcott
Farm C	S 33° 26' 22.93" E 25° 41' 30.24"	Star Ruby

Leaf samples were collected by cutting them from the node using a Foska Scissor (Figure 3. 1). After each cut, a scissor was briefly immersed in a 5% bleach solution, followed by rinsing using tap water (H_2O), and then immediately dried using a paper towel (Figure 3. 1). Leaf samples were stored inside clean medium plastic Zip-lock bags (Figure 3. 1). Furthermore, the bags were tagged and labelled with the collection date and variety/cultivar name (Figure 3. 1). All leaf samples were transferred into a cooler box at room temperature and transported to the laboratory at the Centre for Biological Control, Department of Entomology and Zoology, Rhodes University in Makhanda, Eastern Cape Province, South Africa. All collected citrus leaf samples were removed from the Zip-lock bags and transferred into separate brown envelopes. Samples were dried in Silica Gel sourced from Spellbound Laboratory Solution (Port Elizabeth, EC, South Africa) and stored at room temperature until completely dried for DNA extraction.

Figure 3. 1: The process of leaf sampling from different mature citrus plants in commercial citrus orchards (**Photo credit**: David Taylor).

3.2.2. Genomic DNA extraction from the field-collected samples.

Genomic DNA (gDNA) from the collected plant and insect samples was separately extracted using two distinct extraction methods, as detailed below. Following the extraction, the concentration and quality of the extracted gDNA from both plants and insect samples were analysed in nanograms per microliter (ng/µL) and recorded in three replicates each time in a spectrophotometer, and their average was considered. Nanodrop™ Lite Plus Spectrophotometer (Thermo Scientific, USA) was used to assess the quality and concentration of insect and plant samples extracted gDNA.

3.2.2.1. Plant DNA extraction:

Genomic DNA was extracted from all the collected 15 citrus leaf samples using the PureLink™ Plant Total DNA Purification Kit (Invitrogen™, Thermo Fisher Scientific, USA) following the manufacturer's standard protocol, with only a few specific modifications introduced to optimize the process. To initiate the process, small leaf-square pieces measuring 2×2 centimetres (cm) were manually excised from the leaf margin, extending towards the midvein using an Iris scissor straight. The scissor was dipped into a 3% bleach solution, rinsed in tap water and then dried using

a paper towel after each leaf incision to avoid contamination of samples. Each small leaf-square piece, ranging from 20 to 45 milligrams (mg) in weight (measured using a weighing scale), was then transferred into separate 2 mL tubes. In each tube, 250 microliters (µL) of the Resuspension Buffer (R2) solution was added and leaf samples were manually hand-crushed using clean sterile micro-pestles to create a homogenate. Following these few modifications, the protocol outlined by the manufacturer was followed accordingly to proceed with the gDNA extraction from each of the small leaf-square pieces in their respective tubes. The extracted gDNA was eluted using 100 µL of Elution Buffer and the gDNA samples were then stored at -20 degrees Celsius (°C) until further analysis.

3.2.2.2. Insect DNA extraction:

Prior to performing gDNA extraction on the selected psylloid samples, initially, four distinct insect DNA extraction methods (Table 3. 2) were tested to determine the most effective approach. *Trioza erytreae* was used as a test sample, which was actively collected from a well-known citrus host-plant, Eureka Lemon. Before molecular analysis or to ensure the reliability of our results, psylloid samples preserved in 1.5 mL tubes containing 70% ethanol were subjected to a whole-body surface sterilization process. Initially, psylloid samples were air-dried on a paper towel for one minute at RT, followed by a thorough rinse with distilled water (dH_2O) and another round of air-drying for a minute at RT. This was implemented to ensure that any targeted plant DNA signal detected was from ingested material rather than any potential contaminants on the body surface of the insects (Cooper *et al.*, 2016, 2019; Diepenbrock *et al.*, 2018; Avanesyan & Lamp, 2020). Following the sterilisation process, five psylloid individuals of the same species, collected from the same plants, were transferred into a 2 mL tube and manually macerated by hand-crushing using a clean micro-pestle to avoid any possible contamination between the samples. The DNA extraction process on sterilised and macerated psylloid samples was then conducted according to the manufacturer's instructions, with specific modifications detailed below under each insect DNA extraction method to enhance efficacy. The visibility and brightness of the gDNA band determined the success of each insect DNA extraction method. The DNA extraction method that yielded the most successful extraction of insect gDNA was therefore selected and used for the subsequent DNA extraction of all remaining insect samples, ensuring consistency throughout the study. Psylloid species selected for gDNA extraction were chosen based on the selection criteria of being the "most common and

abundant". These species were then grouped into batches comprising either four or five species (hereafter referred to as samples), depending on the number of prevalent species collected in the orchards (Table S 3. 1 and Table S 3. 2) and per the protocol from the selected DNA extraction method. Five psyllid species selected for gut analysis were all collected from Eureka Lemon plants (Table S 3. 1), while the remaining groups of psyllid species were collected from different citrus plants (Table S 3. 2).

Table 3. 2: Four distinct insect DNA extraction methods were tested for their effectiveness to select the best method for subsequent analysis.

Insect DNA extraction method	Description	Manufacturer
1	Quick-DNA Tissue/Insect Miniprep Kit*	Zymo Research
2	Quick-DNA Miniprep Plus Kit*	Zymo Research
3	PureLink Genomic DNA Mini Kit*	Thermo Fisher Scientific
4	Salting-Out (TNES) **	Lab-based protocol

(*) represents a commercial kit; (**) represents the lab-based protocol.

Method 1:

750 µL volume of BashingBead^{TM} Buffer (supplied in the Kit) was added into the same tube as the sample, followed by further maceration of the sample to ensure complete lysis of the insects. The tube containing 750 µL of BashingBead^{TM} Buffer and the macerated insect samples was then subjected to vortexing at maximum speed for one minute to facilitate thorough mixing. After these few modifications, the manufacturer's protocol was adhered to, commencing from step 3 onwards, to proceed with the extraction of insect gDNA. Lastly, the insect gDNA was eluted with 35 µL of DNA elution Buffer (supplied in the Kit) and then stored at -20 ℃ until further analysis.

Method 2:

95 µL volume of double-distilled water (ddH_2O) was added into a 1.5 mL tube, followed by manual maceration through hand-crushing using a micro-pestle to break down the insects further. A 95 µL of Solid Tissue Buffer (Blue) and 10 µL of Proteinase K (both supplied in the Kit) were then added to the same tube. The crushed insects together with the added reagents, were mixed by vortexing

at maximum speed for 15 seconds. The resulting mixture was then subjected to incubation at 55 ℃ for 2 hours using a heating block. Post-incubation, the tube was centrifuged at 12,000 ×g (rcf) for one minute to remove insoluble debris. A 200 µL of aqueous supernatant (a mixture) was then transferred into a clean 1.5 mL microcentrifuge tube. A 400 µL volume of Genomic Binding Buffer (supplied in the Kit) was added to the 1.5 mL tube containing the transferred supernatant, followed by vortexing at maximum speed for 15 seconds to ensure thorough mixing. The mixture was then transferred to a Zymo-Spin™ IIC-XLR Column in a Collection Tube and centrifuged at 12,000 ×g (rcf) for one minute. The collection tube containing the flow-through was discarded. A 400 µL volume of DNA Pre-Wash Buffer was added into the spin column in a new Collection Tube, followed by centrifugation at 12,000 ×g (rcf) for one minute. The collection tube was emptied, and 700 µL of gDNA Wash Buffer was introduced to the spin column, followed by centrifugation at 12,000 ×g (rcf) for one minute. The flow-through was then discarded, and 200 µL of g-DNA Wash Buffer was added to the spin column, which was then centrifuged at 12,000 ×g (rcf) for one minute. The collection tube with the flow-through was discarded, and the spin column was then transferred into a new or clean 1.5 mL microcentrifuge tube. To elute the DNA, 60 µL of DNA Elution Buffer was added to the spin column matrix and incubated for 5 minutes at RT, followed by centrifugation at 12,000 ×g (rcf) for one minute. The eluted gDNA sample was then stored at -20 ℃.

Method 3:

180 µL volume of PureLink® Genomic Digestion Buffer, followed by 20 µL of Proteinase K (both supplied in the Kit) was added to the same 1.5 mL microcentrifuge tube with hand-crushed insect samples. The tube containing the mixture was then incubated at 55 ℃ for 2 hours using a heating block, with occasional vortexing (every 30 minutes for 5 seconds) to ensure thorough lysis. Post-incubation, the lysate was subjected to centrifugation at 12,000 ×g for 3 minutes at RT to eliminate any particulate matter, after which 190 µL of supernatant was transferred into a new sterile 1.5 mL microcentrifuge tube. A 20 µL volume of RNase A (supplied in the kit) was added to the lysate in the new tube, and mixed thoroughly through a brief vortexing at a maximum speed for 5 seconds, followed by a 2-minute incubation at RT. Post-incubation period, 200 µL of PureLink® Genomic Lysis/Binding Buffer (supplied in the Kit) was added to the lysate and then mixed thoroughly by vortexing for 5 seconds. This was followed by 200 µL of 100% ethanol being added to the lysate, which was then mixed thoroughly through vortexing for 5 seconds. A 520 µL of the lysate prepared

with PureLink® Genomic Lysis/Binding Buffer and ethanol was then added in a PureLink® Spin Column and centrifuged at 10,000 ×g for one minute at RT. The flow-through, along with the collection tube, was discarded, and the spin column was then transferred into a clean PureLink® Collection Tube (supplied in the Kit). This step was followed by two washing DNA steps: 500 µL of Wash Buffer 1 was added to the same spin column, centrifuged at 10,000 ×g (rcf) for one minute at RT, and then the flow-through and collection tube were discarded, and the spin column was transferred into a clean PureLink® Collection Tube. The same procedure was followed where 500 µL of Wash Buffer 2 was added to the spin column, followed by centrifugation at 10,000 ×g (rcf) for 3 minutes at RT. After discarding the flow-through and collection tube, the spin column was transferred into a new sterile 1.5 mL microcentrifuge tube. Lastly, 35 µL of PureLink® Genomic Elusion Buffer (supplied in the Kit) was added to the spin column and then incubated for one minute at RT. The insect gDNA sample was centrifuged at 10,000 ×g (rcf) for one minute to elute the DNA and then stored at -20 ℃.

Method 4:

300 µL volume of TNES buffer [comprising 50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS] and 100 µg/ML volume of Proteinase K, were added to the same 2 mL tube containing crushed insect samples. The tube was then subjected to an incubation at 37 ℃ for 3 hours using a heating block. After the incubation, 85 µL of 5M Sodium Chloride (NaCl) was added and the tube containing the samples and reagents was vigorously shaken for 15 seconds to ensure a thorough mixing. The resultant solution was then subjected to centrifugation at 14,000 ×g (rcf) for 5 minutes to pellet the proteins, after which 400 µL of the resulting clear supernatant was transferred to a new sterile 1.5 mL microcentrifuge tube without disturbing the pellet. A 400 µL volume of 100% ethanol was then added to a clear supernatant and mixed thoroughly by inverting the tube several times until the white DNA strands precipitated out. The tube was then subjected to centrifugation at 12,000 ×g (rcf) for 10 minutes to pellet the DNA, after which the supernatant was discarded. To wash the pelleted DNA, 200 µL of 70% ethanol was added and the tube was centrifuged at 12,000 ×g (rcf) but for 5 minutes. After the centrifugation, the supernatant was discarded again, and the pelleted DNA was air-dried for more than 3 minutes until completely dry at RT. Lastly, 50 µL of the sterile dH2O was added to resuspend the dried DNA pellet, which was then considered as the template DNA. The gDNA sample was then stored at -20 ℃.

3.2.3. Agarose gel electrophoresis (AGE).

The agarose gel [1% (w/v)] was prepared by dissolving 2 grams (g) of Agarose LE (Benchmark Scientific, USA) in 200 mL of 1× TAE buffer [comprising 40 mM Tris base, 20 mM glacial acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA) at pH 8]. This preparation involved manual mixing of the solution in a 500 mL glass beaker through hand-shaking for 30 seconds, followed by microwaving for 1-2 minutes to ensure complete dissolution. To allow the solution to cool, the glass beaker was placed in a fume hood for approximately 3-5 minutes. Once the solution had cooled, the prepared agarose gel was mixed with 8 µL of ethidium bromide (10mg/mL) and then carefully transferred into a gel casting tray, which already had a comb to create wells or lanes on the agarose gel. After 5 minutes, the agarose gel solidified. The gel casting tray with the solidified agarose gel was placed in a gel electrophoresis tank, and 1× TAE buffer was added into the same tank to submerge the agarose gel completely. The comb was then removed from the solid agarose gel, creating wells or lanes where the DNA samples would be loaded. In the first well of each prepared agarose gel, 5 µL of the GeneRuler 1Kb DNA Ladder (Thermo Fisher Scientific, USA) prepared according to the manufacturer's instruction, was loaded to serve as a reference for estimating size. The DNA samples were loaded from the second well onwards, with each specific DNA sample loaded in its respective well.

The visualisation and analysis of both the plant and insect DNA samples were conducted through agarose gel electrophoresis (AGE) using a prepared 1% agarose gel. The gDNA samples were subjected to separation at a constant voltage of 90 volts (V) for between 30-35 minutes in 1× TAE buffer. Following the specified separation period, the resulting gDNA bands and/or amplicons on agarose gels were visualised using the ChemiDoc™ XRS+ (Bio-Rad Laboratories, USA). Furthermore, the images of the agarose gel were photographed and documented using Image Lab™ Software, also from Bio-Rad Laboratories (USA).

For citrus leaf samples: 10 µL of gDNA from each sample was mixed with 2 µL of 6× loading dye (LD) and loaded into individual wells. Each well contained a unique citrus leaf gDNA sample. The AGE procedure for citrus leaf samples was carried out following the same method as described above, with the only modification being the running time, which was extended to 35 minutes while maintaining a constant voltage of 90 V. The resulting DNA bands were visualised using the same procedure mentioned earlier, and the corresponding agarose gel images were captured.

In the analysis of psylloid samples, the following procedure was adhered to: 5 µL of gDNA from each insect sample was mixed with 2 µL of 6× LD, and then loaded into individual wells, with each well containing a specific or distinct insect gDNA sample. The AGE procedure was performed according to the previously described method, with the insect gDNA samples subjected to separation at a constant voltage of 90 V for 30 minutes. After gel electrophoresis, the resulting insect DNA bands were visualised and the corresponding images of the agarose gel were captured accordingly for analysis following the same procedure previously outlined.

3.2.4. Polymerase chain reaction (PCR) amplification of targeted genome region.

Polymerase chain reactions (PCR) were conducted following the manufacturer's protocol for *Taq* 2× Master Mix RED (Ampliqon, Denmark). The PCR reaction mixtures were prepared in PCR tubes and included the specific reagents (Table 3. 3). The extracted gDNA from either plant or insect samples was used as the template DNA. Furthermore, to test the possibility of contamination during PCR set-up, no-template controls (NTCs) were prepared. These NTCs consisted of the same PCR reagents but with the template DNA substituted by ddH_2O for each set of PCR runs (Table 3. 3). The final volume of each PCR reaction was adjusted to 25 µL using ddH_2O (Table 3. 3). All individual PCR reactions together with their corresponding NTCs were briefly mixed and then subjected to centrifugation at a maximum speed for 10 seconds.

Table 3. 3: PCR reaction set-up for each PCR run.

Reagents	**Test reaction (sample)**	**No-template control (NTC)**
Taq 2× Master Mix RED	12.5 µL	12.5 µL
Forward primer (10 µM)	2.5 µL	2.5 µL
Reverse primer (10 µM)	2.5 µL	2.5 µL
Template (DNA)	2 µL	-
Double-distilled water (ddH_2O)	5.5 µL	7.5 µL
Total	**25 µL**	**25 µL**

For all PCR reactions, the amplifications were conducted using a MiniAmp™ Thermal Cycler (Thermo Fisher Scientific, USA). The thermal cycling parameters for each PCR were as follows: initial denaturation at 95 °C for 3 minutes, followed by 30 cycles consisting of denaturation at 95

°C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 1 minute. The amplification process concluded with a final extension step at 72 °C for 3 minutes, followed by cooling at 4 °C.

The resulting PCR amplicons were visualised and analysed through the AGE using a 1% agarose gel. The gel electrophoresis was carried out at 90 V for 30 minutes, consistent with the previously mentioned procedure in **section 3.2.3**. After this separation period, the resulting amplicons were then visualised and the images of the agarose gel were further photographed.

3.2.4.1. Primer testing and selection for plant gDNA amplification:

Three pairs of universal primers (Table 3. 4), all of which were purchased from Inqaba Biotechnical Industries (Pty) Ltd (South Africa), were initially tested using gDNA extracted from a single citrus leaf sample, specifically the Star Ruby variety. Before initiating the testing process, a 100 micromolar (µM) of stock solution was then prepared for each primer using double-distilled water (ddH_2O) on ice, following the manufacturer's instructions without modifications. To ensure thorough mixing and to prevent settling, each primer tube, containing the prepared 100 µM stock solution, was vortexed at maximum speed for 2 seconds using a VELP® Scientifica vortex and followed immediately by centrifugation at 10,000 ×g (rcf) for 5 seconds. To minimize frequent freeze-thaw cycles that could cause quality degradation of each primer and any possible accidental contamination, working solutions were prepared from the 100 µM stock solutions. A 100 µL of 10 µM working solutions for each primer were prepared by pipetting 90 µL of ddH_2O in 1.5 mL microcentrifuge tubes followed by the addition of 10 µL of the 100 µM stock solution, with these stored at -20 ℃ until required.

Table 3. 4: Three primer pairs used against a gDNA sample extracted from citrus leaves to assess their effectiveness before the selection of the best primer pair for further analysis in the study.

Targeted gene	Primer pair	Primer sequence (5'-3')	Amplicon size (bp)	References
rbcL	rbcLaF	ATGTCACCACAAACAGAGACTAAAGC	530	Avanesyan & Lamp (2020)
	rbcLaR	GTAAAATCAAGTCCACCRCG		
trnL	trnL575F	CGACCCCCTTTCCTTAG G	180	Cooper *et al.* (2016)
	trnL755R	TCGGGAATCATTCAACTAGGGA		
rbcL	rbcL19	AGATTCCGCAGCCACTGCAGCCCCTGCTTC	157	Matheson *et al.* (2008) and Avanesyan & Lamp (2020)
	rbcLaF	ATGTCACCACAAACAGAGACTAAAGC		

To test possible contamination, three NTCs were prepared alongside each test reaction, each for a pair of primers, using the same reagents and volumes as specified above in Table 3. 3. All test reactions containing leaf gDNA samples and the NTCs with ddH_2O were briefly mixed by manual inversion and then centrifuged at maximum speed for 10 seconds. After this preparation phase, all test reactions using the three primer pairs together with gDNA samples and the NTCs with ddH_2O were amplified in a MiniAmp™ Thermal Cycler using the same cycling parameters outlined in **section 3.2.4**. To visualise the PCR products and NTCs, the AGE was conducted using 1% agarose gel and separated at 90 V for 30 minutes. Furthermore, 5 µL of a 1kb GeneRuler DNA Ladder was loaded into the first well of the agarose gel as previously outlined in **section 3.2.3**; however, this was followed by loading 5 µL of the first NTC in the second well, 5 µL of rbcLaF/R in the third well, 5 µL of the second NTC in the fourth well, 5 µL of trnL575F/755R in the fifth well, 5 µL of the third NTC in the sixth well and 5 µL of rbcLaF/19 in the last well. Following the separation period, the amplicons were visualised, and the agarose gel images were photographed as outlined in **section 3.2.3**. The primer pair that produced a single visible and bright amplicon was therefore selected as the primary target plant region for detecting citrus DNA in the guts of psylloid species collected in commercial citrus environments.

3.2.4.2. Leaf DNA sample:

The rbcLaF/R primer pair was selected for the subsequent PCR reactions based on the primer testing and selection process. This PCR reaction was conducted across two separate runs, with both runs following the same cycling parameters detailed in **section 3.2.4**. However, there were modifications applied to the primers and template DNA. In this case, the primers utilised were the rbcLaF/R, and the template DNA was gDNA extracted from each of the 15 citrus leaf samples. Furthermore, two NTCs were included, with each NTC corresponding to one of the two PCR runs. The first NTC was associated with the initial PCR run, which consisted of eight test reactions, whereas the second NTC was used in the second PCR run, which involved the remaining seven test reactions.

Following the completion of the two separate PCR runs, the resulting PCR amplicons were then subjected to visualisation and analysis using a 1% agarose gel, following the procedure outlined in **section 3.2.3**. In this regard, 5 µL of a 1kb GeneRuler DNA Ladder was loaded in the first well of the agarose gel, as described in the same section. The loading sequence proceeded as follows: 5 µL of the first NTC was placed into the second well, followed by 5 µL of each of the eight test reactions generated from the template gDNA samples, loaded from the third well onward. Once all eight test reactions from the first run were loaded, 5 µL of each of the remaining seven test reactions were loaded into the same gel following the same sequence, with the second NTC representing these reactions being loaded into the last well. Finally, the same procedure detailed in **section 3.2.3** was used to visualise the resulting amplicons, and the agarose gel images were captured accordingly.

3.2.4.3. Primer testing and selection for insect gDNA amplification:

Three pairs of universal primers (Table 3. 5) also sourced from Inqaba Biotechnical Industries (Pty) Ltd (South Africa), were subjected to evaluation for their efficacy. This assessment involved testing each primer pair with genomic DNA extracted from a test sample, *Trioza erytreae*, utilising four distinct insect DNA extraction methods as described in **section 3.2.2.2**. Before commencing the testing process, a stock solution at a concentration of 100 µM for each primer was prepared using ddH2O, adhering strictly to the manufacturer's instructions without any modifications. Each primer tube containing 100 µM stock solution was vortexed at a maximum speed for 2 seconds

using a VELP® Scientifica vortex, followed by centrifugation at 10,000 ×g (rcf) for 5 seconds to ensure thorough mixing and prevent settling. To mitigate potential quality degradation due to the frequent freeze-thaw cycles and to minimize the risk of accidental contamination, 10 µM working solutions were then prepared from the 100 µM stock solutions, as detailed in **section 3.2.4.1**.

Table 3. 5: The universal primer pairs tested on gDNA test samples from *Trioza erytreae* to assess their effectiveness in amplifying psylloid gDNA samples before selecting the best-performing primer pair.

Targeted gene	Primer pair	Primer sequence (5'-3')	Amplicon size (bp)	References
mtCOI	DCITRI COI-L	AGGAGGTGGAGACCCAATCT	834	Boykin *et al.* (2012)
	DCITRI COI-R	TCAATTGGGGGAGAGTTTTG		
mtCOI	Te-6U30	ATTTTTAAGCACTAATCATAAAATTATTGG	714	Pérez-Rodríguez *et al.* (2019)
	Te-720L26	TATACTTCAGGATGTCCAAAAAATCA		
mtCO1	CO1-F3	TACGCCATACTAGCAATCGG	500	Swisher *et al.* (2012)
	CO1-R3	GAGTAACGTCGTGGTATTCC		

Since four different insect DNA extraction methods and three COI (mitochondrial cytochrome c oxidase subunit I-*mtCOI* or *mtCO1*) primer pairs were assessed for their effectiveness in extracting and amplifying insect DNA, respectively, the PCR reactions were then prepared accordingly for each insect DNA sample extracted with a specific extraction method using the corresponding COI primer pairs. To ensure the absence of contamination during PCR setup, NTCs were included in each PCR run, replacing template DNA with ddH_2O. These NTCs, corresponding to a specific primer pair, were prepared as outlined in Table 3. 3. Following NTC preparation, test reactions corresponding to a specific insect DNA extraction method were prepared, with 2 µL of insect DNA sample added to each test reaction to test primer pair effectiveness in amplifying insect DNA samples. All test reactions with insect DNA and NTCs were mixed and centrifuged according to the procedure previously outlined in **section 3.2.4.1**. After the mixing and centrifugation process,

all test reactions using the three primer pairs, along with insect DNA and NTCs, were subjected to PCR amplification in a MiniAmp™ Thermal Cycler, using the same consistent cycling parameters outlined in **section 3.2.4**. The analysis of the resulting PCR amplicons was then performed through 1% AGE at 90 V for 30 minutes, as previously outlined in **section 3.2.3**. Gel loading followed a specific pattern: 5 µL of 1kb GeneRuler DNA Ladder was loaded in the first well as outlined in **section 3.2.3**; however, this was then followed by 5 µL of the first NTC loaded in the second well and 5 µL of DCITRI COI-L/R in the third well, 5 µL of the second NTC in the fourth well and 5 µL of Te-6U30/720L26 in the fifth well, 5 µL of the third NTC in the sixth well and 5 µL of CO1-F3/R3 in the last well. After separation, PCR amplicons were visualised, and agarose gel images were photographed following the procedure previously outlined in **section 3.2.3**. Lastly, the primer pair that produced a single visible and bright PCR amplicon was selected for further amplification of insect DNA from the remaining psylloid species.

3.2.4.4. Insect DNA sample:

The Te-6U30/720L26 primers were selected as the most suitable candidates for subsequent PCR reactions. These reactions were carried out across multiple runs, which were determined by the quantity of the insect DNA samples processed for plant DNA detection. Despite variations in the primer selection and template DNA, all PCR reactions adhered to the same and consistent cycling parameters outlined in **section 3.2.4**. Specifically, the Te-6U30/720L26 primers were used, while the insect DNA samples served as the template DNA. To ensure accuracy, NTCs were included in each PCR run, with each NTC corresponding to a specific insect DNA sample.

Upon completion of the PCR amplification, the resulting amplicons were subjected to visualisation and analysis via 1% AGE as described in **section 3.2.3.** The loading sequence on the agarose gel was arranged as follows: 5 µL of a 1kb GeneRuler DNA Ladder was loaded into the first well, in line with the procedure outlined in the same section. However, this was then followed by 5 µL of the first NTC loaded in the second well and 5 µL of the first DNA sample in the third well, followed by 5 µL of the second DNA sample in the fourth well and 5 µL of the third DNA sample in the fifth well, and so forth. This loading pattern continued or proceeded until all the DNA samples were loaded in their respective wells. Finally, the visualisation of the resulting PCR amplicons and

capturing of the corresponding agarose gel images were conducted according to the procedure outlined in **section 3.2.3**.

3.2.5. Plant DNA detection in the gut contents of the filed-collected insect samples.

To detect ingested plant DNA in the guts of selected psyllids, PCR reactions were conducted with modifications made to both reagents and their volumes (Table 3. 6). Furthermore, a "spike" was included in each test reaction to validate the success of the PCR amplification process. This spike served as a positive control (PC) to ensure optimal PCR performance by confirming the absence of inhibitors including the contamination, which could impede the PCR amplification of the targeted plant DNA region in the insect gut. The spike in each test reaction consisted of both the extracted insect and plant gDNA samples and the selected *rbcL* primer pair.

Table 3. 6: PCR reaction set-up for detecting plant DNA in the gut contents of psylloid species.

Reagents	**Test reaction (sample)**	**Spike**	**No-template control (NTC)**
Taq 2× Master Mix RED	12.5 µL	12.5 µL	12.5 µL
Forward primer (10 µM-rbcLaF)	2.5 µL	2.5 µL	2.5 µL
Reverse primer (10 µM-rbcLaR)	2.5 µL	2.5 µL	2.5 µL
Template (insect DNA)	5 µL	5 µL	-
Template (plant DNA)	-	2 µL	-
Double-distilled water (ddH_2O)	2.5 µL	0.5 µL	7.5 µL
Total	**25 µL**	**25 µL**	**25 µL**

The resulting amplicons were subjected to visualisation and analysis via AGE using a 1% agarose gel, and gel electrophoresis was conducted at 90 V for 30 minutes, maintaining consistency with the previously mentioned procedure in **section 3.2.3**. However, modifications were made to the loading sequence, starting with a 5 µL of a 1kb GeneRuler DNA Ladder loaded into the first well of the agarose gel, followed by the NTC in the second well. Subsequently, the loading sequence proceeded as follows: 5 µL of the first test reaction loaded into the third well, accompanied by 5 µL of the corresponding spike in the fourth well, followed by 5 µL of the second test reaction and its corresponding loaded spike in the fifth and sixth wells, and so forth, until all test reactions and

their corresponding spikes were loaded into their respective wells. After separation, resulting PCR amplicons were visualised and the corresponding images of agarose gel were captured following the same procedure previously outlined in **section 3.2.3**.

3.3. Results

3.3.1. Analysis of genomic DNA extracted from collected samples.

The gDNA concentration of both citrus plant leaf and psylloid species samples was quantified in triplicate measurements using a Nanodrop™ Lite Plus Spectrophotometer (Thermo Scientific, USA). The gDNA samples extracted from citrus plant leaves showed concentrations ranging from 5.28 ng/µL to 17.73 ng/µL, while, for psylloid species, the extracted gDNA samples demonstrated a wider concentration range, ranging from 5.71 ng/µL to 70.93 ng/µL.

3.3.1.1. Genomic DNA extracted from plant leaf samples:

The genomic DNA (gDNA) extraction process was successfully carried out on all 15 citrus leaf samples using the PureLink™ Plant Total DNA Purification Kit (Invitrogen™, Thermo Fisher Scientific, USA). The success of citrus leaf gDNA extraction is confirmed by the produced gDNA bands (Figure 3. 2). All the gDNA bands demonstrated a consistent pattern, each producing a single faint band in its respective well (Figure 3. 2). Despite their observed faint appearance, these gDNA bands showed a high molecular weight. This is evident from the gDNA bands in wells 1 to 16 that are consistently positioned slightly above the 10,000-base pair (bp) ladder band on the gel (Figure 3. 2). There are limitations in gel resolution, therefore, the specific size of the extracted DNA could not be determined due to its large size. Furthermore, a minor pipetting error occurred during the loading process on the agarose gel, which led to the gDNA extracted from the Orri variety being loaded into two wells, well 3 and well 4 (Figure 3. 2).

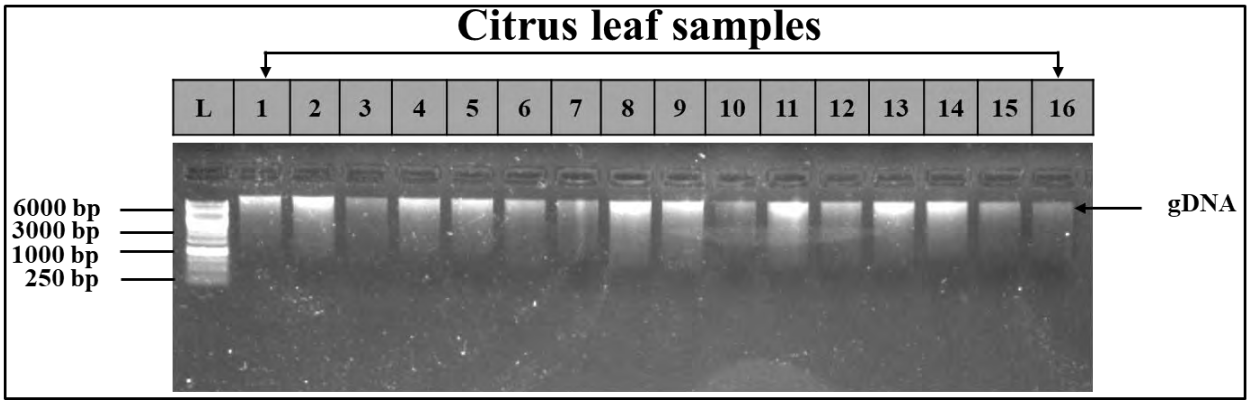

Figure 3. 2: Agarose gel image showing the extracted gDNA from 15 citrus leaf samples collected from mature citrus plants. Well L, represents the GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, USA). Wells 1 to 16 represent the individual wells containing the extracted gDNA from each of the 15 citrus leaf samples, respectively, with duplication in well 3 and 4 for the same variety: Well 1-Star Ruby; Well 2-Eureka Lemon; Wells 3 and 4-Orri; Well 5-Lemon; Well 6-Turkey; Well 7-2PH Lemon; Well 8-Midknight Valencia orange; Well 9-Nadorcott; Well 10-Mandarin; Well 11-Delta; Well 12-Navel; Well 13-Valencia orange; Well 14-Lemon SDL RL; Well 15-Delta Valencia; and Well 16-Palmer navel.

3.3.1.2. Insect DNA extraction method testing and selection:

Among the four DNA extraction methods tested on *Trioza erytreae* (used as a test sample), two methods failed to yield positive results, whereas others showed effectiveness in extracting insect gDNA. There were no visible gDNA bands evident in well M1 (representing **Method 1**) and well M4 (representing **Method 4**) (Figure 3. 3). However, in well M2 (representing **Method 2**) and well M3 (representing **Method 3**), two visible gDNA bands were evident in each, both running slightly above the 10,000 bp ladder, indicating a high molecular weight (Figure 3. 3). The specific or exact size of each gDNA band could not be determined due to limitations in gel resolution. Although both gDNA bands were visible, variations in appearance were observed; the gDNA band observed in well M2 appeared faint and slightly dim, whereas the gDNA band observed in well M3 appeared brighter. Therefore, considering the enhanced visibility and brightness of the gDNA band in well M3 compared to M2, **Method 3** was selected for further analysis in the study, whereas **Method 2** was rejected.

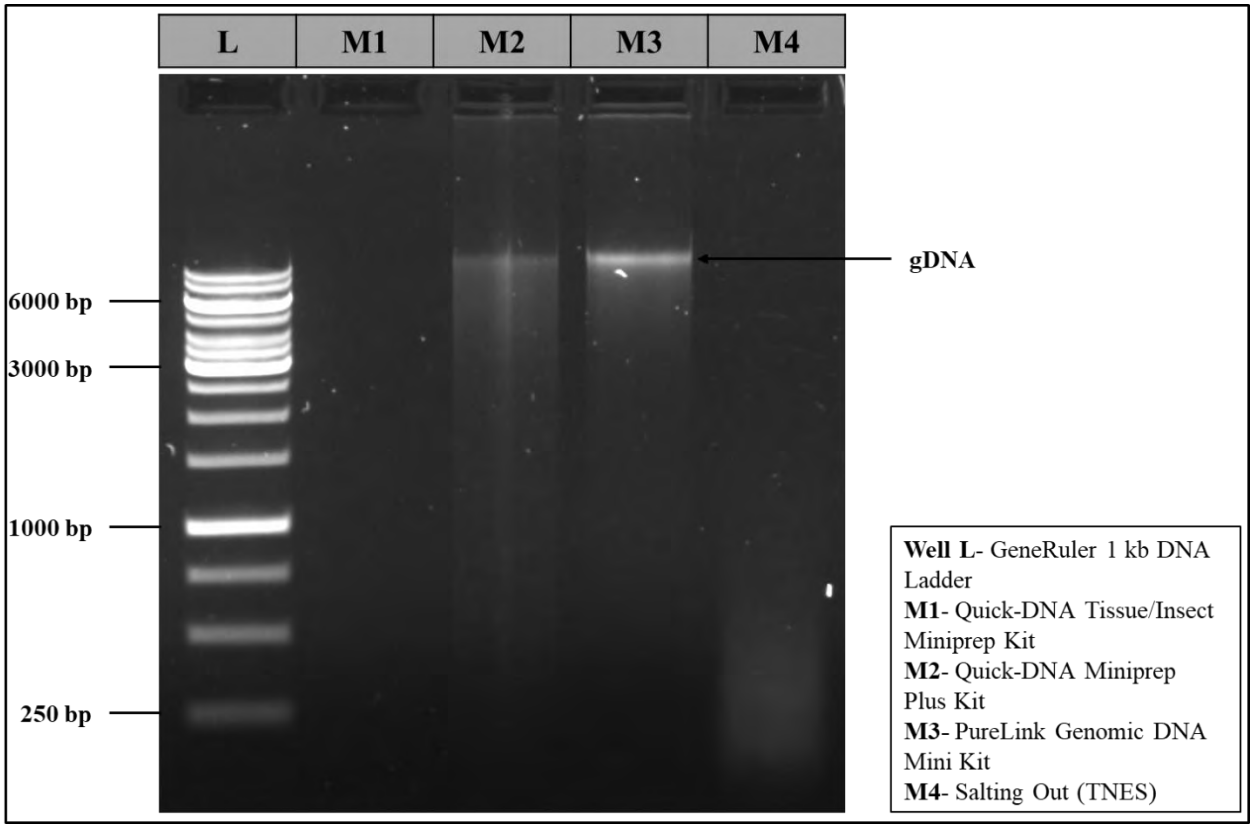

Figure 3. 3: Agarose gel electrophoresis image illustrating the comparative effectiveness of four different insect DNA extraction methods in extracting the insect gDNA. Wells **M1** to **M4** represent the gDNA sample extracted using specific insect DNA extraction methods.

3.3.1.3. Genomic DNA extracted from insect samples:

The gDNA extraction from each of the initial five psyllid species was consistently successful, as evidenced by the visibility and intensity of the gDNA bands in their respective wells (Figure 3. 4). However, variations in the brightness intensity were observed among the gDNA bands. The gDNA bands for *Diaphorina punctulata* and *Pauropsylla trichaeta* (in wells 1DP and 4PT, respectively) demonstrated a relatively high brightness, while those for *Diaphorina virgata* and *Trioza erytreae* (in wells 2DV and 5TE, respectively) showed the highest brightness. However, the gDNA band for *Euryconus* sp. (in well 3EU) appeared slightly to almost dim in comparison (Figure 3. 4).

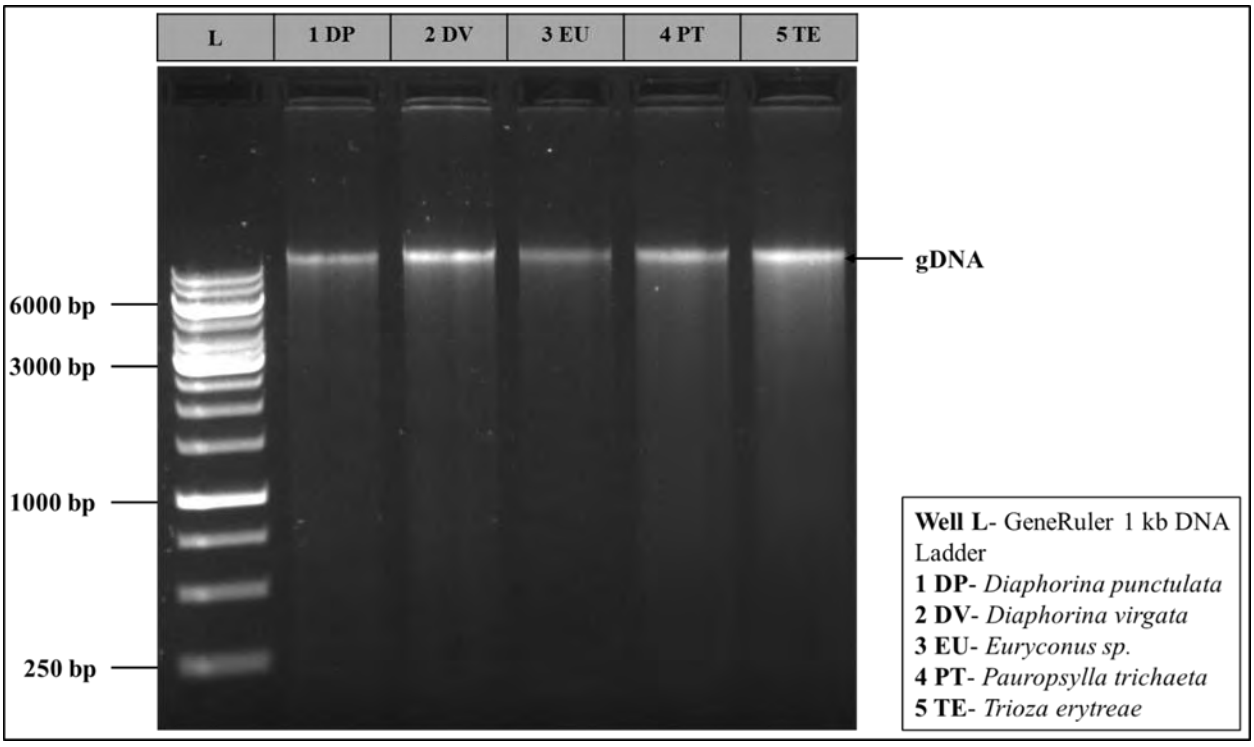

Figure 3. 4: Agarose gel electrophoresis analysis showing the gDNA samples extracted from the initial batch of five predominant psyllid species, selected based on their commonality in different citrus plants and abundance in Eureka Lemons. Each well in the gel represents the gDNA sample extracted from a specific psyllid species.

The gDNA was extracted from selected psyllid species, as confirmed by the visible gDNA bands in their respective wells, with each band representing a specific species and consistently running slightly above the 10,000-bp ladder, highlighting their high molecular weight (Figure 3. 4 and Figure 3. 5). The success of the gDNA extraction across different groups of individual psyllid species, with each species collected from a specific citrus plant (Table S 3. 2), was confirmed through the visible gDNA bands with each observed in its respective well (Figure 3. 5). The appearances of the gDNA bands across all wells were evident; however, varied among each other. In Figure 3. 5A, all gDNA bands were visible, however, the band for *Diaphorina punctulata* (in well 2DP) appeared brighter, followed by the band for *Trioza erytreae* (in well 5TE), which had a relatively lower brightness. However, the gDNA bands for *Acizzia* sp.2, *Diaphorina virgata* and *Psylla loranthi*-group (in wells 1ACZ2, 3DV and 4PLG, respectively) appeared dimmer (Figure 3. 5A). Similar variations persisted in Figure 3. 5B, where only two visible gDNA bands appeared bright, each observed for *Diaphorina virgata* and *Trioza erytreae* (in wells 2DV and 5TE,

respectively), with the band for *Diaphorina virgata* (in well 2DV) showing a high brightness. However, the remaining gDNA bands, each for *Acizzia* sp.1, *Diaphorina zebrana* and *Retroacizzia mopanei* (in wells 1ACZ1, 3DZ and 4RAM, respectively), appeared fainter (Figure 3. 5B). Similarly, in Figure 3. 5C, two visible gDNA bands with each representing *Diaphorina virgata* and *Diaphorina zebrana* (in wells 2DV and 3DZ, respectively), appeared faint yet visible, whereas for *Euryconus* sp. (in well 4EU), the band was slightly faint and dim. However, the gDNA bands for *Diaphorina punctulata* and *Trioza erytreae* (in wells 1DP and 5TE, respectively) appeared bright, with the band for the latter psyllid species (in well 5TE) showing a higher brightness (Figure 3. 5C). In Figure 3. 5D, although all gDNA bands were visible, those that represent *Ciriacremum capense* and *Diaphorina punctulata* (in wells 1CR and 2DP, respectively) were dim, contrasting with the bright bands for *Diaphorina punctulata* and *Trioza erytreae* (in wells 3DP and 4TE, respectively), with the band for the former psyllid species (in well 3DP) being bright (Figure 3. 5D).

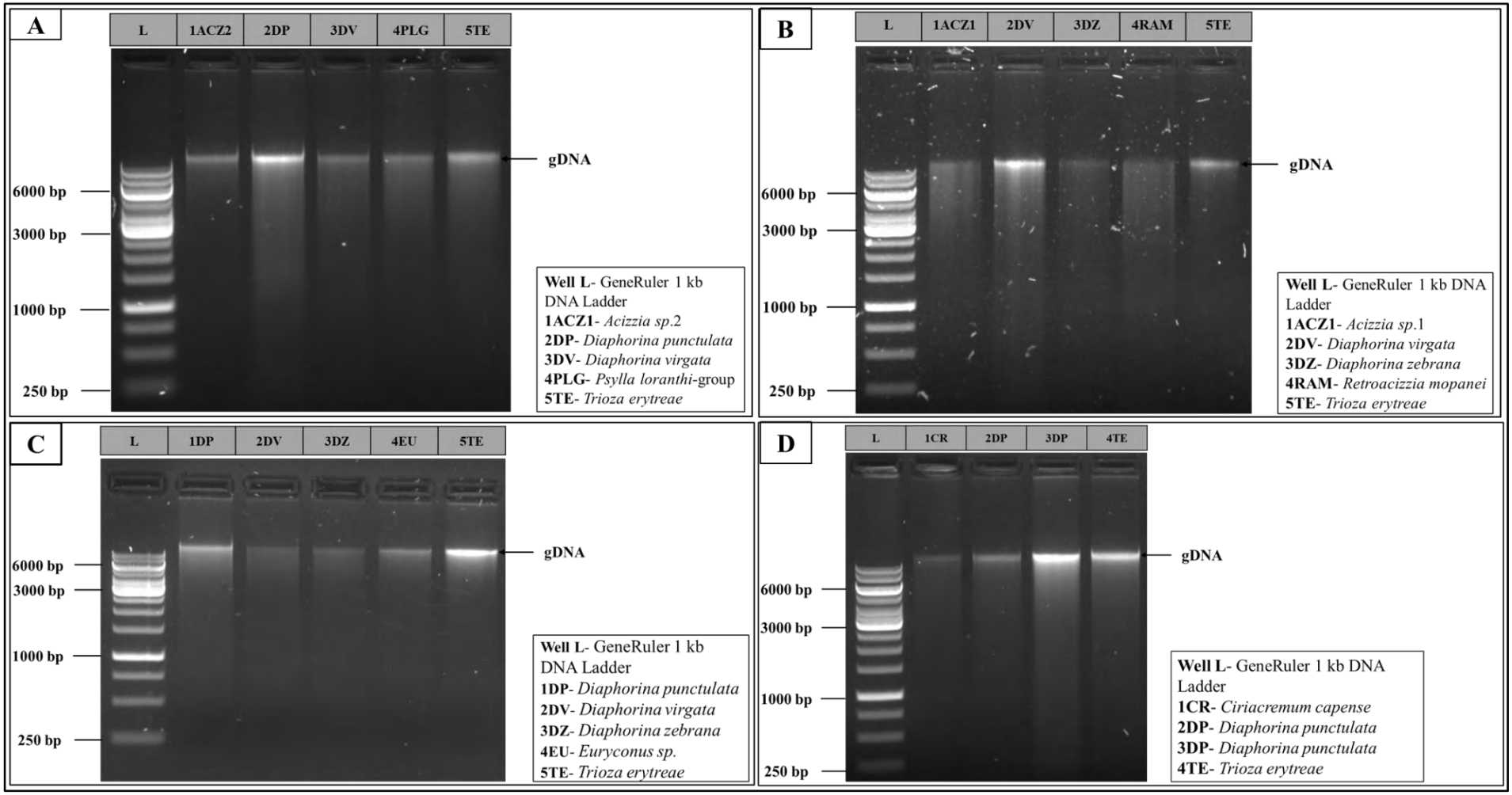

Figure 3. 5: Agarose gel electrophoresis images illustrating the successful gDNA extraction from multiple batches of individual psylloid species, collected directly from diverse citrus plants in citrus orchards. Each well represents a gDNA from a specific psyllid species.

3.3.2. Polymerase chain reaction (PCR) amplification of targeted genome region.

3.3.2.1. Primer testing and selection for plant leaf gDNA amplification:

Among the three primer pairs tested for their amplification efficiency against the gDNA extracted from a leaf sample of the Star Ruby variety, the rbcLaF/R in well 2 and trnL575F/755R in well 4, were the only primer pairs that successfully produced visible amplicons of approximately 530-bp and 180-bp, respectively (Figure 3. 6). However, the rbcLaF/19 primer pair as observed in well 6 failed to produce the expected amplicon (Figure 3. 6). Although the former primer pairs produced visible amplicons, the amplicon produced by the rbcLaF/R primer pair appeared brighter than the amplicon generated from the trnL575F/755R primer pair, which appeared dimmer yet remained visible (Figure 3. 6). Therefore, the rbcLaF/R primer pair which targets only the *rbcL* gene, was chosen, while the trnL575F/755R primer pair were excluded from further consideration in the study. Therefore, all subsequent analyses and findings presented herein were based on the use of the selected rbcLaF/R primer pair. In addition, none of the three NTCs, each representing a specific primer pair, produced an amplicon during testing (Figure 3. 6; Wells 1, 3 and 5). This lack of amplicons in the wells of NTCs indicates the absence of contamination during the PCR set-up for these primer pairs.

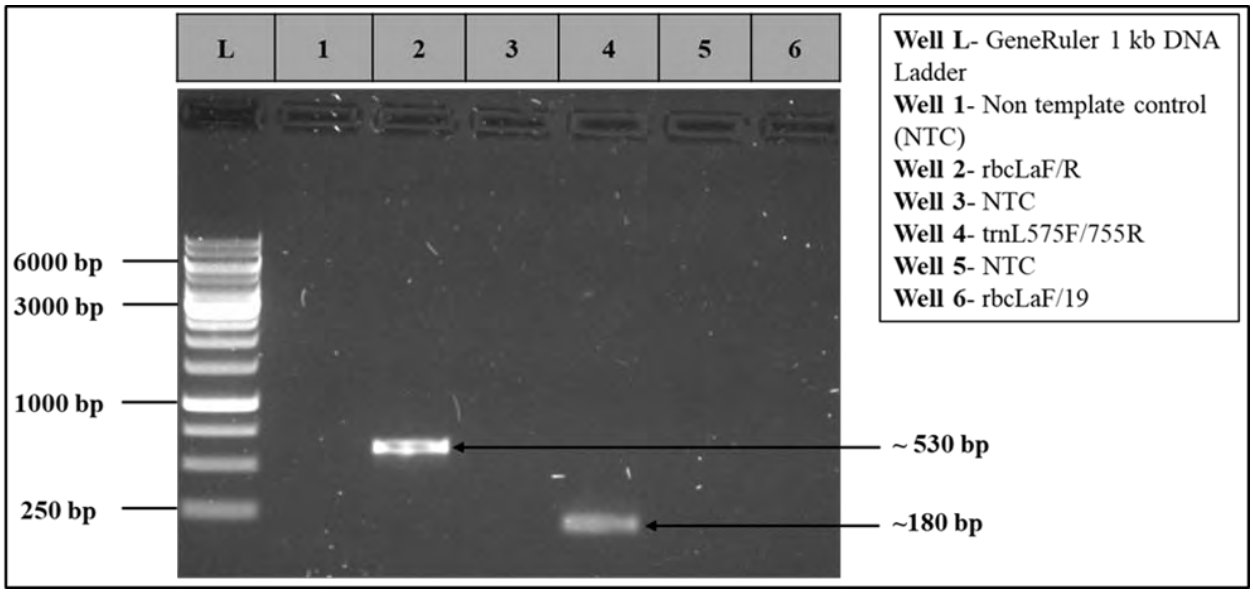

Figure 3. 6: Agarose gel electrophoresis image illustrating the PCR amplification of each tested primer pair using the extracted gDNA sample from the Star Ruby variety. The PCR amplicons in

their respective wells represent the amplified gDNA, confirming the effectiveness of each primer pair.

3.3.2.2. Polymerase chain reaction amplification of plant leaf gDNA samples:

Polymerase chain reaction (PCR) amplifications were successfully carried out using the extracted gDNA from all 15 citrus leaf samples as the template DNA (Figure 3. 7). The rbcLaF/R primer pair was selected for this amplification. The resulting PCR amplicons produced from the *rbcL* region and template DNA amplification were visualised via electrophoresis on a 1% agarose gel stained with ethidium bromide, and the agarose gel image was captured for documentation (Figure 3. 7). In the agarose gel image, the *rbcL* gene in the plant DNA template amplifications produced visible and intensely bright amplicons for all 15 citrus leaf samples. However, the amplicon brightness varied, with the PCR amplicon in well 2 (representing Star Ruby) showing a slightly diminished intensity (Figure 3. 7). Despite variations in amplicon brightness, the sizes of all produced amplicons fell within the range of 500-bp to 750-bp (Figure 3. 7), and the estimated size of the targeted *rbcL* plant region is 530-bp for all 15 citrus leaf samples (Table 3. 4). In addition, the PCR set-up was maintained without any possible contamination, as evident in wells 1 and 17, representing the two NTCs, which produced no amplicons. Instead, these wells remained clear, thus confirming the absence of contamination in the experimental process (Figure 3. 7; Wells 1 and 17).

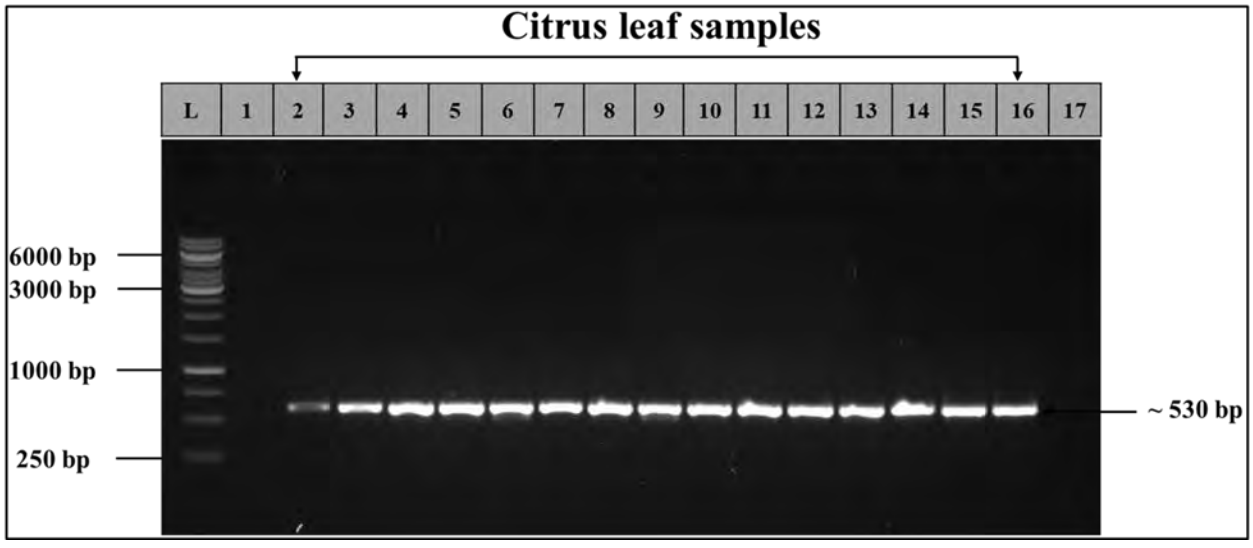

Figure 3. 7: Agarose gel of PCR-amplified gDNA samples extracted from all 15 collected citrus

leaf samples using the rbcLaF/rbcLaR primer pair. Well L, represents the GeneRuler 1 kb DNA Ladder. Wells 1 and 17 represent two NTCs. Wells 2 to 16 represent the amplified gDNA samples from each corresponding citrus leaf sample targeting the *rbcL* region: Well 1-NTC; Well 2-Star Ruby; Well 3-Eureka Lemon; Well 4-Orri; Well 5-Lemon; Well 6-Turkey; Well 7-2PH Lemon; Well 8-Midknight Valencia orange; Well 9-Nadorcott; Well 10-Mandarin; Well 11-Delta; Well 12-Navel; Well 13-Valencia orange; Well 14-Lemon SDL RL; Well 15-Delta Valencia; Well 16-Palmer navel; and Well 17-NTC.

3.3.2.3. Primer testing and selection for insect gDNA amplification:

Among the three primer pairs evaluated against gDNA samples extracted from *Trioza erytreae* test samples using four distinct extraction methods, only the Te-6U30/720L26 primer pair successfully amplified all the extracted gDNA samples. This was evident from the consistently brighter and visible PCR amplicons observed in well 4 across all agarose gel images, each representing gDNA samples extracted using a specific method (Figure 3. 8A-D). The amplified PCR amplicon from the Te-6U30/720L26 primer pair observed in well 4 across all gel images was estimated to be approximately 750-bp (Figure 3. 8A-D). However, the DCITRI COI-L/R and CO1-F3/R3 primer pairs in wells 2 and 6, respectively, failed to amplify the gDNA samples, and no PCR amplicons were observed in their respective wells across the two agarose gel images (Figure 3. 8A and D). Although DCITRI COI-L/R and CO1 F3/R3 could not amplify the gDNA samples extracted using **Method 1** and **Method 4**, the gDNA samples extracted using **Method 2** and **Method 3** were successfully amplified using these primer pairs (Figure 3. 8B and C). However, the amplification using DCITRI COI-L/R and CO1-F3/R3 in wells 2 and 6, respectively, produced less visible and dim PCR amplicons. Furthermore, the estimated sizes of the PCR amplicons from DCITRI COI-L/R in well 2 and CO1 F3/R3 in well 6 were approximately 749-bp and 500-bp, respectively (Figure 3. 8B and C). Due to their inconsistent amplification and dim PCR amplicons, the DCITRI COI-L/R and CO1-F3/R3 primer pairs were then excluded (rejected) from further analysis, and all subsequent results were based only on the primer pair Te-6U30/720L26.

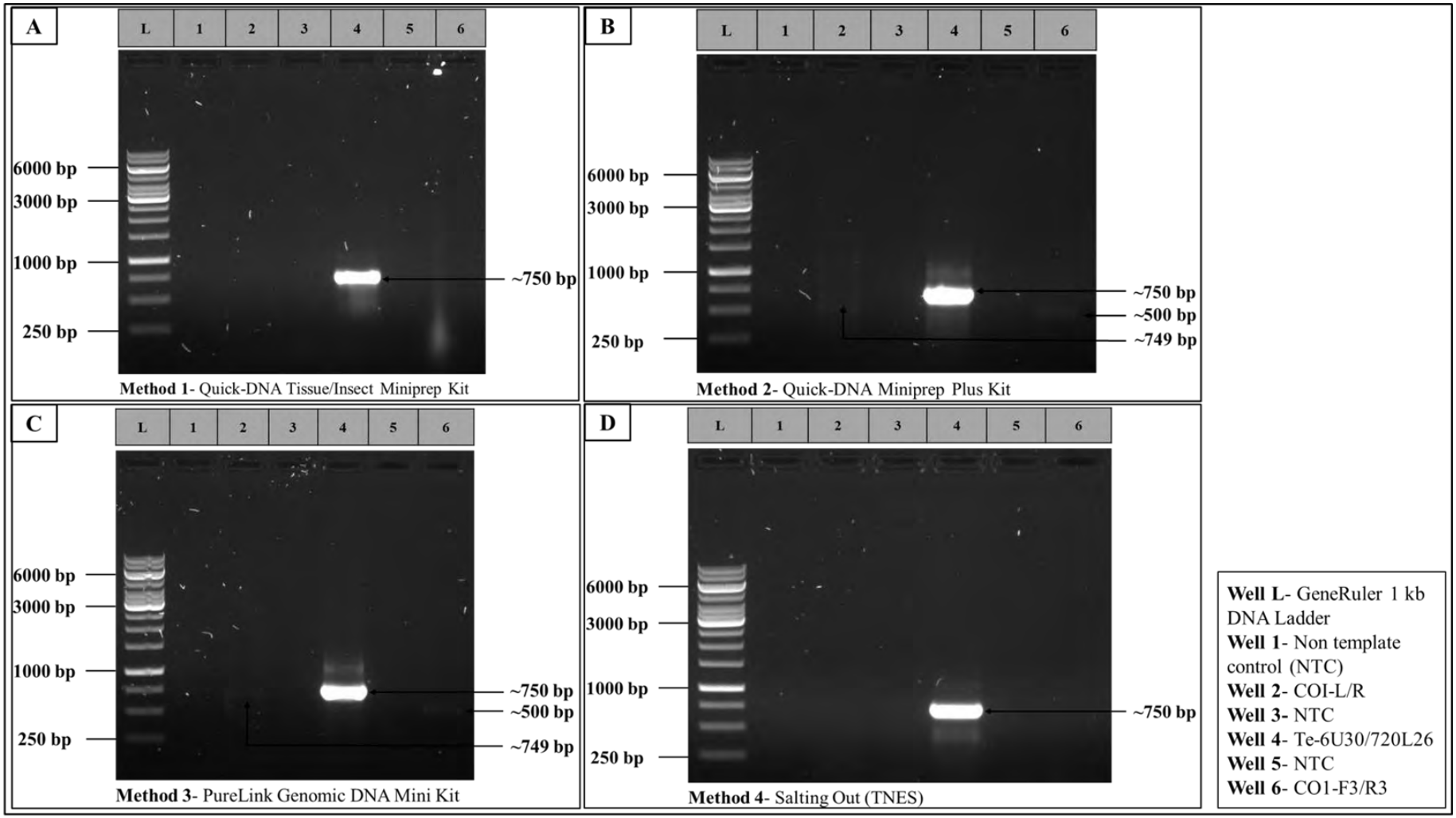

Figure 3. 8: Agarose gel electrophoresis images illustrating the PCR amplification of each gDNA sample extracted from *Trioza erytreae*, serving as the positive control, using four different extraction methods: **Method 1** (A), **Method 2** (B), **Method 3** (C) and **Method 4** (D). Each gDNA sample extracted using a respective method was subjected to PCR amplification using three primer pairs: COI-L/R, Te-6U30/720L26 and CO1-F3/R3.

3.3.2.4. Polymerase chain reaction amplification of insect gDNA samples:

The gDNA amplification using the Te-6U30/720L26 primer pair was successful for most of the psylloid samples, as confirmed by the visible PCR amplicons observed in their respective wells across all agarose gel images (Figure 3. 9 and Figure 3. 10). However, there were variations in the appearances of these amplicons, with the majority showing a bright appearance while a few appeared dim (Figure 3. 9 and Figure 3. 10). In wells where this primer pair failed to amplify the extracted gDNA samples, no corresponding PCR amplicons were observed, consistently evident across all agarose gel images (Figure 3. 9 and Figure 3. 10). Despite either successful or failed PCR amplification of the gDNA samples, each PCR reaction set-up was not contaminated as confirmed by the absence of PCR amplicons in the NTC wells in all gel images (Figure 3. 9 and Figure 3. 10).

In Figure 3. 9, the gDNA samples extracted from *Diaphorina punctulata*, *Diaphorina virgata* and *Trioza erytreae* were successfully amplified, as confirmed by the visible and bright PCR amplicons observed in wells 1DP, 2DV and 5TE, respectively, with each demonstrating a consistently high bright appearance. Furthermore, these PCR amplicons maintained a uniform size estimated at approximately 750-bp. However, within each of the two following wells, 3EU and 4PT, the Te-6U30/720L26 primer pair could not amplify the extracted gDNA samples of *Euryconus* sp. and *Pauropsylla trichaeta*, respectively, as no PCR amplicons were observed in the respective wells (Figure 3. 9).

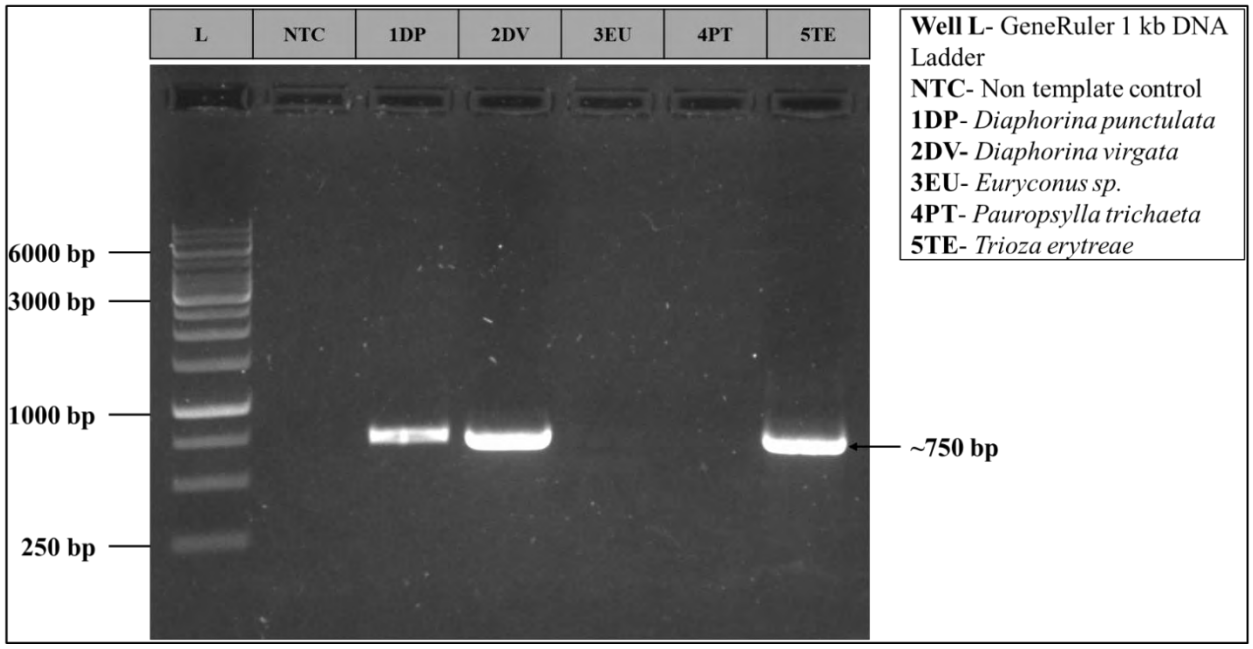

Figure 3. 9: Agarose gel electrophoresis image demonstrating PCR amplifications of the gDNA samples extracted from different psyllid species, using the specific Te-6U30/720L26 primer pair. Each well, except well L and NTC, represents the gDNA sample of a specific psyllid species. The visible PCR amplicons represent amplified gDNA samples.

The PCR amplification was observed consistently across multiple wells, indicating the successful amplification of gDNA samples extracted from specific psyllids using the Te-6U30/720L26 primer pair (Figure 3. 10). The visible PCR amplicons, each estimated to be approximately 750-bp in size, were observed in most wells. In Figure 3. 10A, the Te-6U30/720L26 primer pair amplified the gDNA samples of *Diaphorina punctulata*, *Diaphorina virgata*, *Psylla loranthi*-group and *Trioza erytreae*, with each in wells 2DP, 3DV, 4PLG and 5TE, respectively, resulting in visible PCR amplicons with different bright intensities. The PCR amplicons, each observed in wells 2DP and 3DV, appeared highly bright, followed by the amplicon in 4PLG, which demonstrated a relatively lower brightness. In contrast, the PCR amplicon in 5TE appeared slightly dim (Figure 3. 10A). In well 1ACZ2, no PCR amplicon was observed, indicating unsuccessful gDNA amplification of *Acizzia* sp.2 using the Te-6U30/720L26 primer pair (Figure 3. 10A). In Figure 3. 10B, visible PCR amplicons were observed for four amplified gDNA samples of *Diaphorina punctulata*, *Diaphorina zebrana*, *Retroacizzia mopanei* and *Trioza erytreae*, each in wells 2DV, 3DZ, 4RAM and 5TE, respectively, with varying brightness. For example, the PCR amplicon observed in well 2DV appeared highly bright, followed by those in 3DZ and 5TE, whereas the amplicon in well 4RAM

appeared dimmer (Figure 3. 10B). Similar to well 1ACZ2 in Figure 3. 10A, the gDNA sample of *Acizzia* sp.1 in well 1ACZ1 also failed to amplify using this primer pair, with no PCR amplicon observed (Figure 3. 10B).

Furthermore, in Figure 3. 10C, the visible and bright PCR amplicons were also observed in wells 1DP, 2DV, 3DZ and 5TE, with high brightness, indicating successful amplification of extracted gDNA samples from *Diaphorina punctulata*, *Diaphorina virgata*, *Diaphorina zebrana* and *Trioza erytreae*, respectively. However, no PCR amplicon was observed in well 4EU, indicating failed amplification of the gDNA sample of *Euryconus* sp. with the Te-6U30/720L26 primer pair (Figure 3. 10C). Similarly, in Figure 3. 10D, the Te-6U30/720L26 primer pair could not amplify the gDNA sample of *Ciriacremum capense* in well 1CR, as no PCR amplicon was observed within this well. The gDNA samples of *Diaphorina punctulata*, *Diaphorina punctulata* and *Trioza erytreae* in wells 2DP, 3DP and 5TE, respectively, were successfully amplified, as confirmed by the visible and bright PCR amplicons, each in its respective well, with amplicons observed in wells 2DP and 3DP showing a higher brightness than the amplicon in well 5TE (Figure 3. 10D).

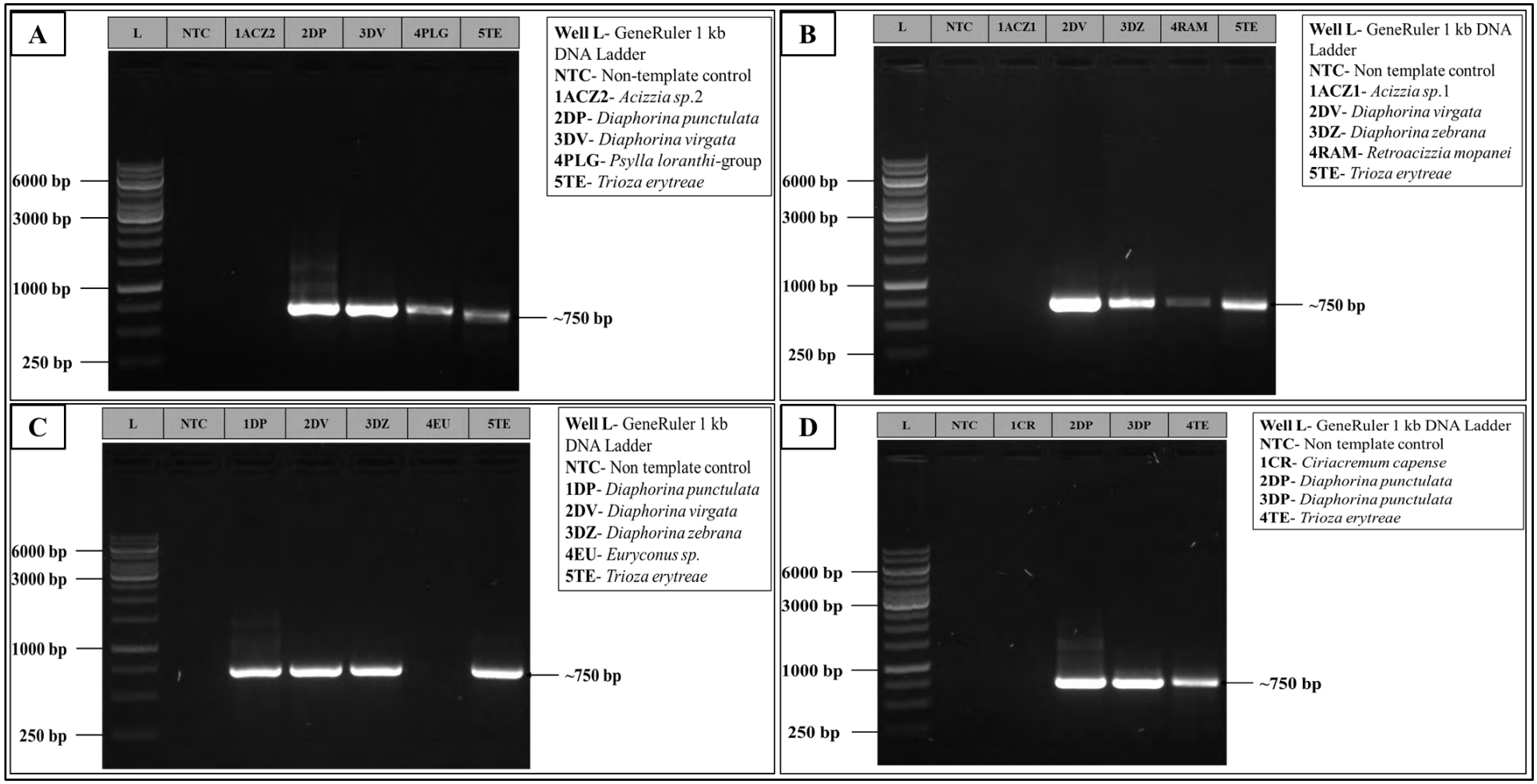

Figure 3. 10: Agarose gel electrophoresis images demonstrating the PCR amplification results of gDNA samples extracted from various psylloid species, utilising the Te-6U30/720L26 primer pair. Each agarose gel well, except well L and NTC, represents a psyllid species collected from a specific citrus plant, with a batch of five species per gel, with the exception of the gel presented in Figure 3.10D.

3.3.3. Plant DNA detection in the gut contents of the field-collected insect samples.

The PCR amplification of the targeted plant DNA region in some psyllid species using the selected rbcLaF/R primer pair was successful, as confirmed by the visible PCR amplicons in the respective wells, each representing a specific psyllid species (Figure 3. 11 and Figure 3. 12). Although the amplified plant DNA region produced visible PCR amplicons, there were variations in both their appearance and size, ranging from 500-bp to 700-bp. However, in the gut samples of other psyllid species, the targeted plant DNA could not be amplified with the same primer pair (Figure 3. 11 and Figure 3. 12). Despite the varied outcomes, the PCR reaction setup remained uncontaminated, as confirmed by the absence of the amplicons in NTC wells across each agarose gel image (Figure 3. 11 and Figure 3. 12).

In the gut samples of *Diaphorina virgata* (well 2DV) and *Euryconus* sp. (well 3EU), the rbcLaF/R primer pair amplified the plant DNA, as evidenced by the visible PCR amplicons (Figure 3. 11). However, these amplicons appeared dim, and their bp sizes varied; the size of the PCR amplicon in well 2DV was estimated to be approximately 750-bp, whereas the expected size of 530-bp was observed for the amplicon in well 3EU. However, in the gut samples of *Diaphorina punctulata* (well 1DP), *Pauropsylla trichaeta* (well 4PT) and *Trioza erytreae* (well 5TE), the rbcLaF/R primer pair failed to amplify the plant DNA, as confirmed by the absence of PCR amplicons within the respective wells (Figure 3. 11). Despite the success or failure of the plant DNA amplification in the gut sample of each psylloid species, no inhibitors were detected, as confirmed by both the visibility and brightness of the PCR amplicons observed in the "spike" (SPK) wells, with each SPK well corresponding to a specific psyllid sample (Figure 3. 11).

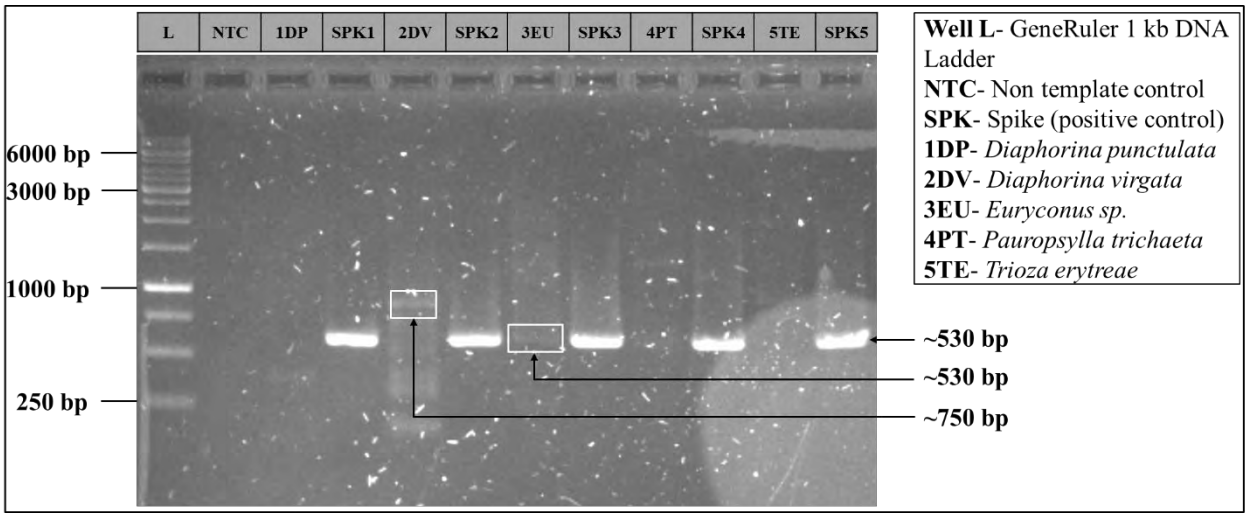

Figure 3. 11: Agarose gel electrophoresis image demonstrating the PCR amplification of plant DNA within the gut contents of selected psylloid species. The visible PCR amplicons marked with white square boxes indicate the presence of plant DNA in the gut of each analysed psyllid species.

The rbcLaF/R primer pair amplified plant DNA in the gut samples of *Diaphorina virgata* and *Trioza erytreae*, as confirmed by the visible yet faint PCR amplicons respectively in wells 3DV and 5TE (Figure 3. 12A). However, the sizes of these amplicons varied, with the PCR amplicon in well 3DV estimated to be approximately 540-bp and the amplicon in well 5TE estimated to be approximately 500-bp (Figure 3. 12A). In contrast, in the gut samples of *Acizzia* sp.2, *Diaphorina punctulata* and *Psylla loranthi*-group, no plant DNA was amplified, as evidenced by the absence of the amplicons in wells 1ACZ2, 2DP and 4PLG, respectively (Figure 3. 12A). Despite variations in successful plant DNA amplification among the gut samples of psylloid species, no inhibitors were detected, as evidenced by the consistent visibility and brightness of the amplicons in the SPK wells, each corresponding to a specific sample (Figure 3. 12A). The brightness of these amplicons varied, with those observed in the first four SPK wells demonstrating higher brightness compared to the amplicon in the last SPK well, which appeared dim (Figure 3. 12A).

Furthermore, in Figure 3. 12B, the plant DNA was amplified within the gut samples of *Diaphorina virgata*, *Diaphorina zebrana* and *Trioza erytreae*, with the visible PCR amplicons observed within wells 2DV, 3DZ and 5TE, respectively. These amplicons also appeared faint, with variations in their bp sizes; amplicons observed in wells 2DV and 3DZ were estimated at approximately 540-bp each, whereas the amplicon in well 5TE was estimated at approximately 700-bp. However, in the gut samples of *Acizzia* sp.1 and *Retroacizzia mopanei*, the plant DNA could not be amplified

using the same primer pair, as confirmed by the absence of PCR amplicons in wells 1ACZ1 and 4RAM, respectively (Figure 3. 12B). Irrespective of the varied outcomes across the samples, the visibility and brightness of PCR amplicons observed across all SPK wells remained consistent, confirming the absence of inhibitors (Figure 3. 12B).

In Figure 3. 12C, the rbcLaF/R primer pair could not amplify the plant DNA within the gut samples of *Diaphorina punctulata*, *Diaphorina virgata*, *Diaphorina zebrana*, *Euryconus* sp. and *Trioza erytreae*, as no PCR amplicons were observed in each of the following wells, 1DP, 2DV, 3DZ, 4EU and 5TE, respectively. However, the PCR amplicons observed across all SPK wells showed consistent visibility and brightness, confirming that no inhibitors were present (Figure 3. 12C). In Figure 3. 12D, the plant DNA amplification using the rbcLaF/R primer pair was evident in the gut samples of *Diaphorina punctulata* and *Trioza erytreae*, with visible but faint PCR amplicons observed respectively in wells 2DP, 3DP and 5TE, and an additional bright amplicon observed in well 3DP. Furthermore, the sizes of these amplicons varied; the amplicon in well 2DP was estimated to be approximately 540-bp, while the dim and bright amplicons from well 3DP were estimated to be 530-bp and 750-bp, respectively. The size of the amplicon observed from well 5TE was estimated to be approximately 760-bp (Figure 3. 12D).

The plant DNA in the gut of *Ciriacremum capense* failed to amplify using the same primer pair, as no PCR amplicon was observed in well 1CR (Figure 3. 12D). No inhibitors were present, and the consistent visibility and brightness of PCR amplicons were observed across all SPK wells, despite the differences in the amplification success of plant DNA in the gut samples of each psyllid species (Figure 3. 12D).

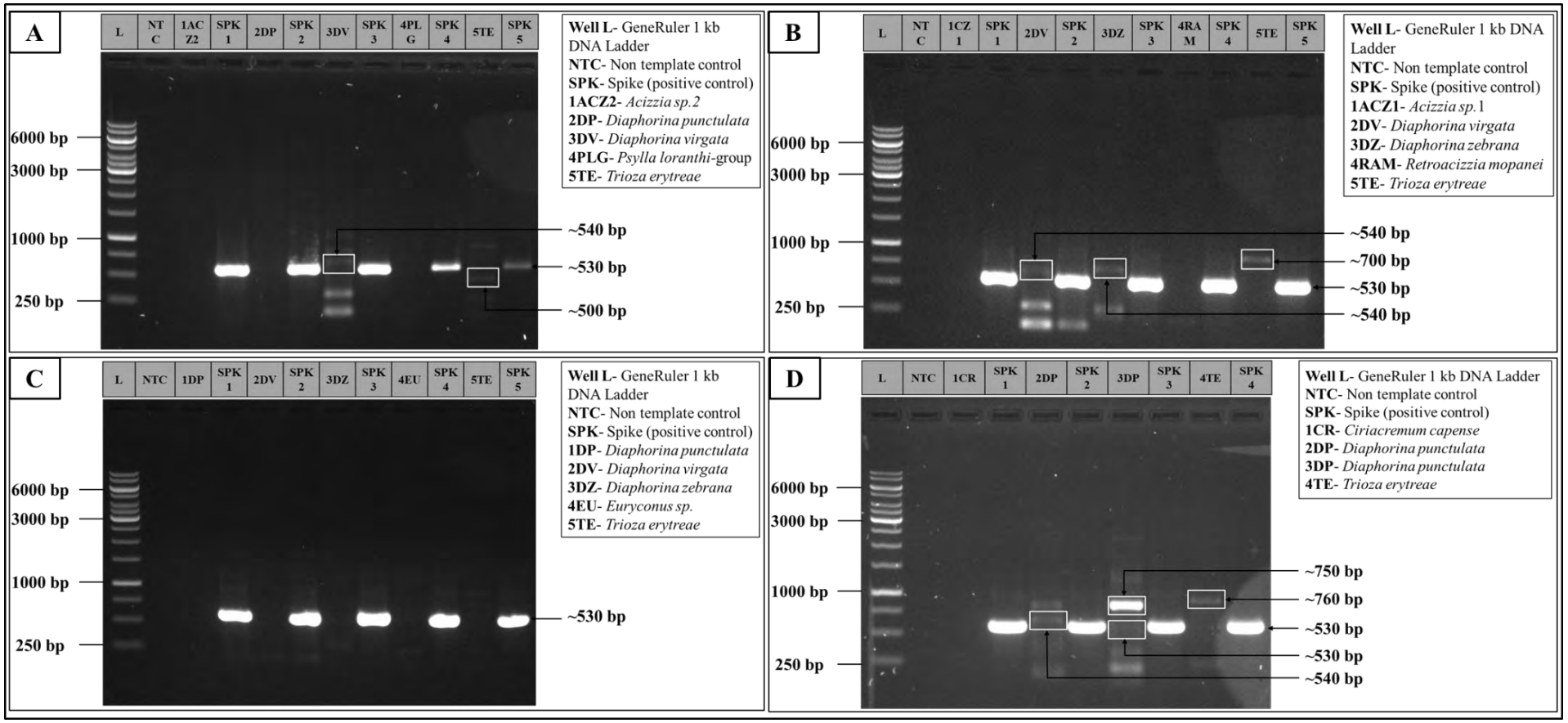

Figure 3. 12: Representative agarose gel electrophoresis images demonstrating the amplification results of plant DNA in the gut contents of various psylloid species via PCR. Each PCR amplicon, marked with a white square box, serves as evidence of potential amplification of plant DNA present in the gut of the respective analysed psyllid species.

3.4. Discussion

Understanding the feeding habits of phytophagous insects can be challenging when there are pests of economically significant in agriculture (Avanesyan, 2014). Direct-field observations of foraging behaviour, microscopical identification of ingested plants and controlled feeding experiments conducted in laboratory settings are among the most common methods that have been employed in investigating the dietary preferences of phytophagous insects (Matheson *et al.*, 2008; Pompanon *et al.*, 2012; Avanesyan, 2014). Although these methods have proven useful, some studies have outlined their limitations in accurately confirming or identifying the consumed plant species by these insects, especially for those feeding on multiple plants (Bafeel *et al.*, 2012; De la Cadena *et al.*, 2017). García-Robledo *et al.* (2013) study found that in controlled feeding experiments, insects tend to nibble on non-host plants, as insects might be forced to feed on available plant resources under the laboratory conditions, which may not coincide with their preferences in the natural field environments. Furthermore, Valentini *et al.* (2009) reported that direct-field observations can be difficult or even impossible. This method is often time-consuming and impracticable when dealing with either elusive or nocturnal insects, or herbivores feeding in complex environments with many spatially intermixed plant species, increasing the risk of misidentifying plants involved in insect-plant interactions.

However, molecular analysis of gut contents has become the most effective, accurate and time-efficient method for identifying previously colonised and fed upon plant species or confirming host-plant utilisation by phytophagous insects prior to capture (Jurado-Rivera *et al.*, 2009; García-Robledo *et al.*, 2013; Avanesyan, 2014; Cooper *et al.*, 2016; Huang *et al.*, 2017). Therefore, in the current chapter, the gut contents of the selected psylloid species (actively collected on citrus in the orchards) were analysed through DNA sequencing techniques to determine their interactions with citrus.

3.4.1. Genomic DNA extraction from plant leaf and insect samples.

3.4.1.1. Plant leaf samples:

Several DNA extraction methods have been established to obtain pure and intact gDNA from plant tissues (Abu Almakarem *et al.*, 2012; Cen *et al.*, 2012; Avanesyan, 2014). Aboul-Maaty & Oraby

(2019) successfully extracted DNA from different plant seeds and crops from seven different orders using a modified Cetyl trimethylammonium bromide (CTAB) method, yielding pure, high-quality DNA suitable for molecular analysis. Similarly, Guo *et al.* (2018) compared the CTAB method and the DNAsecure Plant Kit across three temperate woody bamboo and three tropical woody bamboo species with different preservation methods over different periods of up to three years. They found that gDNA extracted using the modified CTAB method showed clear main bands, whereas DNA from the DNAsecure Plant Kit resulted in slight degradation. Sahu *et al.* (2012) also found that the CTAB method gave better DNA yield in terms of quality and quantity than a Plant genomic DNA extraction Kit, particularly for mangrove and salt marsh plant leaves.

In this study, the gDNA of 15 citrus leaf samples was extracted using the PureLink™ Plant Total DNA Purification Kit (Invitrogen™, Thermo Fisher Scientific, USA). The gel electrophoresis, as anticipated, revealed a single gDNA band in each well, indicating successful extraction from these plant leaf samples. To further validate the success of our citrus leaf gDNA extraction, we observed all visible bands, each corresponding to a specific leaf gDNA sample, running above the 10,000-bp DNA ladder band, aligning with our expectations. Previous research by Gmitter *et al.* (2012), citing Arumuganathan & Earle (1991) and Ollitrault *et al.* (1994), and more recently by Kumar *et al.* (2024), cited Inglese & Sortino (2019), estimated the citrus genome size to range between 372 to 398 Megabase pairs (Mb). Therefore, due to the large size of citrus DNA, determining the exact size of the gDNA extracted from the leaf samples proved challenging.

Although the gDNA bands were visible on the agarose gel, their visibility was accompanied by a faint appearance, which suggests possible degradation or insufficient quantity/concentration of the extracted gDNA loaded on the gel. Factors that could have contributed to the degradation of citrus leaf gDNA samples might have been the preservation method and storage duration. To confirm this hypothesis, we re-extracted gDNA from the same leaf samples and visualised them on a new agarose gel. Unfortunately, the degradation pattern persisted across the new gDNA samples (data is not included here). Guo *et al.* (2018) compared different methods for preserving plant materials and their impact on DNA integrity. In their study, the plant gDNA was extracted using the modified CTAB protocol and DNAsecure plant kit from the leaves of six woody bamboos preserved in silica gel at room temperature and cryopreservation for varying durations (6, 12, 24 and 36 months). They found that the degradation of gDNA samples became noticeable after 12 months of storage

in both room-temperature and cryopreservation-stored samples. The gDNA samples from both storage methods were found to be completely degraded by months 24 and 36.

However, Chase & Hills, (2012) and Wilkie *et al.* (2013) found that leaf samples preserved inside zip-lock bags stored in silica gel were effective for the long-term preservation of gDNA samples and minimised degradation. The current study, however, complements Guo *et al.* (2018) as plant gDNA degradation was encountered, which affected the leaf gDNA quality. Therefore, based on our findings and related literature, it is recommended to preserve the plant leaf samples for less than a month, ideally, one to two weeks, to prevent gDNA degradation and to ensure optimal quality for future DNA analysis.

3.4.1.2. Insect DNA extraction method testing and selection:

The second objective of this chapter focused on extracting gDNA from psylloid species collected from the citrus environments. For this purpose, actively collected psylloid specimens using sweep-netting were chosen for gDNA extraction. These specimens were preferred because they were still in good condition and collected directly from the citrus plants. However, psylloid species passively collected through yellow sticky traps were excluded. This exclusion was due to the majority of the specimens being dry and often breaking off during isolation, attributed primarily to the traps being left in orchards for extended periods. Ballare *et al.* (2019) found that insect specimens collected directly into a preservative yield higher-quality DNA, further supporting the decision to prioritise sweep-netted insects. Furthermore, several studies have previously shown that insect specimens exposed to environmental factors, such as sunlight, high temperatures and humidity, among others are more likely to experience break-off and rapid gDNA degradation (Lindahl, 1993; Mandrioli, 2008; Zimmermann *et al.*, 2008). Butterwort *et al.* (2022) indicated that materials used in sticky traps and clearing agents for isolating trapped insect specimens can further impact DNA quality. Therefore, actively collected psylloid samples were prioritised for gDNA extraction in this study to ensure reliable DNA analysis, particularly for detecting plant DNA in their guts, to confirm their potential to feed on citrus.

Four distinct extraction methods (see **section 3.2.2.2**) were initially tested to determine the most effective method across all selected field-collected psylloid specimens. To achieve this, the *Trioza erytreae* samples served as a test control, with five individuals assigned to each method. Among

the four tested methods, only two extraction methods [i.e. Quick-DNA Tissue/Insect Miniprep Kit and Salting-Out (TNES)] failed to extract gDNA from the insect samples, as no gDNA bands were observed in the corresponding wells. However, the other remaining methods, such as Quick-DNA Miniprep Plus Kit and PureLink Genomic DNA Mini Kit, successfully extracted gDNA, with two bands observed in their respective wells. Although both methods were effective, the PureLink Genomic DNA Mini Kit demonstrated higher effectiveness as the gDNA band extracted using this method appeared brighter (indicating better gDNA extraction) than the band from the Quick-DNA Miniprep Plus Kit. Consequently, the Quick-DNA Miniprep Plus Kit was excluded from further use in the study, and all insect gDNA extraction results in this chapter are based on the PureLink Genomic DNA Mini Kit.

3.4.1.3. Insect samples:

The insect gDNA bands were visible above the 10,000-bp DNA ladder band on the agarose gel, confirming both the success of the extraction process and the effectiveness of the method utilised. Similar to other hemipteran insects, such as pea aphids *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) and whiteflies *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Chen *et al.*, 2016; Li *et al.*, 2019), among others, psylloids generally have large genomes, with estimated sizes ranging from 508.27-Mb to 724-Mb (Nakabachi *et al.*, 2010; Kwak *et al.*, 2023; Lei *et al.*, 2024). Therefore, given their substantial genome sizes, the exact size of each gDNA fragment could not be determined in this study.

There were significant variations in gDNA quality among the gDNA samples extracted from the initial five-psyllid species actively collected from Eureka lemons (Figure 3. 4). *Euryconus* sp. had lower quality gDNA as compared to other psyllid species that had a high quality (Figure 3. 4). A similar trend persisted among gDNA samples from psylloids also collected in the citrus orchards but from different citrus plant species or varieties, as illustrated in Figure 3. 5A-D. *Acizzia* sp.2, *Diaphorina virgata* and *Pauropsylla trichaeta* had lower-quality gDNA with much dimmer bands (Figure 3. 5A). Similar variations in gDNA quality were also observed across other psyllid species (Figure 3. 5B), where only the gDNA samples extracted from *Acizzia* sp.1, *Diaphorina zebrana* and *Retroacizzia mopanei* were of lower quality. Furthermore, in Figure 3. 5C, the gDNA samples from *Diaphorina virgata* and *Diaphorina zebrana* had significantly lower quality compared to the

gDNA sample extracted from *Euryconus* sp. having a slightly lower quality. Figure 3. 5D further confirmed these variations, where the gDNA samples from only two psyllid species; *Ciriacremum capense* and *Diaphorina punctulata* (well 2DP), had a significantly lower quality, indicated by the dim appearance of their respective bands.

Several reasons may explain the significant variations in the quality of the extracted gDNA from psylloid samples, including factors related to preservation methods and specimen characteristics. A study by A'Hara *et al.* (1998) investigated storage methods for preserving spiders before gDNA extraction. They found that storage in ethylene glycol or 70% ethanol at room temperature resulted in significant DNA degradation after three weeks, wherease storage at -80 ℃ proved most effective for preserving specimens for up to a year. This is supported by Hajibabaei *et al.* (2005), Vink *et al.* (2005) and Haelewaters *et al.* (2015), who previously found that preserving insect samples in 70% ethanol can result in the degradation of DNA, and further affect PCR amplification due to acidification, especially after prolonged storage without ethanol refreshment. Similarly, another study by Haelewaters *et al.* (2015) reported a significant decrease in DNA quality when insects were stored in 70% ethanol. In the current study, all psylloid samples, whether actively or passively collected, were preserved in 70% ethanol immediately after being collected and stored at room temperature for varying durations, ranging from several months to nearly a year before DNA extraction. Surprisingly, our results contradicted previous findings regarding the impact of ethanol preservation on gDNA quality. High-quality gDNA was obtained from the specimens regardless of insects preserved in 70% ethanol at room temperature, with some samples stored for nearly a year still showing the highest quality gDNA. This suggests that, in the context of psylloid samples, ethanol preservation may not significantly affect the gDNA quality over time, although this was not the focus of this study. Hazir & Bock (2019) on sand bees revealed that ethanol-preserved samples yielded better-quality DNA for analysis and PCR amplification compared to dry-mounted specimens. Furthermore, other studies have reported that factors, such as specimen size, time since collection, presence of DNA-degrading contaminants, etc., can also influence gDNA quality and PCR amplification (Andersen & Mills, 2012; Hazir & Bock, 2019). Although in the current study variations in psylloid sizes were observed, suggesting a potential impact on gDNA quality, further studies specifically investigating whether psylloid size directly affects gDNA quality are required to validate this hypothesis and enhance our understanding of DNA extraction from these insects.

3.4.2. Primer testing and selection for plant leaf gDNA amplification.

The third objective of this chapter was to identify the most effective primer pair for amplifying gDNA samples extracted from 15 citrus leaf samples. The universal primers capable of amplifying specific regions of DNA across a wide range of organisms were used in the amplification of plant DNA in molecular systematic studies (Sang *et al.*, 1997; Tate & Simpson, 2003; Kress & Erickson, 2007; Kress *et al.*, 2009; Haider, 2011). In the current study, three universal primer pairs were tested using gDNA extracted from a small leaf-square sample of the Star Ruby variety. Among the tested primer pairs, only rbcLaF/19 failed to amplify the targeted region of the *rbcL* chloroplast gene. However, the reason for this failure remains unknown, as PCR set-up contamination was ruled out, as indicated by the corresponding NTC. Trujillo-Argueta *et al.* (2022) in their study found that several factors, such as methodological failures, reactant issues, equipment problems or degradation of plant DNA, might contribute to plant DNA amplification failure in some samples, as well as the universal primer pairs as they might not work uniformly across all plant species. Therefore, following this literature, it is possible that the rbcLaF/19 primer pair was not suitable for amplifying gDNA samples from the Star Ruby variety but can work effectively on other citrus leaf gDNA samples.

The primer pairs, rbcLaF/R and trnL575F/755R, successfully amplified the plant gDNA, yielding PCR amplicons of the expected size of about 530-bp and 180-bp, targeting the *rbcL* and *trnL* chloroplast genes regions, respectively. These primer pairs are commonly used in ecological and molecular studies due to their efficient amplification of their respective target DNA regions (see Matheson *et al.*, 2008; Kress *et al.*, 2009; Kajtoch & Mazur, 2015; Avanesyan *et al.*, 2021). These primer pairs efficiently amplified extracted gDNA from the Star Ruby variety, suggesting their potential applicability to other leaf gDNA samples. Although both primer pairs proved effective, the rbcLaF/R primer pair was considered the most effective compared to trnL575F/755R, primarily attributed to the brighter appearance of its PCR amplicon. The *rbcL* gene, targeted by this primer pair, is highly conserved and commonly used in plant taxonomic and phylogenic studies due to its ability to resolve relationships across diverse plant species (Kress *et al.*, 2009; Kang *et al.*, 2017; Sundari & Papuangan, 2019). However, the current study does not rule out factors, including PCR setup and volume of the PCR amplicon loaded (5 µL) in agarose gel, that might have contributed to these differences in the appearance of PCR amplicons. Although the trnL575F/755R primer pair

was not chosen for further analysis, it does not rule out that it can efficiently amplify the citrus gDNA (Cooper *et al.*, 2016).

3.4.2.1. Polymerase chain reaction amplification of plant leaf gDNA samples:

Following the primer testing and selection, gDNA samples from each of the 15 citrus leaf samples were successfully amplified using the rbcLaF/R primer pair. The success of PCR amplification for all leaf gDNA samples using these primers was confirmed by the produced PCR amplicons, each corresponding to a specific gDNA sample extracted from a particular leaf sample. Furthermore, these amplicons had an expected size of approximately 530-bp region, representing the targeted *rbcL* gene. To further confirm the success of PCR amplification across all leaf gDNA samples, the resulting amplicons were analysed for brightness uniformity on an agarose gel. Consequently, the majority of the amplicons showed a consistent brightness, confirming the successful amplification. However, an exception was observed with the amplicon representing the Star Ruby variety, which appeared slightly dimmer. The dim appearance of this amplicon may indicate that a lower volume of this amplicon was loaded onto the agarose gel. This may have occurred possibly due to a small portion of this amplicon remaining on the loading tip during the gel-loading process, leading to its dim appearance. Therefore, to enhance the reliability of future results, it is important to prevent any residue from remaining on the loading tips during the amplicon loading process and adjust the loading volumes as necessary based on sample characteristics, consequently, maintaining uniform brightness across agarose gel wells.

3.4.3. Primer testing and selection for insect gDNA amplification.

The fourth objective of this chapter aimed to select a primer pair capable of efficiently amplifying all the gDNA samples extracted from the chosen psylloid species. This step was crucial to confirm that the extracted gDNA was only from these insects, eliminating the possibility of contamination from other organisms, including but not limited to plants, viruses or fungi before proceeding with the psyllid gut content analysis. We initially tested three universal primer pairs: DCITRI COI-L/R; Te-6U30/720L26; and CO1-F3/R3, against gDNA samples extracted from a test sample, *Trioza erytreae*, using the four previously described extraction methods. These primer pairs were selected based on their reported insect specificity in previous studies (Boykin *et al.*, 2012; Swisher *et al.*, 2012; Pérez-Rodríguez *et al.*, 2019). Recent molecular studies have successfully utilised these

primers to confirm the identification of psyllid pests in non-native regions, such as *Trioza erytreae* and *Diaphorina citri* in regions like Benin and Ghana. Visual identification, especially by non-taxonomists, often results in misidentification due to the morphological similarities among insect species (Piper *et al.*, 2019; Martoni *et al.*, 2020, 2022; Aidoo *et al.*, 2023; Sétamou *et al.*, 2023).

Among these primer pairs, only Te-6U30/720L26 successfully amplified all four gDNA samples from *T. erytreae* samples, each extracted using a specific method, producing consistent and visibly bright PCR amplicons. Furthermore, these amplicons had an estimated size of approximately 750-bp, slightly larger than the expected size of 714-bp (Pérez-Rodríguez *et al.*, 2019). Although the amplicon size was slightly larger than expected, our results confirmed that the gDNA samples extracted using the four methods originated from *T. erytreae*. However, the remaining primer pairs: namely DCITRI COI-L/R and CO1-F3/R3, failed to amplify gDNA samples extracted from *T. erytreae* samples using the Quick-DNA Tissue/Insect Miniprep Kit and Salting-Out (TNES) methods, indicating potential limitations in their insect specificity. Several factors could explain the failure of these primer pairs to amplify the gDNA samples of *T. erytreae*, considering the sensitivity of PCR methods where even minor variations in setup or the presence of inhibiting agents post-extraction can influence the results (Chen *et al.*, 2000; Agustí *et al.*, 2003; Juen & Traugott, 2005). To ensure that the PCR amplification failure using these primer pairs was not due to either primers, PCR setup or contamination, all corresponding NTCs remained clear, confirming that no contamination during PCR setup. However, one possible factor that might have contributed to the failure of *T. erytreae* gDNA amplification could be the methods used for gDNA extraction. This is supported by the absence of the corresponding gDNA bands, as discussed in **section 3.3.1.2** on insect DNA extraction method testing and selection.

Although the DCITRI COI-L/R and CO1-F3/R3 primer pairs could not amplify *T. erytreae* gDNA samples extracted using the Quick-DNA Tissue/Insect Miniprep Kit and Salting-Out (TNES) methods, those extracted using the Quick-DNA Miniprep Plus Kit and PureLink Genomic DNA Mini Kit methods were amplified with the same primer pairs. Despite the successful amplification, the produced PCR amplicons from the DCITRI COI-L/R and CO1-F3/R3 primer pairs were faint, yet visible, with an estimated size of 749-bp for the DCITRI COI-L/R primer pair, contrary to the expected 834-bp size according to Boykin *et al.* (2012). The CO1-F3/R3 primer pair produced a PCR amplicon of the expected size of 500-bp, aligning with the literature findings (Swisher *et al.*,

2012). Despite these findings, the Te-6U30/720L26 primer pair was chosen for the downstream experiments in the current study due to the consistent effectiveness in amplifying all *T. erytreae* gDNA samples extracted using each of the four methods, as well as the bright appearance of each PCR amplicon it produced.

3.4.3.1. Polymerase chain reaction amplification of insect gDNA samples:

The Te-6U30/720L26 primer pair successfully amplified gDNA samples extracted from selected psylloid species. This PCR amplification not only demonstrated successful gDNA amplification of the selected psylloid species but also confirmed the specificity of these gDNA samples. The Te-6U30/720L26 primer pair successfully amplified all gDNA samples extracted from the following species: *Diaphorina punctulata*, *Diaphorina virgata*, *Diaphorina zebrana*, *Psylla loranthi*-group, *Retroacizzia mopanei* and *Trioza erytreae*. To the best of our knowledge, this is the first study to report the successful amplification of gDNA of other psyllid species using the Te-6U30/720L26 primer pair, which was initially documented to be specific to *T. erytreae* (Pérez-Rodríguez *et al.*, 2019), therefore, expanding its known specificity beyond this psyllid species.

Amplified gDNA samples yielded PCR amplicons with a uniform estimated size of approximately 750-bp. However, there were significant variations in the visual appearances of amplicons, with most amplicons demonstrating a consistently higher brightness appearance, confirming their high-quality gDNA, compared to a few that appeared relatively dimmer which indicates a low-quality gDNA (see **section 3.3.2.4**). A study by Ye *et al.* (2012) has previously found that PCR amplicon outcomes can be influenced by several factors, including but not limited to DNA template quality, PCR reaction conditions or PCR cycling parameters. In this study, the PCR cycling parameters were consistent across all PCR runs. However, variability in insect DNA template quality and/or concentration during PCR setup may have contributed to the observed differences in the brightness of PCR amplicons.

Despite the successful amplification from most psylloid species, the Te-6U30/720L26 primer pair failed to amplify gDNA samples of the following five psyllid species: *Acizzia* sp.1, *Acizzia* sp.2, *Ciriacremum capense*, *Euryconus* sp. and *Pauropsylla trichaeta*. This failure was consistently observed in gDNA samples extracted from *Acizzia* sp.1, *Acizzia* sp.2 and *Euryconus* sp. Although previous sections have discussed several factors contributing to the inability of the primer pairs to

amplify the gDNA samples (though this may not apply to Te-6U30/720L26), one possible reason that might have led to its failure to amplify gDNA from the aforementioned psyllid species could be its lack of specificity to these species, indicating that it is species-specific (based on the existing literature) and possibly genus-specific (according to the findings of this current study). Therefore, it is possible that this primer pair does not work for these five species. For future studies, it would be beneficial to explore the amplification ability of DCITRI COI-L/R or CO1-F3/R3 primer pairs (rejected primer pairs in this study) on gDNA from the aforementioned psyllid species to ascertain their specificity and effectiveness in amplifying their gDNA.

3.4.4. Plant DNA detection from the gut contents of the field-collected insect samples.

The detection of plant DNA from the gut contents of collected phytophagous insect samples is an important aspect of understanding their feeding behaviour and host-plant interactions in the natural field environment. Previous research has primarily focused on detecting plant DNA in the guts of leaf-chewing insects, including beetles, moths, weevils and other orthopterans (Miller *et al.*, 2006; Jurado-Rivera *et al.*, 2009; Kitson *et al.*, 2013; Wallinger *et al.*, 2013, 2015; Avanesyan, 2014; Avanesyan & Culley, 2015; Gonella *et al.*, 2020). This focus was primarily due to the abundance of chloroplast DNA, which serves as a target for molecular analyses and is often found in the guts of these insects, or due to their consumption of large quantities of plant tissues (Bennett & Moran, 2013; Avanesyan, 2014). Although past studies primarily examined leaf-chewing insects for plant DNA detection in their gut contents, recent studies have expanded this focus by conducting gut content analysis in hemipteran phloem-feeding insects (Briem *et al.*, 2018; Cao & Dietrich, 2021). For example, several studies on potato/tomato psyllid *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae), potato leafhopper *Empoasca fabae* (Harris) (Hemiptera: Cicadellidae) and the spotted lanternfly *Lycorma delicatula* (White) (Hemiptera: Fulgoridae) feeding preferences have been identified through the molecular gut content analysis, which revealed the presence of plant DNA in their guts (Pearson *et al.*, 2014; Mustafa *et al.*, 2015; Cooper *et al.*, 2016; Avanesyan & Lamp, 2020).

In this study, the *rbcL* gene, a targeted plant DNA region, was successfully amplified in the guts of only five of the 11 analysed psylloid species, including *Diaphorina punctulata* (collected from Orri and Valencia), *Diaphorina virgata* (Eureka Lemons, Leanri and Orri), *Diaphorina zebrana*

(Orri), *Euryconus* sp. (Eureka Lemons) and *Trioza erytreae* (Nadorcott, Eureka Lemons and Mandarin) (see **section 3.3.3**). However, the appearance of the amplicons produced from the amplified plant DNA in the guts of these psyllids was relatively dim. Studies by Pumariño *et al.* (2011) and Staudacher *et al.* (2011) have previously reported that sap-sucking insects generally have a lower amount of plant DNA in their guts compared to the leaf-chewing insects, and further highlighted that the enzymatic processes in their digestive system may breakdown plant sap more rapidly compared to chewed plant tissue. The dim appearance of the PCR amplicons in this study might have been attributed to a lower quantity of amplified plant DNA in the guts of these insects. Furthermore, Briem *et al.* (2018) reported that plant DNA undergoes either partial or complete degradation during the digestive process in the guts of the insects. Although the current study did not analyse the digestive process in psylloid samples as this was beyond the focus, it cannot be ruled out that the ingested plant DNA might have experienced some degree of degradation within the guts of these insects. Results in this study could suggest a potentially low plant DNA quantity available for the amplification in the guts of psylloids.

The analysis of insect gut contents revealed the presence of multiple PCR amplicons in some wells, suggesting non-specific binding (NSB) (see Figure 3. 11 and Figure 3. 12). Symes *et al.* (2019) indicated that due to plant species having varying base pair lengths between primer binding sites, multiple bands could arise due to the presence of diverse plant DNA in the insect gut. In this study, the host-plants of most psylloid species in the surveyed citrus environments were either abundant or accessible to these insects. This suggests that these insects may have initially fed on their main host-plants before being caught on citrus in the orchards. A similar study by Barthel *et al.* (2020) focused on investigating the feeding habits of the psyllid, *Cacopsylla picta* (Förster) (Hemiptera: Psyllidae), also found a wide range of plant DNA from different genera in their gut. Their study further suggested that psyllids may either feed on or probe plants beyond their primary host even while present in a particular habitat, as observed in apple orchards. Similarly, Hereward & Walter (2012) investigated host-plant interactions of green mirid *Creontiades dilutus* (Stål) (Hemiptera: Miridae) and found that this highly motile bug frequently feeds on other plants than the one from which it was collected, indicating potential movements and multiple plant usage. Erjavec (2020) recently indicated that another contributing factor to NSB could be annealing temperature during PCR, which affects primer binding to targeted plant DNA regions. Recent findings by Yunita *et al.* (2023) emphasise the critical role of annealing temperature, with higher temperatures resulting

in specific primer binding to the targeted plant DNA regions and lower temperatures leading to NSB and/or non-specific amplicons. Although the annealing temperature of 55 °C was successful for the targeted plant DNA amplification within the guts of some psyllid samples, the presence of NSB suggests that the primer pair used may require a different annealing temperature for specific binding to target DNA regions across different plant species. Future studies should address this to optimise the PCR conditions for accurate plant DNA detection in insect gut contents.

The successful plant DNA amplification confirms the presence of plant DNA in the guts of field-collected insect samples. Plant DNA was detected in the guts of some psyllid species: *Diaphorina punctulata*, *Diaphorina virgata*, *Diaphorina zebrana*, *Euryconus* sp. and *Trioza erytre*ae, which were all sampled from different citrus plants as previously outlined. The detection of plant DNA in the guts could suggest that these insects had recently ingested plant phloem-sap, likely within a few hours before their capture in citrus orchards. These findings complement a recent study by Avanesyan & Lamp (2020), who also investigated the presence of the ingested plant DNA in the guts of the spotted lanternfly, an invasive pest of agricultural and forestry crops in the eastern US. They used a molecular gut content analysis approach to determine whether plant DNA ingested by these insects corresponds to the plants from which these insects were collected. Their results showed reliable detection of up to 534-bp of the chloroplast *rbcL* gene region from ingested plants in the gut contents of these insects. Interestingly, they found that the ingested plant species often did not match the plants from which the insects were collected, indicating broader range of ingested plant species than sampled plants, including both native and non-native plant species, woody and non-woody plants, and even plant species not previously documented as host-plants for the spotted lanternfly.

However, our study differs slightly in the methodology focusing on analysing the gut contents of adult psylloids, whereas Avanesyan & Lamp (2020) focused on third and fourth-instar nymphs of the lanternfly, causing damage to a wide range of plant species. Pumariño *et al.* (2011) detected tomato plant DNA in the gut contents of multiple insect species, including *Macrolophus pygmaeus* (Rambur) (Heteroptera: Miridae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), and proposed a recent ingestion of the plant material by these insect species based on decreasing DNA detection over time. Although Pumariño

et al. (2011) employed the tomato-specific *ITS* (internal transcribed spacers) primers, the current study used the *rbcL* primers capable of amplifying a broader range of plant species.

Several studies have documented that plant DNA can still be detected in the guts of field-collected insects even after being subjected to treatment or sterilisation before analysing their gut contents. This implies that the detected plant DNA in the guts was ingested by the insects rather than being a surface contaminant (Cooper *et al.*, 2016; Briem *et al.*, 2018). In the current study, all selected psyllid species were subjected to sterilisation before analysing their gut contents, and plant DNA was still detected in the guts of some of the sterilised psyllid species/samples. This complements previous studies, indicating that the targeted plant DNA detected was likely present in the ingested contents rather than being a surface contaminant on the insect body. The gut contents of *Euryconus* sp. and *Diaphorina punctulata* collected from Eureka lemons and Valencia, respectively, had amplified targeted plant DNA regions in their guts that produced PCR amplicons of the expected size of 530-bp (Figure 3. 11 and Figure 3. 12), indicating that these psyllids had likely fed on these plants in citrus orchards. These findings suggest that *Euryconus* sp. and *Diaphorina punctulata* may utilise citrus as an alternative feeding host, given their observed abundance on these plants during the field surveys in citrus environments. Although the known host-plants of *Diaphorina punctulata* and *Euryconus* sp. are marula [*Sclerocarya birrea* (A. Rich) Hochst. (Anacardiaceae)] and diesel tree [*Copaifera langsdorfii* Desf. (Fabaceae)], respectively (Capener, 1970a, 1970b; Burckhardt & de Queiroz, 2012), there currently is no existing literature on *Euryconus* sp. feeding on any plant species other than its known host-plant. Therefore, this study is the first to document or report that *Euryconus* sp. may also feed on other plants not previously reported as its host.

Our findings further complement previous research by Catling & Atkinson (1974), which cited Catling (1970b), documenting *Diaphorina punctulata* and *Diaphorina zebrana* feed on citrus in South Africa and Swaziland. Unlike the earlier study, our research involved analysing the gut contents of *Diaphorina punctulata* and *Diaphorina zebrana*, and other selected psylloid species actively collected from the citrus orchards, providing more accurate findings related to the feeding habits or behaviour of these psyllids on citrus. It is plausible that the citrus plants whose DNA was detected in the guts of *Diaphorina punctulata* and *Euryconus* sp. could serve as shelter plants, providing either refuge or food resources, supporting the life behaviour of these psyllid species in citrus environments (Burckhardt *et al.*, 2014), particularly in the absence of their primary host-

plants. Furthermore, the amplified plant DNA in the guts of most psyllid species produced PCR amplicons with estimated sizes ranging from 500-bp to 750-bp (see Figure 3. 11 and Figure 3. 12). This could suggest the potential feeding of these psyllids on plant species surrounding the orchards, either hosts or non-hosts rather than on citrus. However, some amplicons (500-bp and 540-bp in size) closely matched the targeted size of 530-bp region (see Figure 3. 12A, B and D), suggesting that these insects might have indeed fed on these citrus plants.

Although plant DNA was successfully detected in the guts of some psylloid species, suggesting their potential to feed on either citrus or adjacent plants, plant DNA could not be detected in the guts of other analysed psyllid species, such as *Acizzia* sp.1 and *Acizzia* sp.2 (both collected from Orri), *Ciriacremum capense* (Star Ruby), *Diaphorina punctulata* (Eureka Lemons, Delta swingle, Nadorcott and Valencia), *Diaphorina zebrana* (Valencia), *Euryconus* sp. (Valencia), *Psylla loranthi*-group (Delta Valencia), *Pauropsylla trichaeta* (Eureka Lemons), *Retroacizzia mopanei* (Late Valencia) and *Trioza erytreae* (Eureka Lemons), despite being collected from the citrus. This suggests that these particular psyllids; (1) may not have fed on citrus plants or any other available plants at the time of collection in citrus orchards, or (2) might have interacted with these citrus plants for alternative purposes rather than feeding, including but not limited to resting. However, it is still possible that plant DNA in their guts was too degraded or the quantity was too small for detection in the guts. Matheson *et al.* (2008) and Wang *et al.* (2017), cited by Avanesyan & Lamp (2020), found that different plant DNA can degrade at varying rates in insect guts and that some plants the insects consumed might have been digested quickly, leading to complete degradation of plant DNA, thus, making it undetectable in their guts. This could therefore explain why plant DNA in this study could not be detected in the guts of other psylloid species, suggesting that the plants these particular psyllids fed on might have undergone rapid digestion or rapidly degraded before collection, resulting in undetectable plant DNA in their gut contents. For example, within the cell rupture-feeding bug *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae), the plant DNA was amplifiable up to 20 hours post-feeding, whereas the spotted wing drosophila *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) retained detectable plant DNA in the guts for up to 7 days post-ingestion (Li *et al.*, 2016; Wang *et al.*, 2017b; Diepenbrock *et al.*, 2018). Therefore, this suggests that it might be best to extract plant DNA from the psylloid guts immediately after being captured to prevent further digestion and degradation of any plant DNA material present in their guts.

The choice of targeted plant DNA region (530-bp) for plant DNA detection might not have been optimal for gut content analysis in this study. Previous studies emphasised the importance of using shorter DNA regions for molecular analysis of insect gut contents, as they are more amenable to amplification even when the DNA is degraded, thereby enhancing detection rates of ingested DNA (Hoogendoorn & Heimpel, 2001; McMillan *et al.*, 2007; Staudacher *et al.*, 2011). For example, previous research by the CBOL (2009) found that the *rbcL* region (599-bp) resulted in low plant DNA amplification rates in mirid bugs due to degradation. In contrast, Wang *et al.* (2019) used smaller DNA regions (*ITS* and *trnH-psbA*) suitable for the PCR amplification of the degraded plant DNA, successfully identifying host plants to genus and species levels. This suggests that targeting shorter DNA regions could improve the probability of successful plant DNA detection in psylloid guts, especially considering that plant DNA degradation increases with increased digestion time post-feeding.

In conclusion, the lack of knowledge concerning the digestion time post-feeding, the choice of targeted plant DNA region, and other factors, including but not limited to feeding time and size of the insect (not discussed in this current chapter) may have influenced the plant DNA detection in the psyllid gut samples analysed in the current study. Therefore, future research should consider these factors to enhance the reliability of detection and identification of plant DNA in the guts of psylloids, which has implications for pest management strategies and understanding insect-plant interactions. Increasing our understanding of the association between psylloids and citrus plants is important for pest management and the classification of pests. This knowledge will inform citrus farmers or growers concerning the necessity of developing effective pest management strategies to suppress psylloid populations, ultimately safeguarding citrus crops and promoting sustainable agricultural practices.

This current study, through molecular gut content analysis, found that plant DNA can be detected in the gut contents of some adult psyllid species collected from diverse plants in commercial citrus environments. This suggests that insects may use different resources when their host-plants are not available, further expanding our knowledge on the feeding habits or behaviour of some psyllid species in these environments. However, the PCR-amplified plant DNA samples from the psylloid guts could not be sequenced using Sanger sequencing due to time constraints. For future research, sequencing the PCR samples using Sanger sequencing would be beneficial to confirm or identify

the plant species whose DNA was ingested and/or detected within the guts of these psylloids. The detection of citrus DNA in the guts of some psyllid species raises significant concerns about their potential role as vectors of plant-pathogenic bacteria, a current knowledge gap that requires further research (possibly infection trial studies) to address these critical questions. However, the detection of non-citrus plant DNA in other psylloid species is less concerning, potentially reducing concerns about their threats to the citrus industry. Therefore, there is a need to evaluate potential biocontrol agents targeting psyllid species that showed evidence of feeding on citrus, particularly considering that one of these species belongs to a genus that includes *Diaphorina citri* (Asian citrus psyllid), a well-known citrus pest and vector of citrus greening agents.

CHAPTER 4

GENERAL DISCUSSION

4.1. Introduction

There is currently a significant gap in the existing literature concerning psylloid species occurring in commercial citrus environments (i.e. citrus orchards and their adjacent natural vegetation) and their ecological role in these specific environments (Chapter 2). Some of these psylloid species are considered serious pests, posing significant threats to the health of fruit trees and overall crop productivity, resulting in major economic losses (Burckhardt, 1994; Ouvrard *et al.*, 2015a, 2015b). Given the role played by citrus as a globally significant cultivated fruit crop for food and nutritional security (Franco-Vega *et al.*, 2016), filling these knowledge gaps within the existing literature is substantial for addressing challenges that might potentially arise from psylloids in citrus orchards and further facilitate the development of effective pest management strategies against these insects within the citrus industry, thereby enhancing its overall sustainability. Therefore, this thesis sought to address these knowledge gaps by identifying and documenting psylloid species occurring across varied citrus environments, and further uncovering their ecological roles in these environments, with a specific focus on their potential as citrus plant feeders. In order to achieve the study aim, two primary objectives were set out: firstly, to determine the diversity and community structure of psylloid species in citrus environments (Chapter 2); and secondly, to determine the presence of citrus DNA in the guts of psylloid species to confirm their potential to feed on citrus plants or other alternative plants (Chapter 3).

4.2. Diversity of psylloid species in commercial citrus environments

Field surveys for the collection of psylloids conducted in commercial citrus environments revealed a significantly high diversity of psylloid species, which was represented by 47 species recorded within these environments, as documented in Chapter 2. This richness in psylloid species was strongly correlated with the diversity of plant species present in citrus environments. Studies have consistently highlighted that insect diversity and distribution in any particular environment are influenced by several factors, including the host-plant availability and environmental conditions (Dangles *et al.*, 2009; Sollai *et al.*, 2017; Reside *et al.*, 2018; Ashlee *et al.*, 2021). A recent study by Yang & Chen (2021) further emphasised the significance of host-plant availability in supporting

the diversity of insect species, as these plants act as suitable oviposition sites for adult females and vital food sources for immature stages, significantly contributing to species establishment. Thus, it is not surprising that the rich diversity of psylloid species found in citrus environments could be attributed to high plant diversity. These findings further agree with those of Yager *et al.* (2016), although their focus was on butterfly species in a forest nursery ecosystem in Nigeria. They discovered a rich diversity of butterfly species (17 species), attributing it mainly to the abundance and accessibility of diverse flowering and ornamental trees in this environment. The butterfly study was undertaken over two months while this study continued for nine months, and the extended timeframe likely contributed to the recording of a higher number of psylloid species. Similarly, research by Chiawo *et al.* (2017) examined bee composition and floral resources in the forest core and adjacent farmlands in Kenya, recording a total of 41 bee species over six months of sampling, and correlated this with the species richness of annuals and overall floral diversity present.

Despite our study duration being nine months, the analysis of species accumulation curves (SACs) indicated that the sampling effort was inadequate to capture or sample all psylloid species present in the surveyed citrus environments. The SACs suggested that additional sampling, particularly using the sweep-netting method, could lead to the discovery of more new psylloid species in citrus environments. This is a common phenomenon in ecological studies (Work *et al.*, 2002; Scharff *et al.*, 2003). However, the alternative method appeared to have captured a sufficient number of psylloid species in citrus environments, particularly in citrus orchards, suggesting that continuous sampling efforts using this method may not be necessary. Extending the sampling periods in the butterfly and bee studies beyond two and six months, respectively, would likely increase the chances of capturing new unique species. Therefore, longer sampling periods should be considered when assessing the insect diversity in different environments to ensure more accurate records.

Although a rich diversity of psylloid species was recorded across the surveyed citrus environments, the abundance of psylloids varied between citrus orchards and adjacent natural vegetation when using a sweep-net, with higher abundance recorded in the latter habitat. Several ecological studies have consistently outlined that the structure of vegetation in an environment may influence both the abundance and diversity of insects (Abdullah & Sina, 2009; Khadijah *et al.*, 2013; Landsman & Thiel, 2021; Harris & Ratnieks, 2022). Sandeep *et al.* (2023) recently found that the abundance of insects is strongly influenced by the quantity and quality of food available in any habitat. When

there is an ample food supply, insect populations can increase. Research by Curtis *et al.* (2015) also found significant positive correlations between butterfly abundance and the presence of food resources, with both the availability of host-plants and nectar serving as important predictors of butterfly population density. In our study, the vegetation was more diverse in plant species than the citrus orchards, which could explain the higher psylloid abundance in this habitat. Comparable results were reported in a study by Herdiawan *et al.* (2021), who investigated the effects of Surjan and conventional rice field ecosystems on the diversity and abundance of detritivores, pollinators and other insects. Their results indicated that the abundance of these insects was higher in Surjan rice fields, which included both rice and horticultural crops, compared to conventional rice fields that were solely planted with rice. Furthermore, another factor that might have contributed to the differences in the abundance of psylloids between these two habitats might be the application of insecticides in the surveyed citrus orchards. In our study, no psylloids were sampled in the orchards treated with insecticides, especially when sampling occurred a few days post-treatment, consistent with the findings of Thomson & Hoffmann (2006) and Park *et al.* (2015), who also reported insect population decline following insecticide application in the orchards. Therefore, the abundance of psylloids in commercial citrus environments is influenced by a combination of factors, such as the structure and diversity of vegetation, availability of food resources, insecticide use and potentially other ecological variables.

The similarity in species richness and composition between Limpopo and Mpumalanga provinces contrasts with the distinct abundance of psylloids collected within these regions. These provinces share ecological characteristics such as rich natural vegetation and extensive agricultural activities, which support diverse arthropod populations. Despite these similarities, psylloid abundance was consistently higher in the surveyed citrus environments of Limpopo compared to Mpumalanga. As discussed in recent studies (Reside *et al.*, 2018; Minor *et al.*, 2021; Sandeep *et al.*, 2023; Abid *et al.*, 2024), different biotic and abiotic factors influence the diversity, distribution and abundance of insect species. However, in the current study, we discovered that among the possible factors that might have significantly contributed to the higher psylloid abundance in Limpopo could be the number of field sites surveyed. A total of eight different citrus environments were surveyed in Limpopo, compared to four in Mpumalanga, each site with a diverse range of plant species. Studies have shown that psylloids are often host-specific, and their presence or establishment in terrestrial environments is influenced by the availability of associated host-plant species (Hodkinson, 2009;

Burckhardt *et al.*, 2014). Therefore, surveying more field sites in Limpopo allowed for sampling a wider variety of plants, resulting in a higher number of psylloids recorded than in Mpumalanga with fewer sites surveyed, which led to a lower psylloid count. However, suppose the number of field sites selected for the surveys was equal between Limpopo and Mpumalanga provinces. In that case, we believe that high psylloid abundance would have also been recorded in the latter province as most plants would have also been sampled, and the majority of plant species sampled in Limpopo were also sampled in Mpumalanga. Furthermore, the slight difference in the number of host-plants sampled (six in Limpopo and five in Mpumalanga) may have also contributed to the observed differences in psylloid abundances between these provinces. Future research should aim to balance the number of field sites and host-plants surveyed in different provinces to provide more accurate comparisons of psylloid abundance and distribution in citrus environments.

In citrus environments, several psylloid genera were recorded, with only a few, such as *Cacopsylla*, *Diaphorina*, *Glycaspis* and *Trioza*, recognised for harbouring major pests of economic importance globally (Halbert & Manjunath, 2004; Martínez *et al.*, 2018; Jarausch *et al.*, 2019; Morrow *et al.*, 2020; Zhao *et al.*, 2023). Among these, psylloid species in the *Cacopsylla*, *Diaphorina* and *Trioza* genera are identified as major pests in agricultural ecosystems, particularly affecting cultivated crops as outlined in Chapter 1, while those belonging to *Glycaspis* primarily pose threats within forest ecosystems (Brennan *et al.*, 1999; Martínez *et al.*, 2018). Interestingly, none of the psylloid species documented in these environments has been reported as pests of cultivated plants, except for *Trioza erytreae* and *Glycaspis brimblecombei*a (pest of eucalyptus trees) (Brennan *et al.*, 1999; Halbert & Manjunath, 2004; Martínez *et al.*, 2018). However, despite the lack of existing literature concerning the pest status of these psyllids in citrus environments, their presence raises concerns regarding their potential to feed on citrus trees. This concern is based on the fact that many psylloid species are either monophagous or oligophagous, meaning they feed and complete their life cycle on a single or a few host-plant species (Hodkinson, 1974).

4.3. Host-plants associated with psylloid species in commercial citrus environments

In the surveyed citrus environments, a variety of plant species were sampled, among which *Ficus* sp., marula, mopane and *Vachellia* spp. were identified as host-plants for some collected psyllid species, as outlined in Chapter 2. A previous study by Capener (1970a, 1970b) documented marula as a host-plant for *Diaphorina punctulata* and *Diaphorina virgata*. This aligns with our findings,

as both *Diaphorina punctulata* and *Diaphorina virgata* were among the psylloid species sampled across diverse plants in citrus environments. Interestingly, although these *Diaphorina* species were sampled on multiple plant species, such as citrus, *Ficus* sp. and marula, a higher abundance of both psyllids was consistently associated with marula. Furthermore, their distribution in the surveyed citrus environments correlated significantly with the prevalence of marula plants, thus confirming marula as a primary host-plant for both psyllid species. This was further confirmed by collecting their immatures on the same marula plants where adults were found. The term "host-plant" refers to a plant species that supports the complete development of a psyllid life stages, from eggs and immature stages to adults (Burckhardt *et al.*, 2014), Thus, our findings confirm marula as a host-plant for *Diaphorina punctulata* and *Diaphorina virgata*.

Several studies have reported associations between *Acizzia* species and *Vachellia* spp. and to a lesser extent, some *Albizia* spp. (Webb & Moran, 1974; Yen, 2002; Taylor & Moir, 2009). Our findings support these reports, as *Acizzia russellae*-group was mostly sampled from *Vachellia* spp. with fewer occurrences on citrus and mopane plants. However, its highest abundance across all surveyed citrus environments was consistently recorded on *Vachellia* spp., and its distribution was closely correlated with the prevalence of this plant species; the same inline context was discovered by Powell *et al.* (2012). Furthermore, the immatures of this psyllid were collected from the same plant species as the adults, further confirming *Vachellia* spp. as a host-plant. These findings are consistent with those of Webb & Moran (1974), who also documented both adults and immatures of *Acizzia russellae* on *Vachellia karroo* in southern Africa. In the current study, we could not classify the sampled *Vachellia* sp. to the species level but rather to the genus level due to the lack of botanical classification materials. However, given the presence of immatures on the sampled *Vachellia* spp., it is possible that this plant is *V. karroo*, but further confirmation is required to verify this.

Similarly, a previous research by Oppong *et al.* (2010, cited Ernst & Sekhwela, 1987) documented mopane as a host-plant for several insects, including *Retroacizzia mopanei*. The current study complements this previous research, as immatures of *Retroacizzia mopanei* were collected from mopane in adjacent natural vegetation. Furthermore, during field surveys in citrus environments, adults of *Retroacizzia mopanei* were collected on both mopane (same as the immatures) and some citrus plants. Despite its occurrence on two distinct plant species, our study revealed a significantly

higher abundance of this psyllid species on mopane plants compared to citrus, with its distribution closely associated with the presence of mopane in the surveyed citrus environments.

4.4. Monitoring methods of psylloids in commercial citrus orchards

Monitoring pest populations is a crucial component of Integrated Pest Management (IPM), as it is fundamental for assessing pest dynamics and determining pest status in the field (Miranda *et al.*, 2017; Daniel *et al.*, 2018). In the context of citrus orchards, effective pest monitoring ensures that citrus growers are aware of the presence or absence of insect pests attacking their crops, providing an early warning system for decision-making regarding the evaluation of control methods and management actions, and this is common in other agricultural industries (Nandagopal *et al.*, 2010; Miranda *et al.*, 2017). Several sampling methods have been published for monitoring psylloid pests in agricultural fields, including visual inspection, sticky traps, insect sweep-nets, motorised suction devices and vacuum sampling (Sétamou *et al.*, 2008; Hall & Hentz, 2010; Weintraub & Gross, 2013; Monzo *et al.*, 2015). Among these sampling methods, sticky traps have been reported as the most efficient for monitoring populations of adult psylloids and other flying phytophagous insects in diverse agroecosystems globally (Miranda *et al.*, 2017; Sétamou *et al.*, 2019).

In the current study, two sampling methods, such as yellow sticky traps and insect sweep-net, were used to sample psylloids in citrus environments. Among these, yellow sticky traps consistently captured a higher number of psylloids in citrus orchards than sweep-net. However, these findings contradict a previous study by Horton (1994), who sampled pear shoots to estimate the absolute densities of adult pear psyllid using beating trays and yellow sticky traps. Horton used regression analyses to determine whether these two methods were equally effective in predicting the absolute densities of the summer form pear psyllid. They found that beating trays were more effective than sticky traps in predicting the absolute densities of adult pear psyllids on pear shoots. Similarly, Aldini *et al.* (2003) monitored leafhopper *Scaphoideus titanus* (Ball) (Hemiptera: Cicadellidae) and planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) in vineyards in Italy using yellow sticky traps and sweep-nets. They found that sweep-nets were more effective than yellow sticky traps in monitoring both leafhoppers and planthoppers in vineyards, further contradicting the effectiveness of yellow sticky traps reported in the current study. However, more recent studies support the findings of this study. Miranda *et al.* (2018), for instance, in their study of monitoring *Diaphorina citri* within commercial citrus orchards with and without the insecticide application

programs, found that yellow sticky traps were the most effective over other methods at detecting and quantifying the populations of *D. citri*, particularly in citrus orchards with frequent insecticide applications, therefore, contradicting Horton (1994) and Aldini *et al.* (2003). Similar findings were recently reported by Dongiovanni *et al.* (2023) who compared yellow sticky traps and sweep-nets for monitoring spittlebug species, *Philaenus spumariu*s (Linnaeus) and *Neophilaenus campestris* (Fallenin) (both from Hemiptera: Aphrophoridae) in olive orchards and other agricultural crops in southern Italy. Their findings consistently revealed that yellow sticky traps captured significantly higher abundances of these species than sweep-nets, supporting the present study. Therefore, with support from multiple studies, this study confirms yellow sticky traps as the best-recommended sampling method for monitoring psylloid pests in commercial citrus orchards.

Several studies have reported that many phytophagous insects are visual specialists, relying on visual cues for orientation and successful host-plant selection. Visual traps mimicking particular host-plant visual stimuli have been found highly effective in monitoring insect pest populations on cultivated crops (Prokopy & Owens, 1978; Sétamou *et al.*, 2014; Czarnobai De Jorge *et al.*, 2023). Among phytophagous hemipteran insects, such as aphids, whiteflies, bees and hoverflies, psylloids are particularly attracted to yellow and green targets, with a strong preference for yellow visual targets within the 500 to 600 nanometers (nm) wavelength range (Döring & Chittka, 2007; Döring, 2014). Although the focus of this study did not include visual preference analysis, it is possible that the higher abundance of psylloids collected with yellow sticky traps compared to sweep-net could be attributed to their visual attraction to yellow colour. Hall *et al.* (2007) reported that greater numbers of adult *Diaphorina citri* were captured each week by using yellow sticky traps than blue sticky traps in citrus orchards. Despite their effectiveness, the colour of sticky traps can further influence the capture of non-target insects. For example, Knight & Miliczky (2003) found that the colour of sticky delta traps, which were used for monitoring the codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) in their study, also influenced the number of honeybees *Apis mellifera* Linnaeus (Hymenoptera: Apidae) and non-target muscoid flies captured. Similarly, Hall *et al.* (2007) found that diverse species of Diptera, including the love bug *Plecia nearctica* Hardy (Bibionidae), were sometimes captured or trapped in significant numbers on yellow sticky traps, which interfered with the counting, identification and capturing of the adult *D. citri*. In the current study, several non-target insects, including but not limited to tsetse flies, caenflies and snipe flies,

were frequently encountered on sticky traps. This made the identification, isolation and counting of psylloids on yellow sticky traps more challenging in most cases.

To address this issue and enhance the specificity of yellow sticky traps for psylloid monitoring in citrus orchards, the introduction of baits like plant volatile lures could be beneficial. This approach, commonly used in fruit fly monitoring studies where male lures target primarily fruit flies (Umeh & Garcia, 2008; Karki *et al.*, 2023), could reduce the capture of non-target insects and minimise interference with beneficial insect populations. However, several studies have reported that the use of sticky traps (i.e. yellow or lime sticky traps) in pest monitoring programs has a negative impact on the populations of beneficial arthropods, including honeybees and ladybird beetles, potentially reducing the number of pollinators and natural enemies of pests, thereby indirectly increasing pest populations (Mondor, 1995; Sétamou *et al.*, 2019). In conclusion, while yellow sticky traps show effectiveness for psylloid monitoring in citrus orchards, their use should be complemented with strategies to reduce capturing of non-targets and minimise adverse effects on the population of the beneficial insects. Future studies should consider the incorporation of specific lures to improve the specificity of these yellow sticky traps to psylloids, thereby improving monitoring accuracy.

In agricultural landscapes, the edges of habitats, usually the first two rows, host a higher abundance of insects coming from the surrounding natural vegetation compared to the interior of the habitats (Olson & Andow, 2008; Sétamou & Bartels, 2015). This phenomenon is often attributed to several factors, including insect behaviour, host-plant distribution, availability of oviposition and feeding sites, distribution and densities of natural enemies, the presence of windbreaks and microclimates (Olson & Andow, 2008; Sétamou & Bartels, 2015). In the current study, yellow sticky straps were deployed along the edges of the citrus orchards, with the second row of each orchard chosen as the placement site, which likely contributed to the capturing of a significant abundance of psylloids in the orchards. These findings complement Sétamou & Bartels (2015), who monitored the densities of all developmental stages of *Diaphorina citri* on three sentinel trees (edge, adjacent and interior) every two weeks using lime-green sticky traps and visual observation. Despite differences in sampling methods and study sites, Sétamou & Bartels (2015) found that significantly more psyllids were present on the edge trees in both mature grapefruit and sweet orange orchards throughout the study period, suggesting a strong edge effect in the distribution of *D. citri* in orchards. Furthermore, they reported that flush-shoot infestation levels and densities of *D. citri* were consistently highest

on edge trees and gradually declined towards the interior of the orchard. Similar edge effects have been noted in the spatial distribution of other insect species. For example, higher densities of the Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) were recorded on the edges of soybean fields compared to the interior, especially in downwind hedges, which harboured more beetles than the other edges (Sara *et al.*, 2013). Therefore, the current study supported by previous studies, highlights the importance of considering the edge effects when monitoring and managing psylloid populations in commercial citrus orchards.

4.5. The role of psylloids collected in the commercial citrus environments

Insect pests pose a significant threat to global crop production, particularly within agroecosystems, with chewing and sap-feeding insects among the most economically impactful (Vanti *et al.*, 2018). Sap-feeding insects, classified under the Hemipteran order, are considered the most economically important pests (Oerke, 2006; Fereres & Raccah, 2015). This status is primarily due to their potential to vector plant-pathogens (bacteria, fungi and viruses), rather than direct feeding damage. These pathogens cause devastating diseases that threaten overall plant health and lead to significant crop losses, particularly in agricultural ecosystems (Fereres & Raccah, 2015; Vanti *et al.*, 2018). Therefore, understanding the feeding habits of plant-feeding insects in agricultural environments is vital for accurate assessment of their pest status and for developing effective pest management strategies. Several methods have been employed to determine the feeding habits of diverse insect pests, such as visual observation of pest presence and plant damage, microscopic evaluation of gut contents, molecular gut content analysis and laboratory feeding trials (Matheson *et al.*, 2008; Valentini *et al.*, 2009; Pompanon *et al.*, 2012; Avanesyan, 2014). Among these, molecular analysis of gut contents has become the most effective, accurate and time-efficient method for identifying plants that insects have previously fed on or confirming host-plant use before capture in the natural field environment (Jurado-Rivera *et al.*, 2009; Valentini *et al.*, 2009; García-Robledo *et al.*, 2013; Avanesyan, 2014; Cooper *et al.*, 2016; Huang *et al.*, 2017). Previous studies focused on detecting plant DNA in the guts of leaf-chewing insects due to the abundance of chloroplast DNA in their guts (Miller *et al.*, 2006; Jurado-Rivera *et al.*, 2009; Wallinger *et al.*, 2013; Avanesyan, 2014; Avanesyan & Culley, 2015; Gonella *et al.*, 2020). However, some recent studies have shown that plant DNA can also be detected in the guts of sap-feeding insects (see Pearson *et al.*, 2014; Mustafa *et al.*, 2015; Cooper *et al.*, 2016; Avanesyan & Lamp, 2020).

In Chapter 3 of this study, the molecular gut content analysis was conducted on actively collected psylloids from citrus plants in orchards to determine their potential feeding preferences on citrus or alternative plants adjacent to orchards (see Table S 3. 1 and Table S 3. 2). Actively collected psylloid species or specimens were preferred for gut content analysis because these insects were still in good condition, and most importantly, were collected directly from citrus plants. Ballare *et al.* (2019) emphasised that insect specimens preserved immediately after collection yield high-quality DNA. In this study, all field-collected psylloid samples were preserved in 70% ethanol immediately after collection. However, psylloids collected through yellow sticky traps were not considered for gut analysis for several reasons. Studies have shown that insect specimens exposed to environmental factors, such as sunlight, high temperatures and humidity, often dry out, which increases the likelihood of breakage during the isolation process and results in rapid degradation of genomic DNA (gDNA) (Lindahl, 1993; Mandrioli, 2008; Zimmermann *et al.*, 2008). Since yellow sticky traps were left in orchards for extended periods, most psyllids collected using sticky traps were already dried and prone to breakage during the isolation process, which then led to their exclusion from gut content analysis in this study.

4.5.1. Genomic DNA (gDNA) extraction from leaf samples and the PCR amplification.

In preparation for the gut content analysis of the selected psyllid species to ascertain their potential feeding on citrus or other adjacent plants to the orchards, genomic DNA (gDNA) was extracted from 15 citrus leaf samples collected from the same plants as adult psyllids. The PureLink™ Plant Total DNA Purification Kit (Invitrogen™, Thermo Fisher Scientific, USA) was utilised for this purpose, as it is widely recognised as one of the most effective methods for obtaining pure and intact gDNA from plant tissues (Abu Almakarem *et al.*, 2012; Cen *et al.*, 2012; Avanesyan, 2014; Aboul-Maaty & Oraby, 2019; Pineda-Rodriguez *et al.*, 2023). As expected, successful extraction of gDNA from all 15 leaf samples was achieved, as confirmed by the presence of distinct gDNA bands representing each specific leaf sample on agarose gel electrophoresis. However, the quality of the gDNA was poor, as indicated by the faint appearance. This could suggest potential gDNA degradation or insufficient quantity/concentration of the extracted gDNA loaded on agarose gel. A recent study by Saraswathi & Mullainathan (2020), which cited Porebski *et al.* (1997), indicated that mature plant leaf materials/samples usually contain higher quantities of polysaccharides and polyphenols, which could adversely affect DNA quality. In this study, all the collected leaf samples

were not fresh, which could explain the observed poor gDNA quality. Hence, the use of fresh and young plant leaf samples is highly recommended for future studies to obtain high-quality gDNA. Furthermore, factors such as the preservation method (silica gel kept at room temperature) and storage duration (1-2 months) may have also contributed to gDNA degradation (e.g. see Guo *et al.*, 2018).

Following gDNA extraction, PCR amplification was performed on all gDNA samples obtained from the 15 citrus leaf samples using the rbcLaF/R primer pair. This primer pair is well-known for targeting a 530-base pairs (bp) region of the *rbcL* chloroplast DNA gene (Avanesyan & Lamp, 2020). Several ecological and molecular studies have used the rbcLaF/R primer pair, along with other primer pairs such as trnL575F/755R, due to their efficient amplification of the respective target plant DNA regions (see Matheson *et al.*, 2008; Kress *et al.*, 2009; Kajtoch & Mazur, 2015; Avanesyan *et al.*, 2021). Therefore, it was expected that all PCR-amplified citrus leaf DNA would be obtained using this primer pair. The aim of amplifying the extracted gDNA leaf samples was to confirm the ability of the selected rbcLaF/R primer pair to amplify the targeted citrus plant DNA gene region in the guts of psylloid species. The results of this study showed that all PCR-amplified leaf gDNA yielded amplicons of about 530-bp (see Figure 3. 7). These PCR amplicons represent the targeted *rbcL* gene, thus confirming the efficiency of the rbcLaF/R primer pair in amplifying the targeted citrus DNA region in each psyllid gut.

4.5.2. Genomic DNA (gDNA) extraction from insect samples and the PCR amplification.

Several techniques have been used to extract gDNA from small insects, such as the CTAB method and commercially available kits like the DNeasy® Blood and Tissue Kit and QIAamp PowerFecal DNA Kit (Calderón-Cortés *et al.*, 2010; Gonella *et al.*, 2020). In our study, gDNA samples were extracted from 11 selected psylloid species using the PureLink Genomic DNA Mini Kit. Despite successful extraction, the gDNA quality of most psylloid species was relatively lower. Firmansyah *et al.* (2023) compared different DNA extraction methods (i.e. PureLink Genomic DNA Mini Kit, DNAzol Direct reagent and a microwave-based method) for extracting DNA from adult southern house mosquitoes *Culex quinquefasciatus* (Say) (Diptera: Culicidae) for PCR assays. They found higher DNA concentrations with the DNAzol Direct method than the other methods, suggesting potential loss of DNA yield during extraction with other methods. Jangra & Ghosh (2022) reported

that repeated washing and resuspension steps in standard methods might also contribute to low DNA yields. In the current study, all psylloid gDNA extractions followed the PureLink Genomic DNA Mini Kit protocol, which included repeated washing and resuspension steps. Despite this, some psyllid species yielded high-quality gDNA, indicating that these steps did not consistently affect DNA yield. Furthermore, previous studies by A'Hara *et al.* (1998) and Haelewaters *et al.* (2015) reported decreased DNA quality in insects preserved in 70% ethanol. Interestingly, our results showed that the ethanol preservation method did not negatively impact gDNA quality, as high-quality gDNA was consistently obtained from psylloid specimens preserved in 70% ethanol, thus contradicting previous findings. Despite variations in the gDNA quality, all extracted gDNA samples underwent PCR amplification to confirm their origin and rule out contamination before gut content analysis.

The PCR amplification on the gDNA samples from the selected 11 psylloid species was conducted using the Te-6U30/720L26 primer pair, which targets a 714-bp region of the *mtCOI* gene (Pérez-Rodríguez *et al.*, 2019). Among the tested species, successful PCR amplification was achieved for six out of 11 species: *Diaphorina punctulata*, *Diaphorina virgata*, *Diaphorina zebrana*, *Psylla loranthi*-group, *Retroacizzia mopanei* and *Trioza erytreae*. However, the amplicons generated were slightly larger than expected, approximately 750-bp in size, as compared to the anticipated 714-bp size (Pérez-Rodríguez *et al.*, 2019). However, despite this size difference, the successful amplification using the Te-6U30/720L26 primer pair confirmed the origin of gDNA samples from these psyllid species, thereby ruling out potential contamination from other organisms like plants, viruses or fungi before gut content analysis. Interestingly, the Te-6U30/720L26 primer pair was initially documented to be specific to *Trioza erytreae* (Pérez-Rodríguez *et al.*, 2019); however, the current study found that this primer pair can also amplify the gDNA from diverse psylloid species across different genera in the superfamily Psylloidea.

However, gDNA samples from the remaining five psyllid species, including *Acizzia* sp.1, *Acizzia* sp.2, *Ciriacremum capense*, *Euryconus* sp. and *Pauropsylla trichaeta*, failed to amplify using the Te-6U30/720L26 primer pair. Several studies have shown that variations in the PCR setup or the presence of inhibiting agents after the extraction can influence the PCR results (Chen *et al.*, 2000; Agustí *et al.*, 2003; Juen & Traugott, 2005). To confirm that the PCR amplification failure using these primer pairs was not due to either the PCR setup or contamination, all corresponding NTCs

remained clear, confirming that no possible contamination occurred during the PCR setup process. However, as previously discussed, the Te-6U30/720L26 primer pair is species-specific based on the existing literature, and possibly genus-specific according to the findings of this current study; therefore, it is possible that this primer pair does not work for these five species. However, failure to amplify the gDNA extracted from the above-mentioned psyllid species using this primer pair does not imply that the extracted gDNA samples are not from these insects. As such, it is possible that other primer pairs, such as the DCITRI COI-L/R and CO1-F3/R3, might successfully amplify these gDNA samples.

4.5.3. The presence of plant DNA in the gut contents of field-collected insect samples.

To ascertain if psylloids collected directly from citrus in the orchards were feeding on these plants, which is the primary aim of this study, PCR analysis was conducted using the extracted gDNA samples from these insects and the rbcLaF/R primer pair, targeting the 530-bp region of the *rbcL* chloroplast DNA gene. This analysis is significant for understanding the feeding habits, dietary preferences, and ecological roles of these insects within commercial citrus environments. The PCR analysis revealed successful amplification of plant DNA in the guts of only five of the 11 analysed psylloid species: *D. punctulata* (collected from Orri and Valencia), *D. virgata* (Eureka Lemons, Leanri and Orri), *D. zebrana* (Orri), *Euryconus* sp. (Eureka Lemons) and *T. erytreae* (Nadorcott, Eureka Lemons and Mandarin). Despite this positive amplification, the quantity of plant DNA ingested by these species appeared relatively low, as indicated by the faint appearance of PCR amplicons. A recent study by Cao & Dietrich (2021) focusing on sap-sucking insects (Hemiptera: Cicadellidae) found that plant DNA concentrations in microleafhoppers, which primarily feed on parenchyma cell contents, were significantly higher compared to other leafhopper groups that feed preferentially on phloem or xylem. Bennett & Moran (2013) reported that, unlike phloem or xylem sap, the parenchyma cell contents inherently have higher DNA concentrations; hence, this may explain the lower amount of plant DNA detected in the guts of psylloids in our study. Furthermore, other studies have found that the enzymatic processes within the digestive systems of sap-feeding insects can rapidly break down the plant sap than chewed plant tissue, contributing to lower plant DNA quantities and further reducing the detectable plant DNA (Pumariño *et al.*, 2011; Staudacher *et al.*, 2011). Lastly, the degradation of plant DNA during digestion, as observed in other insect

species (Briem *et al.*, 2018), could further diminish available plant DNA for amplification in the guts of psylloids.

The detection of plant DNA in the gut contents of five psyllid species suggests recent ingestion of plant phloem-sap, likely occurring a few hours before their capture in citrus orchards. This finding is consistent with studies such as Pumariño *et al.* (2011), who detected or identified tomato plant DNA within the guts of several insects, such as *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), thus, indicating recent plant material ingestion based on the observed decrease in DNA detectability over time. Although Pumariño *et al.* (2011) employed the tomato-specific *ITS* (internal transcribed spacers) primers, the current study used the *rbcL* primers, which can amplify a broader range of plant species. Furthermore, a recent study by Avanesyan & Lamp (2020) investigated the presence of the ingested plant DNA in the guts of the spotted lanternfly *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), a significant pest of cultivated crops. They detected a 534-bp chloroplast *rbcL* gene region from ingested plants in the gut contents of these insects. Interestingly, the ingested plant species often differed from those where the insects were collected, suggesting a wider range of ingested plant species than sampled plants. This included both native and non-native plants, woody and non-woody plants, and even species not previously documented as the host-plants for the spotted lanternfly. However, our study differed slightly in methodology; we focused mainly on analysing the gut contents of adult psylloid species, targeting the 530-bp *rbcL* gene region, whereas Avanesyan & Lamp (2020) analysed the gut contents of the third and fourth-instar nymphs of the spotted lanternfly, targeting the 534-bp *rbcL* gene region.

Although plant DNA was amplified in the guts of the aforementioned psyllid species, the targeted plant DNA region of the expected size (530-bp) was only successfully detected from the gut contents of two psyllid species: *Diaphorina punctulata* (collected from Valencia) and *Euryconus* sp. (Eureka Lemons). As previously discussed, this finding could imply that these psyllids might have recently fed on these plants shortly before being captured in the orchards. The host-plants of *D. punctulata* and *Euryconus* sp. have been documented respectively as marula [*Sclerocarya birrea* (A. Rich) Hochst. (Anacardiaceae)] and diesel tree [*Copaifera langsdorfii* Desf. (Fabaceae)] (Capener, 1970a, 1970b; Burckhardt & de Queiroz, 2012). However, there are no existing records of *Euryconus* sp. feeding on any plant species other than its known host. Therefore, this study is

the first to document that *Euryconus* sp. may also feed on other plants not previously documented as its host. This finding complements previous research by Catling & Atkinson (1974), which cited Catling (1970b), that documented *Diaphorina punctulata* and *Diaphorina zebrana* feed on citrus in South Africa and Swaziland. Unlike the earlier study, our research involved gut content analysis of *Diaphorina punctulata* and *Diaphorina zebrana* and other psylloid species collected from citrus orchards, indicating accurate findings related to the feeding habits or behaviour of these psyllids on citrus plants. Catling (1970b) reported that several psylloid species are occasional citrus feeders and that some may inhabit citrus for extended periods when their primary hosts are unattractive or dormant. This could also be the case for *D. punctulata* and *Euryconus* sp., indicating that they may act as occasional citrus feeders, along with other psylloid species as visitors to citrus plants.

Despite the positive amplification of the targeted plant DNA region from the guts of *D. punctulata* and *Euryconus* sp., the PCR analysis also revealed the presence of diverse plant DNA in the guts of the remaining psyllid species. These include *D.virgata* (Eureka Lemons, Leanri and Orri), *D. zebrana* (Orri) and *T. erytreae* (Nadorcott, Eureka Lemons, and Mandarin), and *D. punctulata* (Orri and Valencia), even though the targeted plant DNA region was successfully amplified from this species. The presence of diverse plant DNA resulted in non-specific binding (NSB) during DNA amplification. This issue arises because different plant species often have varying base pair lengths between primer binding sites. Thus, primers designed for a specific plant species may also bind to similar sequences from other plants present in the insect gut, resulting in the amplification of non-target DNA regions (Pompanon *et al.*, 2012; Symes *et al.*, 2019). Several host-plants of most psyllid species were recorded in the surveyed commercial citrus environments (see Chapter 2), and these plants were abundant near the citrus orchards where these psyllids were collected. Therefore, it is plausible that these insects initially ingested plant sap from either their primary hosts or other nearby plants before migrating into the orchards where they were captured. Similar findings have been reported in studies by Hereward & Walter (2012) and Barthel *et al.* (2020). Furthermore, recent studies found that NSB could also be influenced by the annealing temperature during PCR, with higher temperatures resulting in specific primer binding to the targeted plant DNA regions, whereas lower temperatures lead to NSB and/or non-specific amplicons (Erjavec, 2020; Yunita et al., 2023). Although an annealing temperature of 55°C worked well for the PCR amplification of targeted plant DNA regions in some psylloid guts, the presence of NSB suggests that optimising the annealing temperature may be necessary to achieve specific binding to targeted

plant DNA regions across different plant species in the guts of the insects. This optimization could enhance the accuracy and specificity of DNA amplification analyses in future studies involving plant-feeding insects like psylloids.

The PCR analysis further showed that the targeted plant DNA region, including the non-targeted region within the guts of the remaining psyllid species, including *Acizzia* sp.1 and *Acizzia* sp.2 (both collected from Orri), *Ciriacremum capense* (Star Ruby), *Diaphorina punctulata* (Eureka Lemons, Delta swingle, Nadorcott and Valencia), *Diaphorina zebrana* (Valencia), *Euryconus* sp. (Valencia), *Psylla loranthi*-group (Delta Valencia), *Pauropsylla trichaeta* (Eureka Lemons), *Retroacizzia mopanei* (Late Valencia) and *Trioza erytreae* (Eureka Lemons), could not be detected or amplified using the rbcLaF/R primer pair, even though successful plant DNA amplification was obtained within the guts of other psylloids using the same primer pair as previously discussed. The inability raises questions about factors influencing the detectability of host-plant DNA in insect gut samples. Cao & Dietrich (2021) reported that the successful detection of plant DNA in insect gut samples can be attributed to several factors, including the age and state of preservation of the original insect samples, whether or not the insect fed on a plant shortly before being captured and preserved, factors that were beyond our control during the sampling. However, it is plausible that the psylloid species in question might not have actively fed on the respective citrus plants or any other plant available in the surveyed citrus environments, including their known hosts, at the time of sampling or just prior to capture, leading to the absence of plant DNA in their guts. Furthermore, studies on plant DNA degradation, such as those by Hoogendoorn & Heimpel (2001) and Valentini *et al.* (2009), have documented that environmental factors such as high temperatures and the digestive processes of insects can lead to rapid digestion of ingested DNA. This rapid degradation can render plant DNA undetectable or no longer present in gut content analyses, which may explain the lack of detected plant DNA in the psylloid samples examined in this study.

The choice of targeted plant DNA region (530-bp) for plant DNA detection might not have been optimal for gut content analysis in this study, as observed in other studies using different DNA regions for amplification, such as the chloroplast *rbcL* region versus shorter regions like *ITS* and *trnH-psbA* (Hoogendoorn & Heimpel, 2001; McMillan *et al.*, 2007; Staudacher *et al.*, 2011). For example, Staudacher *et al.* (2011) successfully detected or identified the ingested plant DNA in soil-dwelling insect larvae of click-beetles *Agriotes* lineatus (Linnaues) (Coleoptera: Elateridae),

commonly known as wireworms, by using short primer pairs that targeted specific regions of *rbcL* (153-bp) and *trnL* (90-bp). Similarly, Wang *et al.* (2019) demonstrated successful identification of host-plants at genus and species levels using smaller DNA regions like *ITS* and *trnH-psbA*, which are more suitable for PCR amplification even from degraded plant DNA. In the present study, a long primer pair targeting a 530-bp region of the chloroplast *rbcL* gene was employed. However, this approach may have limited the detection capabilities in gut samples of the analysed psylloids, particularly if the ingested plant DNA had completely degraded either before or during the time of sampling, rendering it undetectable or not amplifiable with this long primer pair. Future research in this area could benefit from exploring the primer pairs that target shorter regions of plant DNA, particularly in cases where DNA degradation is likely to occur before sample collection, enhancing the likelihood of detecting and identifying ingested plant species in insect gut contents accurately.

4.6. Conclusion and recommendations for future studies

In summary, the current study addressed a significant gap in the existing literature by investigating the ecological roles of psylloid species in commercial citrus environments. Field surveys were conducted using double-sided yellow sticky traps and an insect sweep-net across different citrus environments, sampling forty-seven psylloid species and revealing their considerable diversity and community structure (Chapter 2). Although some of these species belong to genera known for harbouring pests of cultivated plants globally, none except *Trioza erytreae* are currently classified as pests in the global agricultural industry. Furthermore, several plant species adjacent to the citrus orchards were identified as host-plants for some psylloid species, indicating correlations between their distribution and abundance in these environments and the presence of these plants. Yellow sticky traps proved to be more effective in sampling psylloid species compared to an insect sweep-net, although both methods were complementary as each captured unique species.

Among the field-collected and analysed psylloid species, only two from citrus orchards were found to have citrus DNA in their guts through molecular gut content analysis, suggesting potential citrus feeding (Chapter 3). *Euryconus* sp. was reported for the first time as potentially feeding on citrus or utilising citrus for refuge when its host-plant is depleted. However, both psyllids, *D. punctulata* and *Euryconus* sp., do not lay their eggs nor complete their life cycle on citrus, thus, confirming that citrus is not their primary host-plant. Furthermore, other psylloids species had multiple plant DNA in their guts, indicating a broad host range. However, further molecular gut content analysis

through Sanger sequencing of the PCR-amplified plant DNA is recommended for identifying plant species ingested by psylloids, including those with multiple plant DNA in their guts. Overall, this research contributes significantly to informing citrus growers and communities regarding the need to prioritise the management of indigenous psylloid species due to their role in transmitting plant-pathogenic bacteria responsible for establishing citrus greening disease in citrus. Furthermore, the presence of citrus DNA in the guts of some psyllid species raises concerns about their potential as vectors of citrus greening agents, necessitating early development of effective pest management against potential threats that might arise from these new psyllid citrus-feeders.

Furthermore, laboratory experiments should be conducted where psylloid species are force-fed on available citrus plants to assess/investigate their potential to feed on citrus in the absence of their primary hosts. In conclusion, investigating the potential biocontrol agents for psyllid species found to have citrus DNA in their guts is significant as this could significantly impact managing psylloid pests and reducing the spread of citrus greening disease.

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SUPPLEMENTARY MATERIALS

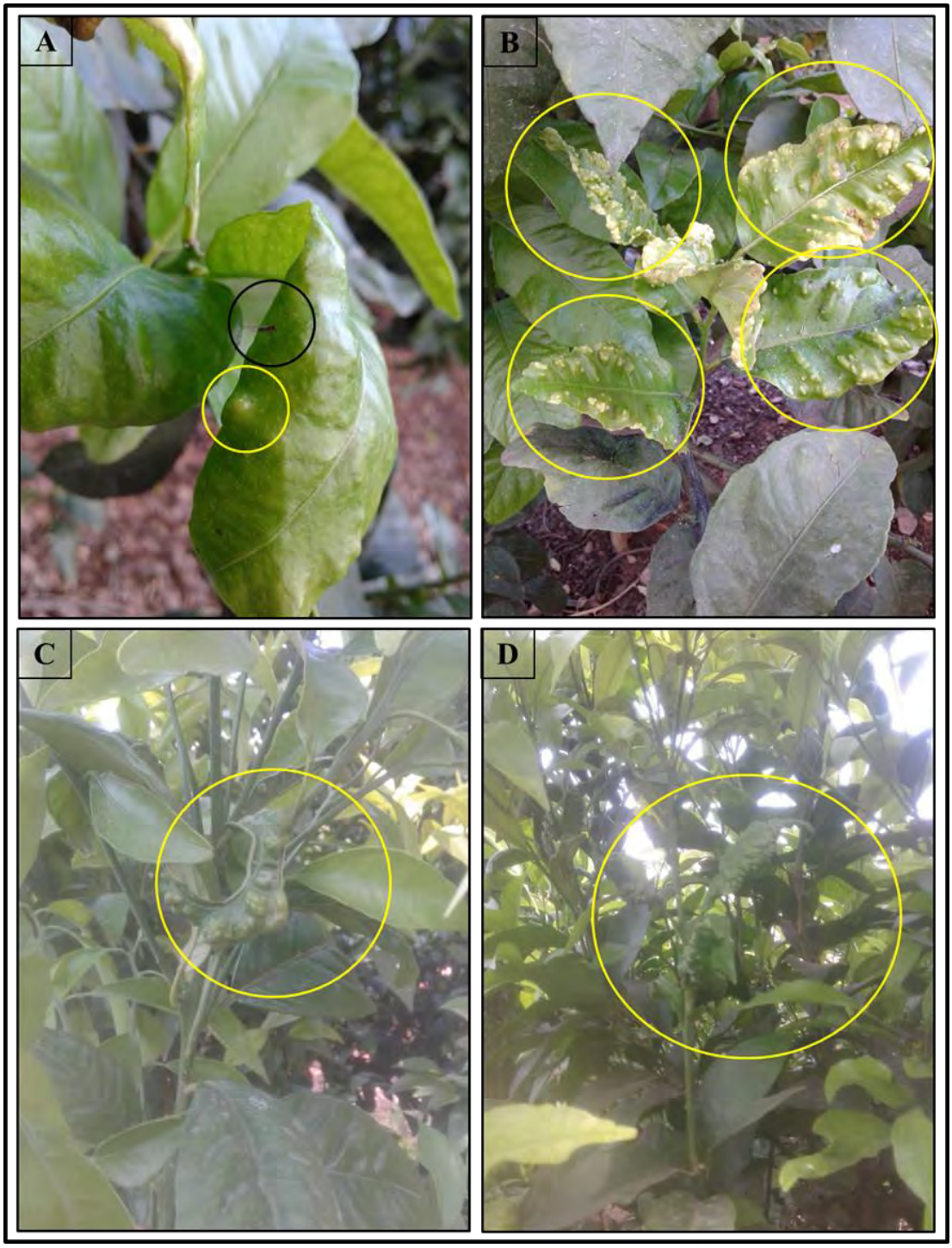

Figure S 2. 1: The feeding damage signs (galls) of *Trioza erytreae* on Eureka lemon (**A** and **B**) and Orri (**C** and **D**) plant leaves in citrus orchards. The adult psyllid is circled with a black colour (**A**), whereas all damaged leaves are circled with a yellow colour (**Photo credit**: Raynold Moagi).

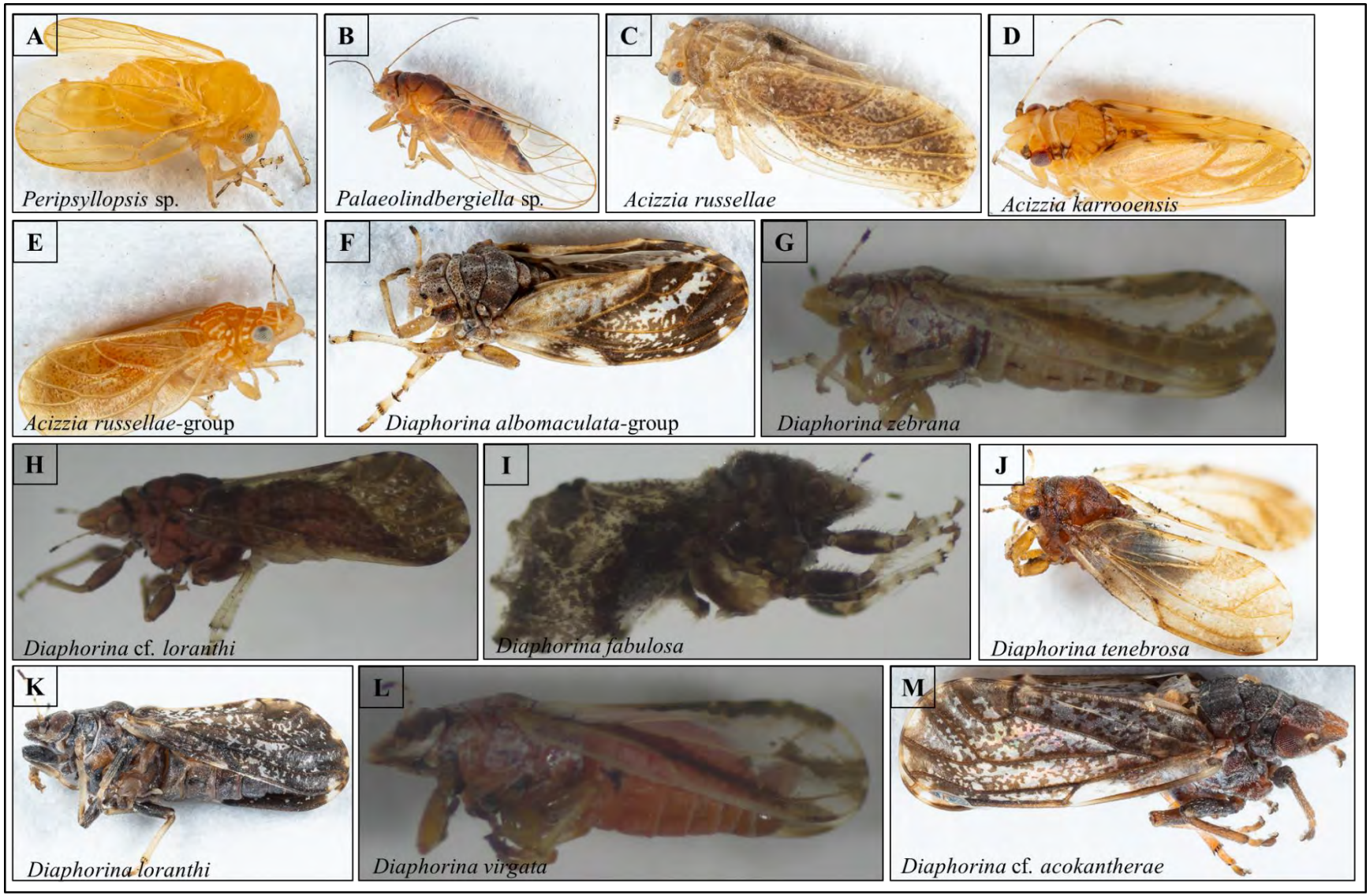

Figure S 2. 2 (**A-M**): Some of the adult psyllid species caught or sampled in commercial citrus environments through passive and active collection methods (**Photo credit**: David Taylor and Raynold Moagi).

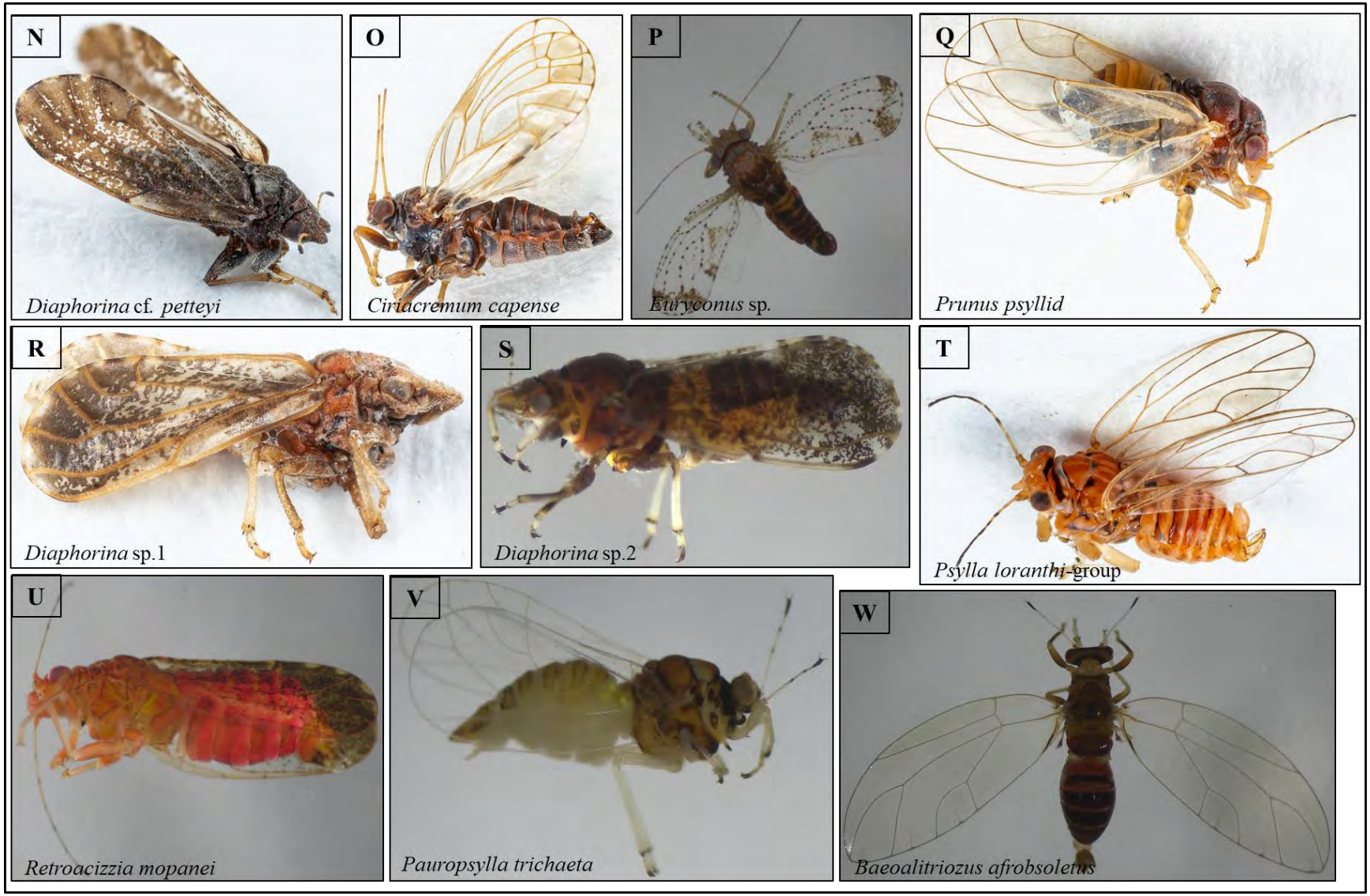

Figure S 2. 2 (N-W) (Continued): Some of the adult psyllid species caught or sampled in commercial citrus environments through passive and active collection methods (**Photo credit**: David Taylor and Raynold Moagi).

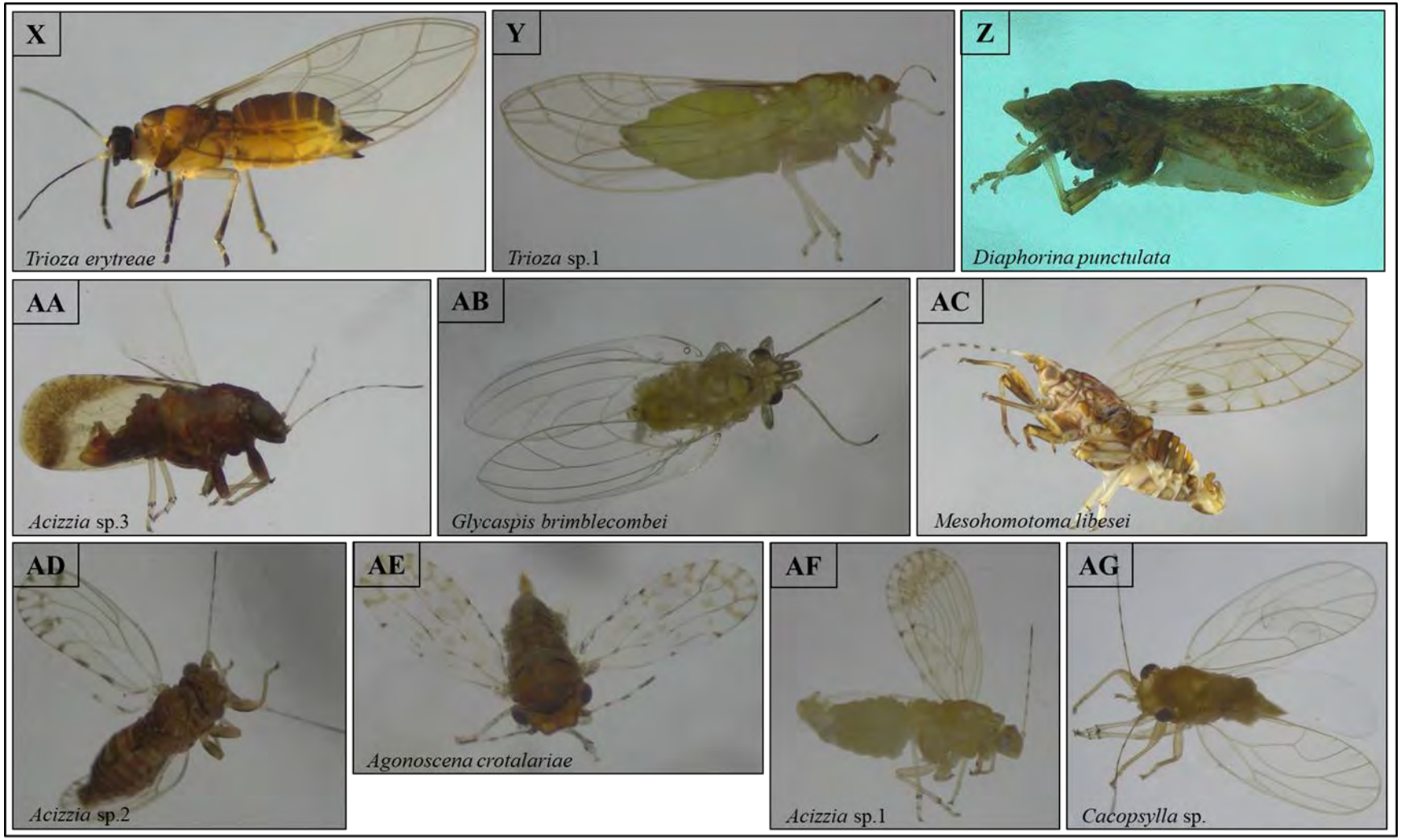

Figure S 2. 2 (**X-AG**) (Continued): Some of the adult psyllid species caught in commercial citrus environments through passive and active collection methods (**Photo credit**: David Taylor and Raynold Moagi).

Table S 2. 1: The presence of psylloid species between two different habitats in citrus environments.

Family	Morphospecies	Citrus orchards		Adjacent natural vegetation
		Yellow sticky traps	Insect sweep-net	Insect sweep-net
Aphalaridae	*Agonoscena crotalariae*	+	-	+
Aphalaridae	*Glycaspis brimblecombei*	+	-	-
Carsidaridae	*Mesohomotoma libesei*	+	-	-
Liviidae	*Euphyllura speciosa*	+	-	+
Liviidae	*Euphyllura* cf. *speciosa*	+	-	-
Liviidae	*Peripsyllopsis* sp.	-	-	+
Psyllidae	*Acizzia* sp.1	+	+	-
Psyllidae	*Acizzia* sp.2	+	+	+
Psyllidae	*Acizzia* sp.3	+	+	+
Psyllidae	*Acizzia* sp.4	+	-	-
Psyllidae	*Acizzia karrooensis*	+	-	+
Psyllidae	*Acizzia russellae*	+	+	-
Psyllidae	*Acizzia russellae*-group	+	-	+
Psyllidae	*Cacopsylla* sp.	+	+	+
Psyllidae	*Ciriacremum capense*	+	+	-
Psyllidae	*Diaphorina albomaculata*-group	+	-	-
Psyllidae	*Diaphorina* cf. *acokantherae*	-	+	-

(+) means Present; (-) means Absent.

Table S 2. 1 (Continued): The presence of psylloid species between two different habitats in citrus environments.

Family	Morphospecies	Citrus orchards		Adjacent natural vegetation
		Yellow sticky traps	Insect sweep-net	Insect sweep-net
Psyllidae	*Diaphorina* cf. *bicolor*	+	-	-
Psyllidae	*Diaphorina* cf. *loranthi*	+	+	+
Psyllidae	*Diaphorina* cf. *petteyi*	+	+	-
Psyllidae	*Diaphorina fabulosa*	+	-	-
Psyllidae	*Diaphorina loranthi*	-	+	-
Psyllidae	*Diaphorina tenebrosa*	+	-	-
Psyllidae	*Diaphorina punctulata*	+	+	+
Psyllidae	*Diaphorina virgata*	+	+	+
Psyllidae	*Diaphorina zebrana*	+	+	-
Psyllidae	*Diaphorina* sp.1	+	-	+
Psyllidae	*Diaphorina* sp. 2	+	-	-
Psyllidae	*Euryconus* sp.	+	+	+
Psyllidae	*Epiacizzia* sp.	-	+	-
Psyllidae	*Parapsylla capensis*	-	+	-
Psyllidae	*Palaeolindbergiella* sp.	+	+	+
Psyllidae	*Prunus psyllid*	+	-	-
Psyllidae	*Psylla loranthi*-group	+	+	-

(+) means Present; (-) means Absent.

Table S 2. 1 (Continued): The presence of psylloid species between two different habitats in citrus environments.

Family	Morphospecies	Citrus orchards		Adjacent natural vegetation
		Yellow sticky traps	Insect sweep-net	Insect sweep-net
Psyllidae	*Psylla* cf. *loranthi*-group	+	-	-
Psyllidae	*Psyllidae* sp.	+	-	+
Psyllidae	*Retroacizzia mopanei*	+	+	+
Triozidae	*Baeoalitriozus afrobsoletus*	+	+	+
Triozidae	*Pauropsylla trichaeta*	+	+	+
Triozidae	*Trioza carvalhoi*	+	-	-
Triozidae	*Trioza erytreae*	+	+	-
Triozidae	*Trioza* cf. *erytreae*	+	-	-
Triozidae	*Trioza* cf. *xylopia*	+	-	-
Triozidae	*Trioza* sp.1	+	-	-
Triozidae	*Trioza* sp.2	+	-	-
Triozidae	*Trioza* sp.3	-	-	+
Unknown	*Psylloid* sp.1	+	-	-

(+) means Present; (-) means Absent.

Table S 3. 1: Analysed psylloid species actively collected on different dates in different commercial citrus orchards from the Eureka lemon plants in Limpopo and Mpumalanga provinces.

Figure	Sample no. (Well no.)	Species name	Plant sampled	Collection date	Processed date
Figure 3.4	1 (1DP)	*Diaphorina punctulata*	Eureka lemon	October 2022	December 2023
	2 (2DV)	*Diaphorina virgata*	Eureka lemon	September and October 2022	
	3 (3EU)	*Euryconus* sp.	Eureka lemon	October 2022	
	4 (4PT)	*Pauropsylla trichaeta*	Eureka lemon	April 2023	
	5 (5TE)	*Trioza erytreae*	Eureka lemon	April 2023	

Table S 3. 2: Analysed psylloid species actively collected on different dates from different citrus varieties/cultivars in different commercial citrus orchards in Limpopo and Mpumalanga provinces.

Figure	Sample no. (Well no.)	Species name	Plant sampled	Collection date	Processed date
Figure 3.5A	1 (1ACZ2)	*Acizzia* sp.2	Orri	October 2022	December 2023
	2 (2DP)	*Diaphorina punctulata*	Delta swingle	September 2022	
	3 (3DV)	*Diaphorina virgata*	Leanri	March 2023	
	4 (4PLG)	*Psylla loranthi*-group	Delta Valencia	April 2023	
	5 (5TE)	*Trioza erytreae*	Nadorcott	April 2023	
Figure 3.5B	1 (ACZ1)	*Acizzia* sp.1	Orri	October 2022	January 2024
	2 (2DV)	*Diaphorina virgata*	Orri	October 2022	

Table S 3. 2 (Continued): Analysed psylloid species actively collected on different dates from different citrus varieties/cultivars in different commercial citrus orchards in Limpopo and Mpumalanga provinces.

Figure	Sample no. (Well no.)	Species name	Plant sampled	Collection date	Processed date
Figure 3.5B	3 (3DZ)	*Diaphorina zebrana*	Orri	October 2022	January 2024
	4 (4RAM)	*Retroacizzia mopanei*	Late Valencia	September 2022	
	5 (5TE)	*Trioza erytreae*	Eureka lemon	January 2023	
Figure 3.5C	1 (1DP)	*Diaphorina punctulata*	Nadorcott	March and April 2023	January 2024
	2 (2DV)	*Diaphorina virgata*	Valencia	August and October 2022	
	3 (3DZ)	*Diaphorina zebrana*	Valencia	August and October 2022	
	4 (4EU)	*Euryconus* sp.	Valencia	October 2022	
	5 (5TE)	*Trioza erytreae*	Eureka lemon	April 2023	
Figure 3.5D	1 (1CR)	*Ciriacremum capense*	Star Ruby	October 2022	January 2024
	2 (2DP)	*Diaphorina punctulata*	Orri	October 2022	
	3 (3DP)	*Diaphorina punctulata*	Valencia	October 2022	
	4 (4TE)	*Trioza erytreae*	Mandarin	November 2022	