**Biological Sciences: Evolution.**

**Phylogeny and microbiome of the New Zealand Psylloidea (Hemiptera: Sternorrhyncha) reveal insect-bacteria co-evolution and a new case of phylosymbiosis*.***

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**Abstract**

Like all phloem- and sap-feeding insects, psyllids (Hemiptera: Psylloidea) have a rather limited diet and depend on symbiotic bacteria for the provision of fundamental aminoacids. A number of studies has focused on the vertically transmitted primary symbiont, *Candidatus* Carsonella rudii, and a number of both vertically and horizontally transmitted secondary symbionts to determine their co-evolutionary relationships with psyllids. However, recent improvements in microbiome sequencing techniques now allow to focus on the pan-microbiome diversity, moving the focus from a few bacterial symbiont species to a broader whole-microbiome context. Furthermore, in the case of psyllids - insects with strong species-specific host plant associations - the study of microbiomes and their comparison with the hosts’ phylogenetics enables a better undestanding of plant-microbe-insect interaction and co-evolutionary relationships. Here, for a better understanding such relationships, we generated a phylogenetic framework for the New Zealand psyllids and compared it with the whole pan-microbiome composition of the New Zealand psyllids. To investigate this, a metabarcoding analysis of the bacterial 16S gene was analysed and tested for correlation with a multi-marker phylogenetic study of the New Zealand Psylloidea, their hostplant and their geographical distribution. The results obtained enhance our knowledge and understanding of the insect superfamnily Psylloidea in New Zealand, its evolution, ecology and microbiome composition. We suggest that today’s New Zealand psyllid fauna originated from at least six ancestral lineages/arrivals, confirming monophyly of the New Zealand genera *Ctenarytaina* and *Psylla* and demonstrating that the present radiation of the New Zealand *Trioza* species and their association with endemic host plantsis the result of a number of host switches. Furthermore, the core microbiome of the New Zealand psyllids species appeared to be strongly associated with the hosts’ phylogenetic relationships, highlighting a new case of phylosymbiosis.

**Significance Statement**

Bacteria living inside certain insects, such as psyllids, may provide advantages to their hosts, including provision of food and increased adaptability to environmental conditions. The same bacteria species can be found in the same psyllid species even when these live in different places, suggesting that psyllids and bacteria have evolved together for a long time, generating a strong bond between species. This co-evolution resulted in each insect species hosting a bacterial community almost as distinct as a finger print. This phenomenon, defined as phylosymbiosis, means that bacterial diversity is connected to the relationships between psyllid species. This will improve our understanding of how bacteria and insects have co-evolved through time and how they have influenced each other in the process.

1. **Introduction**

The association between phloem feeding hemipterans insects, such as aphids and psyllids, and their host plant is partially dependant on bacterial endosymbionts, which provide nutrients to the insects (Douglas 2016, and reference therein). Research suggests that this symbiosis is a major driver of insect diversification, which is a first step towards adaptive radiation (Vavre and Kremer 2014). Variation in the microbiome has been linked to insect phenotypic traits associated with diversification and speciation (Hosokawa *et al.* 2007), while a rapid adaptation of some insects to a new host plant has been linked to modifications of the microbiome (Chu *et al*. 2013). These studies have led to the hypothesis that the switch by insects to novel host plants may be symbiont-mediated (Tsuchida *et al.* 2011, Frago *et al.* 2012). For example, S-symbionts in aphids were proposed to facilitate or restrict the use of certain host plants (Tsuchida *et al.* 2011, Hansen and Moran 2014).

Psyllids are generally host plant specific, with each psyllid species generally associated with a single host plant (Brown and Hodkinson 1988; Burckhardt *et al.* 2014). Such host plant specificity has been associated with the presence of certain bacterial symbionts, allowing psyllids to feed on a specific plant (Hansen and Moran 2014). However, the lack of such specificity allows a few psyllids to feed and live on a high number of plants, making these insects dangerous plant pests (e.g. REF). These factors have largely driven psyllids-associated bacteria studies towards a pathogens-focused path, with the aim to understand insect-pathogen pairings of economic concern (e.g., Saha *et al.* 2012). However, the ever-growing amount of research confirming the interactive roles of pathogenic and insect symbiotic bacteria (e.g. Fagen *et al.* 2012; Nakabachi *et al.* 2013; Ramsey *et al.* 2015; Gonnella *et al.* 2019) is now highlighting the importance of a better understanding of non-pathogenic symbiotic bacteria, especially in non-model organisms (Prosdocimi *et al.* 2015).

A better understanding of what can influence the “pan-microbiome” composition - intended here as the whole composition of the microbiome, as opposed to only a few bacteria - could provide a better understanding of how insects switch host plants and potentially why some groups of psyllids are more invasive than others (Bennett 2013) or more prone to pathogen transmission (REF). The value of this pan-microbiome focus was demonstrated by reports that the plant-microbe-insect interaction may influence pathogen spread (Bennett 2013).

Phylosymbiosis (Brucker and Bordenstein 2012, Brooks *et al.* 2016) is “a significant association between host phylogenetic relationships and host-associated microbial community relationships” (Lim and Bordenstein 2019). This definition can be extended into “an eco-evolutionary pattern where evolutionary changes in the host are associated with ecological changes in its microbiome” (Sanders *et al.* 2014; Brooks *et al.* 2016). Determining the presence of a phylosymbiotic patterns can enable a better understanding of the role played by the microbiome in the genetic and phenotypic variation of the overall insect-bacteria holobionts (Lim & Bordenstein 2019) and, possibly, in the case of hemipterans, in the broader IPM network. Furthermore, phylosymbiosis defines a quantifiable link between the host evolutionary relationships and the microbiome diversity and composition, therefore providing a testable hypothesis (Lim & Bordenstein 2019). Therefore, the presence and degree of phylosymbiosis across an insect group could be implemented in ecological modelling analysis used to predict invasive species movements or likeliness of pathogens-vectoring (i.e. Syfert *et al.* 2017). However, despite having been recorded in a number of organisms, including rodents (Kohl *et al.* 2017), corals (Pollock *et al.* 2018), and insects (Colman *et al.* 2012; Minard *et al.* 2017), phylosymbiosis has not been recorded within the superfamily Psylloidea or the order Hemiptera.

Most studies focusing on non-pathogenic bacteria from this insect superfamily have focused on the primary symbiont of psyllids, “*Candidatus* Carsonella rudii”. All have reported phylogenetic congruence between *Ca* C. rudii and its psyllid hosts as evidence for strict co-speciation (Thao *et al.* 2000a; Thao *et al.* 2001; Sloan and Moran 2012; Hall *et al.* 2016). On the other hand, lack of congruence between the phylogenies of S-endosymbionts and hosts appears to indicate, for example, horizontal transmission of S-symbionts across psyllids (Thao *et al.* 2000b). Nonetheless, due to their role in the synthesis of amino acids, it has been hypothesised that psyllid S-endosymbionts have established long-term, stable associations with their hosts (Sloan and Moran 2012) suggesting a widespread presence of these endosymbionts across different psyllid species and a partial vertical transmission, too. Indeed, a work focusing mostly on the Australian genus *Cardiaspina* confirmed both vertical and horizontal transmission for S-symbionts(Hall *et al.* 2016).

A better understanding of the vertical and horizontal transmission of symbiotic bacteria across a wider set of psyllid taxa, however, has been limited by the lack of a robust phylogenetic framework that could be used to compare the microbial diversity with the phylogenetic relationships of the hosts. However, recent mitochondrial and nuclear genomic analyses have provided a phylogenetic backbone for the world Psylloidea (Percy *et al.* 2018; Cho *et al.* 2019). On the other hand, molecular studies of the Asia-Pacific Psylloidea have been focussing largely on COI DNA barcoding to understand specific aspects of biodiversity, as opposed to large-scale phylogenetic relationships (Taylor *et al.* 2016; Percy 2017; Martoni *et al.* 2018). Therefore, a phylogenetic study of a group of these psyllids, paired with an analysis of their microbiome could not only answer phylogenetic questions, but assess for the first time the microbial diversity of this group on a larger scale.

New Zealand’s landmass has undergone a series of geological events – from the separation from the Gondwanan super continent, to its partial sinking and isolation – that contributed to make its present days’ fauna unique when compared to that of other countries (e.g. Goldberg *et al.* 2008; Buckley *et al.* 2015). Therefore, the psyllid fauna of New Zealand, still relatively poorly studied, may be an ideal candidate to assess the presence of taxa of ancient origin (Goldberg *et al.* 2008) as well as to study the radiation and speciation of ancestral psyllids and their microbiome.

The psyllid fauna of New Zealand offers an example of today’s worldwide diversity within the superfamily Psylloidea, including ancient lineages, recent arrivals and economically relevant species. Indeed, not only New Zealand is home to both native and adventive species (Martoni *et al.* (2016), but some of these are also of economic importance, such as *Arytainilla spartiophila*, a bio-control agent of the Scotch Broom, *Cytisus scoparius* (Syrett *et al.* 2007), and the tomato/potato psyllid (TPP), *Bactericera cockerelli* Šulc, a significant pest (Vereijssen *et al.* 2018). A recent molecular assessment of the New Zealand Psylloidea (Martoni *et al.* 2018), coupling COI barcoding with morphological characters, identified more than 20 new taxa. Three spp have since been formally described (Martoni and Armstrong 2019a and b). The New Zealand psyllid fauna consists of at least 102 taxa, belonging to 24 genera and six families (of the eight worldwide) (Dale 1985; Macfarlane *et al.* 2010; Martoni *et al.* 2018), making it a more manageable case study than the nearby psyllid biodiversity hotspot that is Australia (more than 400 species; Hollis 2004, Ouvrard 2019).

The importance of a phylogenetic analysis of the New Zealand psyllid fauna and its microbiome resides in the high number of endemic psyllid taxa that could answer a number of evolutionary questions (e.g. Ferris and Klyver 1932, Tuthill 1952; Dale 1985). For example, the genus *Trioza* has a very wide range of native host plants across many different families (Martoni *et al.* 2016), which is considered a common trait of the genus worldwide (Ouvrard *et al.* 2015). However, the lack of a complete phylogenetic study of *Trioza* raised concerns on its taxonomical robustness, especially considering it has been deemed a “catch-all genus” (Ouvrard *et al.* 2015), suggesting the wide host plant association may be inflated by the presence of multiple genera. Furthermore, a phylogenetic study on this group would allow to answer taxonomic doubts regarding the position of the New Zealand genera *Atmetocranium* and *Anomalopsylla,* debated for more than 70 years (Ferris and Klyver 1932; Tuthill 1952; Heslop-Harrison 1960; Bekker-Migdisova 1973; Dale 1985; Burckhardt and Ouvrard 2012). At present, *Atmetocranium* is provisionally assigned to the family Calophyidae, together with South American species (Burckhardt and Ouvrard 2012), while *Anomalopsylla* is currently assigned to the subfamily Rhinocolinae (Aphalaridae) (Burckhardt & Ouvrard 2012).

To understand the relationships between New Zealand Hemipteran phloem-feeding insects and their symbionts, here we decided to generate a phylogenetic structure of this group that could be compared with a microbiome analysis in order to answer questions on the psyllids phylogenetics, their microbial diversity and psyllid-plant-bacteria interactions. The main aims of this work were to i) investigate the number of ancestral arrival events that have led to the present day’s native psyllid fauna to determine how many psyllid radiations happened, and ii) what evolutionary processes have led to the current psyllid-plant host relationships in New Zealand. By overlaying the phylogenetic framework obtained to a microbiome metabarcoding analysis, we aimed to enable a better understanding of the insect-bacteria dynamics and the microbiome composition. Hence, an additional aim was to , iii) verify what was hypothesised here, that one of the factors influencing the microbiome composition of the New Zealand psyllids could be the host evolutionary history, as opposed to a co-evolution with the host plants of the insects. Therefore, the psyllid phylogeny was used here to test its impact on the microbial composition of psyllids microbiome.

1. **Materials and methods**
   1. ***Psyllid DNA extraction, amplification and sequencing.***

DNA, from individual adult psyllids, was from (Martoni *et al.* 2018) or a selection of new samples from Australia and United States of America (Table SM1). PCR protocols essentially followed Martoni *et al.* (2018). For the ribosomal 18S, a 544-bp PCR product was amplified from 179 specimens using the primers 18S\_F (CTGGTTGATCCTGCCAGAGT; Ouvrard *et al.* 2000) and 18S\_Rmod, (ACCAGACTTGCCCTCCAAT; modified in this study from Ouvrard *et al.* 2000). Thermal cycling conditions were: an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min, followed by a final extension of 7 min. For COI, PCRs were performed on 16 new individual psyllids including specimens of *Blastopsylla, Cryptoneossa* and two species of Triozidsfrom Australia (*Trioza tricornuta* and *Acanthocasuarina acutivalvis*) (Table SM1). All amplicons were sequenced directly using the Sanger method (Bio-Protection Research Centre, Lincoln University, New Zealand) with the PCR amplification primers.

Twenty partial Elongation Factor-1α sequences (240 bp) were from (Martoni *et al.* 2017). A new EF1 α sequence was isolated here from *Atmetocranium myersi* (Acc. Number MH556913).

* 1. ***DNA sequence variation and phylogenetic analysis***

In total there were 665 psyllid DNA sequences used (plus three aphid sequences used as the outgroup), paired by specimen for each locus (Table SM1). This included sequences from all 90 psyllid taxa identified as present in New Zealand (Martoni *et al*. 2018) with at least two specimens from each species used where possible, plus ten species from Australia, Europe and USA. In addition, 16 sequences for the same COI and 18S gene regions were obtained from GenBank from six psyllid taxa, which were two species of *Trioza* (*T. remora and T. urticae*), two species of *Psylla* (*P. alni and P. buxi*), and the species *Rhinocola aceris* and *Heterospylla texana*. The pea aphid, *Acyrthosiphon pisum*, was used as an outgroup (Table SM1).

For each gene, DNA sequences were manually quality-checked, and alignments performed using MEGA version X (Kumar *et al.* 2018). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (Kumar *et al.* 2018). Maximum likelihood [ML] phylogenetic trees were then constructed using the Kimura-2-parameter [K2P] model (Kimura 1980) with a bootstrap of 10,000 replicates (Figures SM1 and SM2).

The best substitution model for each gene alignment was calculated in MEGA X using the Bayesian information criterion (BIC, Schwarz 1978). The General Time-Reversible (GTR) model (Tavaré 1986) was used for COI, the Tamura and Nei (TN93, Tamura and Nei 1993) model for 18S and the Hasegawa, Kishino, Yano (HKY) model (Hasegawa *et al.* 1985) for EF-1α. A three-gene species tree was developed using the package Starbeast (\*BEAST, Heled and Drummond 2010) in BEAST v2.5.1, with the Markov Chain Monte Carlo (MCMC) method (Drummond *et al.* 2012; Bouckaert *et al.* 2014; Bouckaert *et al.* 2018) and multiple chains of 1 billion replicates each.

Each model was selected together with a gamma distribution with a rate of 4. The mitochondrial gene COI was set to a 0.5 ploidy compared to the 2.0 for both 18S and EF-1α, as suggested for multi-gene analyses (Drummond and Bouckaert 2015). The software Tracer v1.7 (Rambaut *et al.* 2018) was used for visualization and diagnostics of the MCMC output. This confirmed that the Bayesian analysis had reached convergence and the resulting estimated sample size (ESS) was >>200 (508). LogCombiner was used to subsample the number of trees from 500000 to 100000. TreeAnnotator (Drummond *et al.* 2012; Bouckaert *et al.* 2014) was used to summarize the information in a single tree and to set a 10% burn-in based on the information visualized with Tracer. The resulting species tree was drawn using FigTree v1.4.3 (Rambaut 2016).

* 1. ***Microbiome sequencing***

The V3 and V4 regions of the bacterial 16S ribosomal RNA gene were amplified from a total of 220 whole insect specimens (Table S1), encompassing 65 species across 178 populations collected both in New Zealand and in Australia. DNA extractions, amplification and purification were performed in a Physical Containment [PC2] facility in order to minimize the risk of environmental contamination. Sixteen of the 200 individuals were sequenced twice (as technical replicates), in order to confirm the consistency of the results (Table 1). Amplification was conducted using 16S\_F and 16S\_R primers (Klindworth *et al.* 2013), modified with Illumina adapters as per the Illumina 16S Metagenomic protocol 15044223 Rev. B (available at https://support.illumina.com/downloads/16s\_metagenomic\_sequencing\_library\_preparation.html). PCR amplification was performed using an initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C denaturation for 30 s, 55°C annealing for 30 s and 72°C elongation for 30 s. A final 72°C elongation was performed for 5 min. Amplicons were verified on 1% agarose gel and checked for absence of visible bands in control samples, then purified using the Agencourt® AMPure® XP kit (Beckman Coulter, Brea, California, United States). The concentrations of PCR products were measured using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and samples at concentrations between 10 ng/μL and 50 ng/μL were sequenced on an Illumina MiSeq platform using 2x300bp reads at New Zealand Genomics Limited (NZGL).

* 1. **Microbiome bioinformatics**

Demultiplexed MiSeq reads (NCBI SRA acc no: xxxxxxx) were trimmed of PCR primers and sequencing adapters using BBDuK in BBTools v38.01 (Bushnell 2017) and truncated to 280bp. All reads containing ambiguous ‘N’ bases were removed, and sequence quality profiles were used to filter reads with more than two expected errors in the forward read, or 3 in the reverse read. Due to the quality crash at the end of reverse reads typical of 2x300bp Illumina sequencing, all reverse reads further truncated to 200bp to minimize the number of reads violating the error filter. Quality trimmed sequences were then analysed using DADA2 v1.9.3 (Callahan *et al.* 2018). As error rates can vary between flow cells and libraries, the DADA2 error model was determined separately for each MiSeq lane using the “pseudo-pooling” mode for increased sensitivity to rare variants. Following denoising, the inferred amplicon sequence variants (ASVs) from each MiSeq lane were merged into a single table, which was further filtered to remove chimeras (available at xxxxx: acc no). A search through public 16s reference sequence databases indicated that Silva database contained the highest representation of insect associated bacteria. Therefore, hierarchical taxonomy was assigned to the 1254 ASVs to the lowest rank possible with a minimum bootstrap support of 60% using the RDP classifier implemented in the DADA2 R package and a version of the Silva v132 database (Quast *et al.* 2013). This was followed by extra species level assignment using exact matching between the query and reference sequences, which has previously been shown to be the most robust method for assigning species level taxonomy to short 16S reads (Edgar 2018). A ML bacterial phylogenetic tree was also assembled from the 16S reads using the General Time-Reversible (GTR) model (Tavaré 1986) and the phangorn package in R. All samples with below 1000 total reads remaining, and all taxa that were classified as Chrolorplast, Mitochondrial, or non-bacterial were removed. To assess the reproducibility of the workflow, all replicated samples were rarefied to the same read depth and distance checked using a principal component analysis of Euclidian distance. This analysis suggested reproducibility (stats?), and all replicates were merged.

* 1. ***Statistical Analysis***

*Alpha diversity*

As species richness for samples is positively correlated to sequencing depth, this needs to be accounted for to allow comparison across samples. The common procedure of rarefying samples to the same read depth has been found to be statistical inadmissible (McMurdie *et al.* 2014, Willis 2019). Instead the breakaway method was used to estimate the additional unsampled diversity in the environment based on biological replicate, sequence depth and number of rare taxa in the data, (i.e. population of that psyllid species) (Willis & Bunge 2015). To assess how alpha diversity changes across psyllid species, we use then applied a betta model (Willis *et al*.2017) using psyllid species as the predictor, and sequence run as a random effect, in a framework that which accounts for uncertainty in diversity estimates due to unsampled taxa. To further assess if bacterial species richness was associated with psyllid phylogeny, Morans I (Moran 1950), Abouheif's Cmean (Abouheif 1999), Bloombergs K (Blomberg *et al.* 2003), and Pagel's λ (Pagel 1999) were calculated using the breakaway estimates of species richness and a Brownian motion model of evolution with the phylosignal R package (Keck *et al.* 2016).

Community assembly

To first investigate whether there was evidence for selective maintenance of microbial taxa, as opposed random passive acquisition from the environment, the Sloan Neutral Community Model (Sloan *et al.* 2006) was fit to the prevalence of a taxon in each sample compared to its abundance across the wider metacommunity of all psyllid hosts in the study. To further assess if bacterial communities were in the psyllids were assembled from phylogenetically clustered, or overdispersed taxa, the net relatedness index (NRI) and nearest taxon index (NTI) were calculated from the 16S gene tree and ASV relative abundance data using the Picante R package (ref). Significance of phylogenetic clustering within each sample was assessed against a permuted null model generated by randomising ASV’s and their relative abundances across the tips of the phylogeny (null.model = ‘taxa.labels’) across the phylogeny to simulate random draws from the bacterial metacommunity source pool.

*Beta diversity / phylosymbiosis through time*

To assess if microbiome could be predicted by psyllid species, Permutational Multivariate Analysis Of Variance Using Distance Matrices (adonis) tests were used on the microbial community distance matrix with 999 permutations using the vegan R package (ref). To identify patterns of phylosymbiosis, beta diversity dissimilarity matrices were assembled using, Jaccard, Bray-Curtis, weighted and unweighted Unifrac, as well as the compositionally aware Aitchison (Gloor *et al.* 2017) and philr (Silverman *et al.* 2016) distances and compared to psyllid phylogeny, plant genetic distance and geographic distance were using Mantel (Mantel 1967) and procrustean superimposition tests. Partial Mantel tests were further used to measure the correlation between matrices while controlling for a third. To assemble distance matrices for host plant phylogeny, Information on the phylogenetic relationships of the New Zealand plants was obtained from the Landcare Research database, Phylogeny of New Zealand Plants (<http://plantphylogeny.landcareresearch.co.nz/WebForms/Home.aspx>) (Wagstaff *et al.* 2004). Psyllid host plant definitions follow that of Burckhardt *et al.* 2014 and are either obtained from observations in the literature (e.g., Dale 1985, Ferris and Klyver 1932, Tuthill 1952) or, for the undescribed taxa, from direct observations on the host plant that the psyllids were located on (Martoni *et al.* 2018). The host plant evolutionary distance matrix was generated using Phylocom V4.2 (Webb *et al.* 2008). Geographic distance matrices were represented as Great Circle distance between latitude and longitude coordinates from collection locations. For each test with host or environmental factor, the distance matrix was filtered to just those samples for which metadata were available. As a complementary measure to matrix correlation, tree topology between the psyllid phylogeny and a ward.d2 hierarchical clustering of the microbial distance matrix was assessed using both the Robinson-Foulds (ref) and Matching Cluster (ref) metrics. As community assembly models indicated potential disparity In the phylogenetic scale of community assembly patterns, beta diversity through time analysis (BDTT) was used to sample the bacterial community in 0.01 time intervals backwards in evolutionary time and conduct the above tests at each time slice in order to differentiate patterns arising from recent co-diversification from those due to more ancient bacterial evolution. These analyses were conducted both with and without the family Enterobacteriaceae to determine if these patterns were being driven primarily by the known primary symbiont *Candidatus* Carsonella rudii.

Co-phylogeny / Co-speciation

In addition to the well-known association with their primary symbiont *Candidatus* Carsonella rudii, psyllid species can be associated various secondary symbionts with differing rates of vertical inheritance. Microbial taxa with more strict vertical inheritance should show stronger signs of co-phylogeny with the psyllid host. To identify these groups, phylogenetic congruence was first investigated at the scale of the entire community of co-occuring species using the Random Tanglegram Partitions (Random TaPas) algorithm. Given that microbial communities are generally labile assemblages, it is to be expected that certain interactions (such as symbiosis) are more consistent with a hypothesis of phylogenetic congruence than others. Therefore, the dataset was further subset to bacterial clades whose interactions contribute most to cophylogenetic congruence by comparing the residuals from clade level interactions to those of the remainder of global interactions using Welsh’s t test. The Random TaPas algorithm was then fit separately to these individual clades to identify fine-scale co-phylogeny. In addition to psyllid microbiome phylogeny, this approach was further conducted between the psyllid species tree and the hostplant tree, as well as just the Trioza genus and the hostplant tree.

**Results**

* 1. ***Evolutionary relationships amongst the New Zealand psyllid fauna***

The 460 psyllid COI sequences aligned here had 211 parsimony informative sites and showed high intra-generic nucleotide variation resulting in more than 200 haplotypes (Figure 1a). Accordingly, the COI ML tree (Figure SM1) could successfully separate all the taxa included here, as also previously reported for the original subset of sequences (Martoni *et al.* 2018). However, deeper nodes at family and genus level were not well supported.

The 184 18S sequences (from 100 taxa) had 49 parsimony informative sites and a total of 57 haplotypes after excluding five regions within the sequences that had insertions/deletions (Figure 1b). Therefore, while the 18S haplotype network (Figure 1b) could generally separate the different genera (with three exceptions, Figure 1), it often failed to separate different species, especially within the genera *Acizzia, Trioza* and *Ctenarytaina* (Figure 1b). Overall, the 18S ML tree showed a stronger bootstrap value for the nodes separating genera, compared to the COI ML tree that instead had stronger support for the nodes separating species (Figures SM1 and SM2).

The species tree inferred using the three alignments showed a structure with generally high posterior probability values at species, genus and family level (Figure 2). Here, the New Zealand native psyllids (determined as those hosted by New Zealand native plants; Martoni *et al.* 2016) fell into six lineages with the major clades represented by the genera *Ctenarytaina* (Aphalaridae)*, Psylla* (Psyllidae) and *Trioza* (two clades) (Triozidae), plus one lineage each for the genera *Atmetocranium* (Calophyidae) and *Anomalopsylla* (Aphalaridae). The species adventive to New Zealand (all the other genera, Table 1) were divided into a total of six families.

Within the subfamily Spondyliaspidinae (Aphalaridae), the New Zealand ***Ctenarytaina***formed a monophyletic clade, closely related to five Australian *Ctenarytaina* which formed another monophyletic clade diverged by 2.1% (Figure 2). Other genera within the same subfamily were more distant to New Zealand *Ctenarytaina*, ranging between the 4.1% of *Glycaspis* and the 4.75% of *Creiis* and *Cardiaspina* (Figure 2). Within the New Zealand *Ctenarytaina,* three taxa from *Fuchsia* *excorticate* (Onagraceae) were the earliest branching group. The second clade contained *Ctenarytaina* sp. A hosted by *Olearia* (Asteraceae) and the taxa from Myrtaceae.

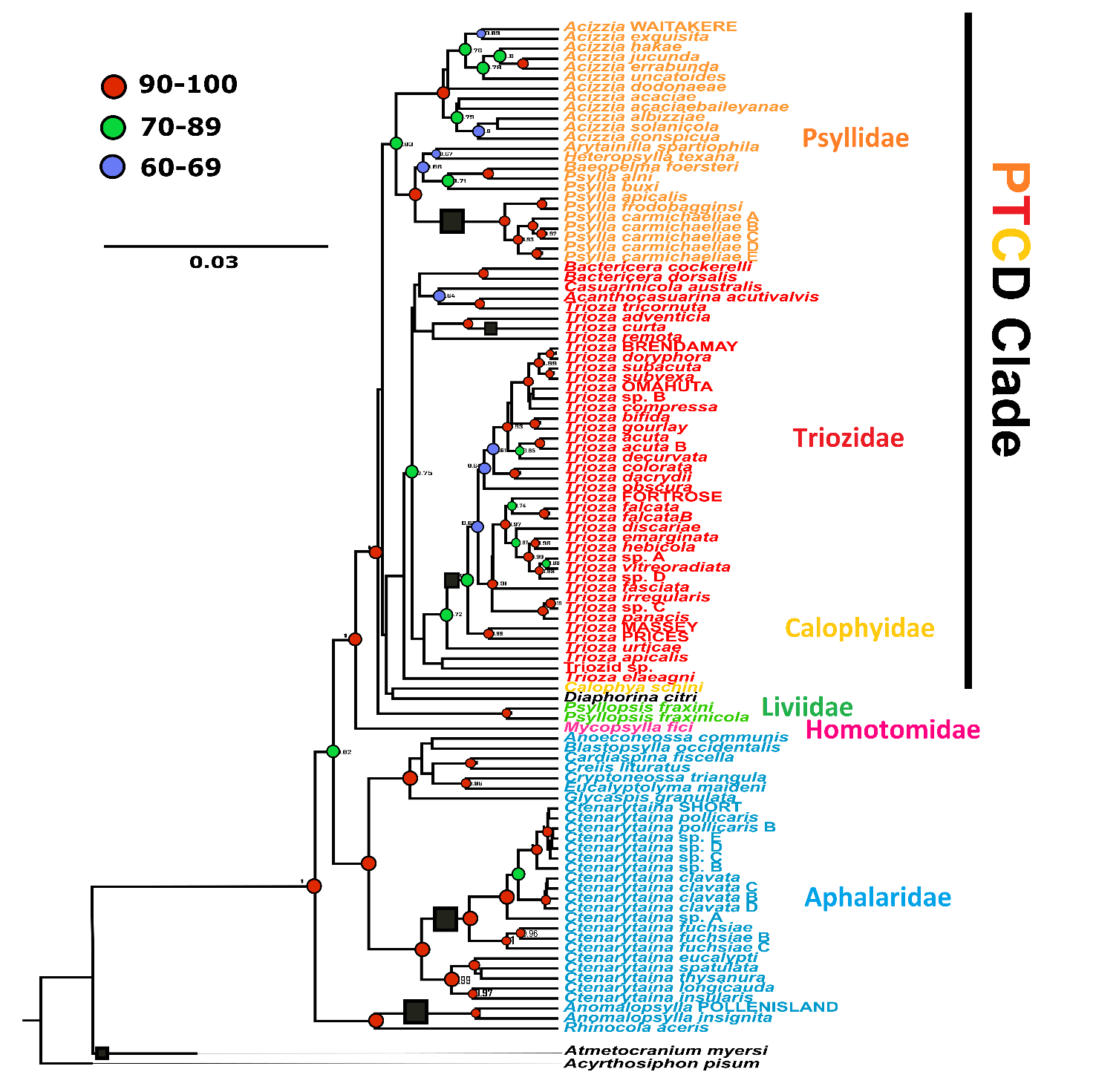
Separated from the other genera of the Aphalaridae, the genus ***Anomalopsylla*** clusters together with European species *Rhinocola aceris.*

The New Zealand *Psylla* formed a monophyletic group. This includes two described species and five undescribed taxa diverging more than 3.2% (3.23%) from the closest *Psylla* species from Europe. The subfamily Psyllinae forms a monophyletic group including the New Zealand *Psylla* and other non-New Zealand species, comprising the adventive genera *Baeopelma* and *Arytainilla*, and European *Psylla buxi* and *P. alni*, but also the North American *Heteropsylla texana* (Ciriacreminae). The Psyllinae are well separated by a 1.9% divergence from the other monophyletic Psyllidae subfamily, the Acizzinae, represented in New Zealand by the Australian genus *Acizzia.*

Within the Triozidae, all but one of the 31 native New Zealand *Trioza* species cluster into a single monophyletic clade (Figure 2). The closest non-New Zealand relative was the European *T. urticae*, although the bootstrap support for this relationship was low. The native *T. curta* diverged from the other New Zealand *Trioza* by 4%, demonstrating a separate ancestral introduction. *Trioza curta* formed a monophyletic association with *T. adventicia,* an adventive species from Australia that is hosted by another Myrtaceae species, *Syzygium* *smithii* (Percy 2017; Martoni 2017). The Australian triozids formed a monophyletic clade (including New Zealand’s *T. curta*) but with very weak affinities to one another. Beside the New Zealand species of the genus *Trioza,* the family Triozidae included the adventive species of the genera *Acanthocasuarina, Bactericera, Trioza* (from Australia and Europe)*, Casuarinicola* and an Australian triozid species collected from the host plant *Casuarina*. *Trioza* species not native to New Zealand included two spp. from Europe (*T. urticae* and *T. remota*) and one from Australia (*T. tricornuta*)*.* With the European *T. urticae* branching at the base of the major New Zealand group (including all but two species) while the New Zealand taxa *T. curta* clustered with *T. adventicia* and the rest of the Triozidae.

**Table 1: Psyllid taxa analysed** divided by families, subfamilies and genera, with number of species and individuals samples. The subfamily Atmetocraniinae (in red) is only tentatively assigned here to none of the current families. For each psyllid genus, the genes used in the analysis are marked with a tick (**).** Details on the psyllid species and their host plants are reported in Table SM1.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Family** | **Subfamily** | **Genus** | **Species** | **Samples** | **COI** | **18S** | **EF-1α** |
| Aphalaridae | Rhinocolinae | *Anomalopsylla* | 2 | 7 | **** | **** | **** |
|  |  | *Rhinocola* | 1 | 1 | **** | **** |  |
|  | Spondyliaspidinae | *Anoeconeossa* | 1 | 3 | **** | **** | **** |
|  |  | *Blastopsylla* | 1 | 6 | **** | **** | **** |
|  |  | *Cardiaspina* | 1 | 3 | **** | **** | **** |
|  |  | *Creiis* | 1 | 1 | **** | **** | **** |
|  |  | *Cryptoneossa* | 1 | 10 | **** | **** | **** |
|  |  | *Ctenarytaina* | 20 | 132 | **** | **** | **** |
|  |  | *Eucalyptolyma* | 1 | 2 | **** | **** | **** |
|  |  | *Glycaspis* | 1 | 2 | **** | **** | **** |
|  | Atmetocraniinae | *Atmetocranium* | 1 | 2 |  | **** | **** |
| Calophyidae | Calophyinae | *Calophya* | 1 | 4 | **** | **** |  |
| Liviidae | Euphyllurinae | *Psyllopsis* | 2 | 7 | **** | **** | **** |
|  |  | *Diaphorina* | 1 | 2 | **** | **** | **** |
| Homotomidae | Macrohomotominae | *Mycopsylla* | 1 | 5 | **** | **** | **** |
| Psyllidae | Acizzinae | *Acizzia* | 12 | 84 | **** | **** | **** |
|  | Psyllinae | *Arytainilla* | 1 | 4 | **** | **** |  |
|  |  | *Baeopelma* | 1 | 2 | **** | **** | **** |
|  |  | *Psylla* | 9 | 39 | **** | **** |  |
|  | Ciriacreminae | *Heteropsylla* | 1 | 1 | **** | **** |  |
| *Triozidae* |  | *Bactericera* | 2 | 7 | **** | **** | **** |
|  |  | Triozid sp. | 1 | 3 | **** | **** |  |
|  |  | *Casuarinicola* | 1 | 3 | **** | **** | **** |
|  |  | *Trioza* | 35 | 130 | **** | **** |  |
|  |  | *Acanthocasuarina* | 1 | 1 | **** | **** |  |



**Figure 1:** **Species tree of the New Zealand Psylloidea included in this study.** The tree was inferred from 668 DNA sequences of partial COI, EF-1α and 18S genes using BEAST v.2.5.0. The families Psyllidae (orange), Triozidae (red) and Calophyidae (*Calophya schini;* yellow), together with *Diaphorina citri* (black) form the PTCD clade, as reported in (Percy *et al.* 2018). *Acyrthosiphon pisum* (Hemiptera) was used as an out group. New Zealand endemic species are indicated with black squares on branches showing ancestral arrivals of native species. Posterior probability values at the nodes are reported in red when ranging between 0.9 and 1; in green when between 0.7 and 0.89; and in blue when between 0.6 and 0.69. Posterior probability values lower than 0.6 are not reported.

* 1. ***Microbiome results***

**Bacterial diversity**

In total, n ASVs were recovered, representing 7 distinct phyla. The bacteria that were recorded with the greatest number of reads and of OTUs from the GreenGenes database belong to the family Enterobacteriaceae. A total of 106 Enterobacteriaceae OTUs, 28 recorded less than 300 reads and were therefore not considered further, while the other 78 were aligned to generate a 16S gene tree to assess the genetic distance between them. The same OTU was found to be often present in different species and families or with multiple OTUs in the same individual insect (e.g. T. falcata). The average genetic distance between these OTUs was 11%, with the greatest of 26%. When comparing the different Enterobacteriaceae OTUs with sequences in NCBI, the closest results were between 92% and 94% similarity to “Sodalis-like sequences” and between 99%-100% similar to other S-symbiont sequences isolated from other psyllids (such as Calophyia schini and Mycopsylla fici).

In addition to the P- and S-symbionts a number of other culturable and unculturable (Candidatus) bacteria were recorded. Wolbachia was the most abundant bacterium after the Enterobacteriaceae, recorded in 135 insects belonging to ten of the 17 psyllid genera across all psyllid families. Rickettsiella (Gammaproteobacterium) was recorded in five insects at a maximum compositional level of 41.2%: Acizzia acaciaebaileyanae, Arytainilla spartiophila, Ctenarytaina longicauda (both the samples analysed) and Glycaspis granulata. Mycoplasma was recorded from 42 samples consisting of eight Trioza, 28 Ctenarytaina, one Casuarinicola and five Acizzia. The seven samples recording the highest levels of Mycoplasma were all Ctenarytaina species. Other bacteria recorded included Candidatus Rhabdochlamydia (in six psyllids), Candidatus Hamiltonella (single psyllids), Candidatus Cardinium (four samples of the same species, Anomalopsylla insignita), Acidovorax (216 psyllids), Pseudomonas (135 psyllids), Agrobacterium (73 psyllids).

3.1. Comparison of different psyllid species from the same individual plant

Variation in the microbiome composition of different psyllid species feeding on the same individual plant was considered in order to assess if there were any microbiome-host plant specific relationships. Two analyses were performed on samples belonging to two psyllid species of the Fraxinus-feeding genus Psyllopsis, P. fraxinicola and P. fraxini, and on two species belonging to the Sophora-feeding genus Psylla, P. apicalis A and P. apicalis B. For Psyllopsis, different P-symbiont OTUs and different Enterobacteriaceae OTUs are consistently recorded between the two psyllid species (Table 2). Conversely, for Psylla the same P-symbiont OTU and two Enterobacteriaceae OTUs are present in both species, although three additional Enterobacteriaceae OTUs (5, 143, 208) appear associated with separate psyllid species (Table 3). Wolbachia appeared to be present at consistent levels in the Psyllopsis fraxini and Psylla apicalis B populations, but vary markedly in the Psyllopsis fraxinicola and Psylla apicalis A populations. Otherwise there were no stark presence/absence or highly variable levels apparent between either of the pairs for the other OTUs (Table 2 and 3).

Community assembly

The amount of variation explained by Sloans neutral model was low (R2 =0.15, m=0.00034), with 45.5% of bacterial taxa over-represented compared to neutral expectations. This indicates that microbial communities within psyllids are not random assemblages drawn from a common pool and may instead be selected for by host or environmental characteristics. This was further supported by models of phylogenetic community assembly with high values of both NRI and NTI (Supp Table x, supp figure x) indicating significantly smaller phylogenetic distances among species than expected under a null model of random assembly. Taken together, this indicates that deterministic filtering by aspects of psyllid physiology, diet, or other environmental factors may select for phylogenetically clustered taxa with overlapping niches. To assess if these patterns were localised to a portion of the psyllid phylogenetic tree, Morans I, Abouheif's Cmean, Bloombergs K, and Pagel's λ were calculated for both the alpha diversity and phylogenetic community assembly metrics, however statistical significance under a Brownian model of evolution was only found for a minority of metrics (table x), further suggesting a global pattern. Finally, the mean NTI value was twice as large as the mean NRI, as NTI is generally thought to be more sensitive to tree wide patterns, which NRI is more sensitive towards the tips of the phylogeny this could further indicate phylogenetic disparity in phylogenetic scale of these patterns.

Phylosymbiosis

To assess for signals of phylosymbiosis between the overall microbial communities and their psyllid hosts, matrix correlations between the beta diversities, as well as topological comparisons of hierarchical clustering and a range of statistics were conducted. Due to previous results indicating a potential disparity in phylogenetic scale for patterns of phylosymbiosis, BTDD analysis was conducted. Mantel tests found psyllid evolutionary distance and microbiome beta diversity to be significantly correlated across all dissimilarity metrics and time slices. Similar results were seen with partial mantel tests where either plant distance or geographic distance were taken into account. In contrasts, the correlation between microbiome and plant genetic distance, as well as microbiome and spatial distance was only significant deeper into evolutionary time, and only for some distance metrics (Fig x). When analyses were repeated with and without the family Enterobacteriaceae, the correlation between microbiome beta diversity and psyllid phylogeny remained significant, indicating these patterns were not being driven by the primary symbiont alone.

|  |  |  |
| --- | --- | --- |
| Statistic | Value | P |
| Cmean | 0.0663762 | 0.168 |
| Morans I | --0.005323148 | 0.207 |
| K | 0.76136029 | 0.009 |
| Lambda | 0.8880115 | 0.001 |





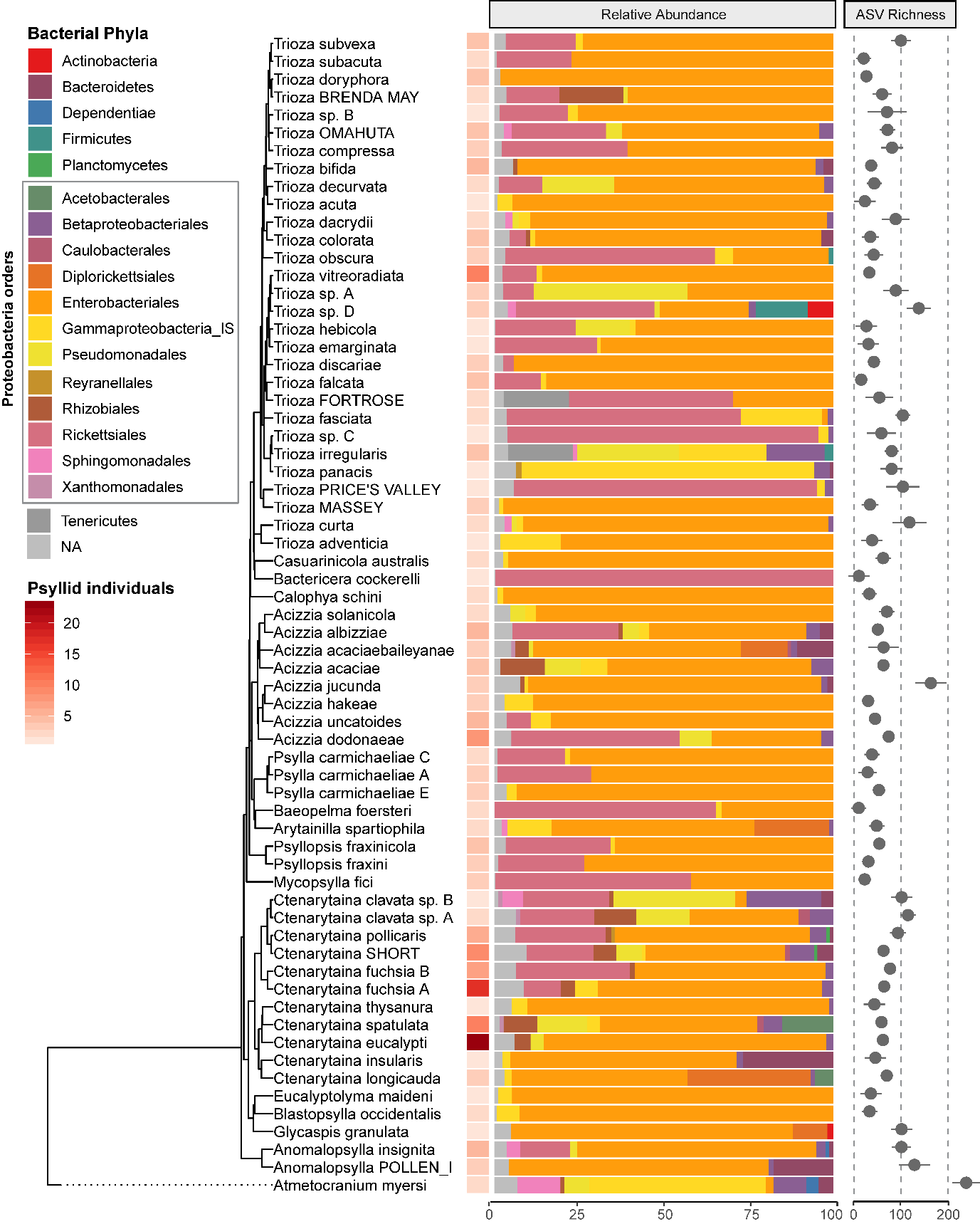




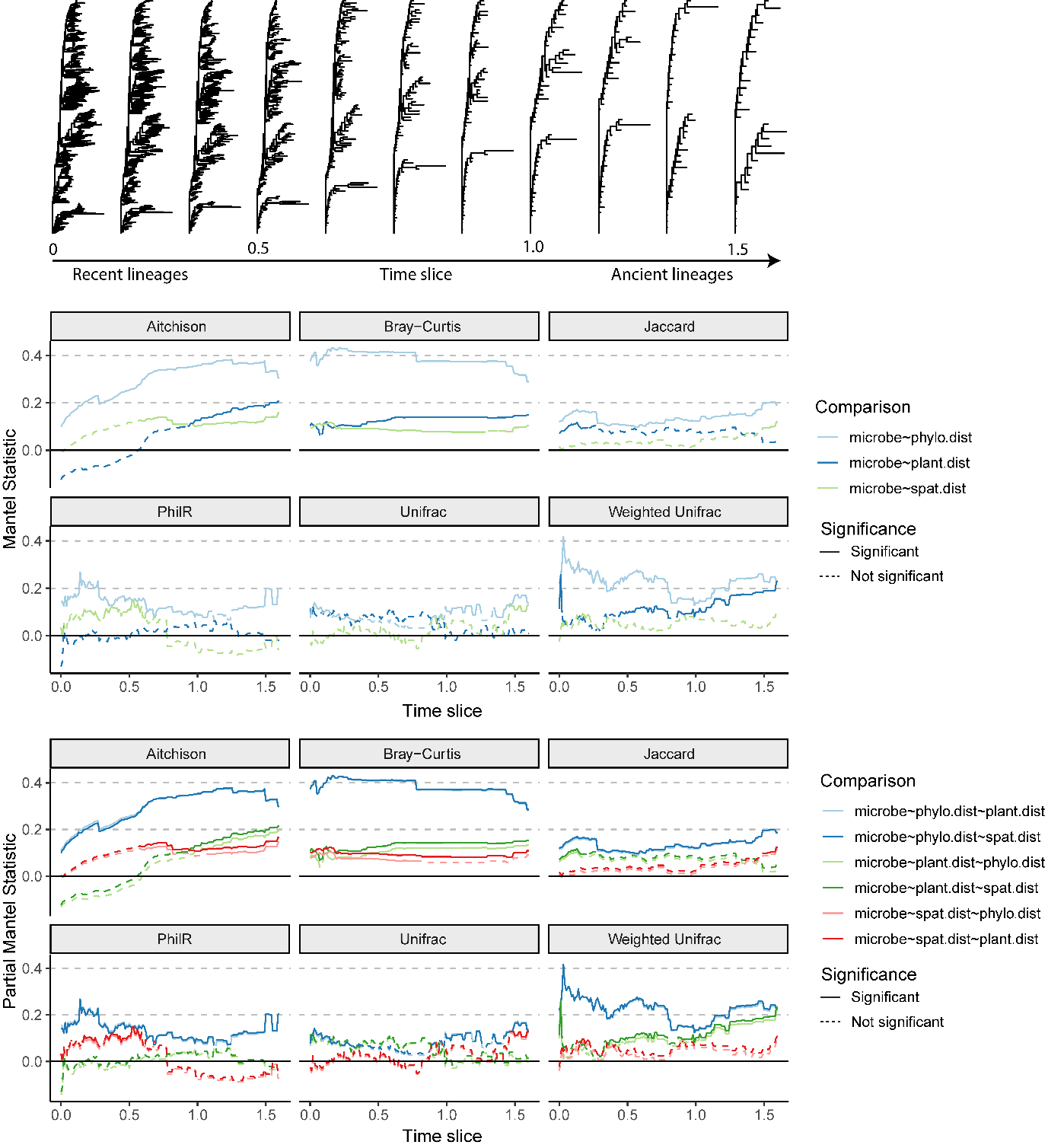












Cophylogeny

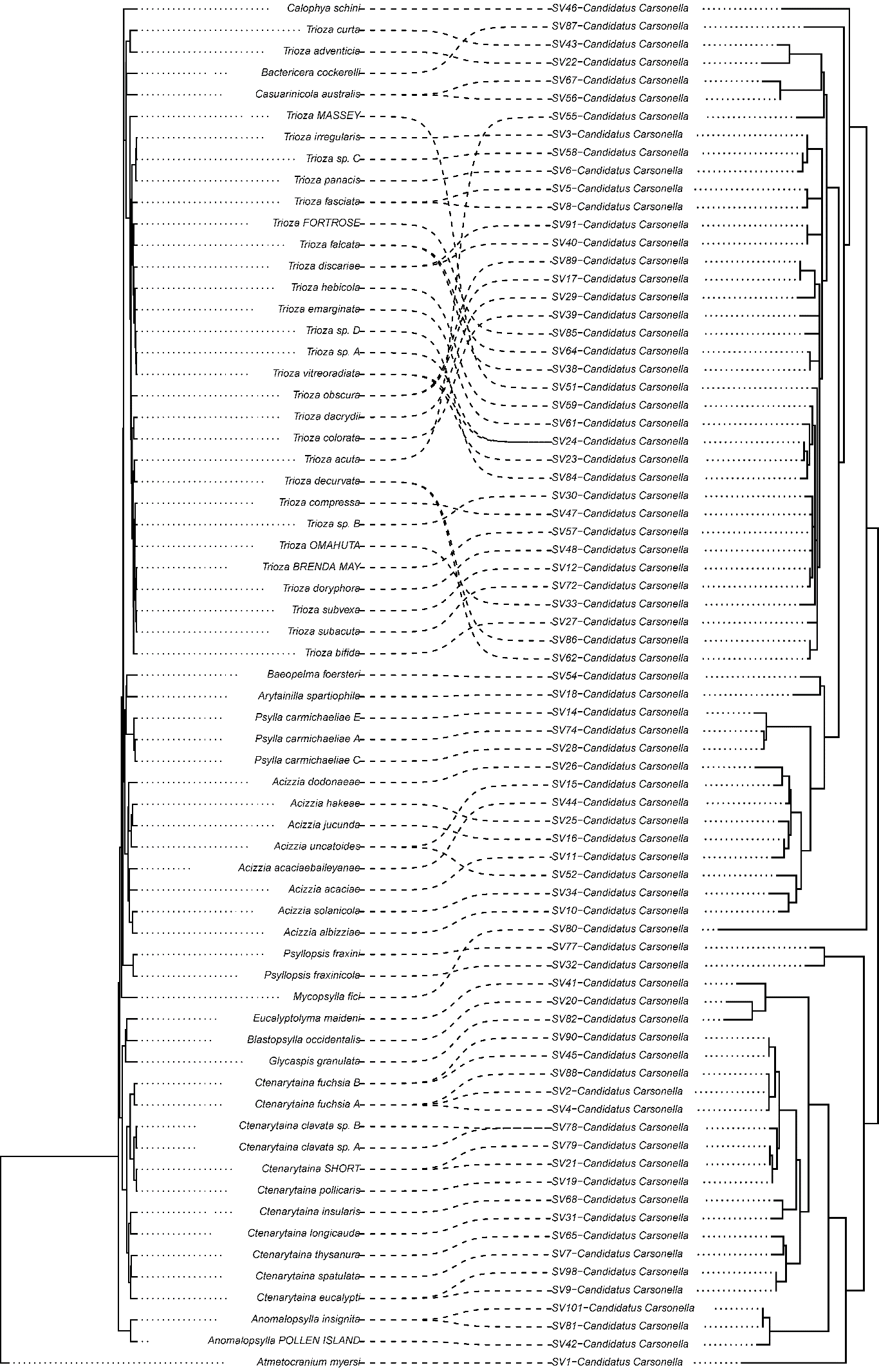
* 1. *Diversity of the primary symbiont: Candidatus Carsonella rudii.*

*Candidatus Carsonella* was the only bacterial genus recorded across all psyllid species analysed, albeit generally at low abundance, with a mean relative abundance of x. Almost all psyllid species analysed contained a unique *Carsonella* haplotype, and no intraspecific diversity in the haplotypes. A few exceptions were the two populations of *Psylla apicalis* and *P. frodobagginsi,* each showing a separate – although very closely related – haplotype, populations of *T. falcata, T. fasciata* and *A. uncatoides.* Similarly, two populations of *Trioza vitreoradiata* showed a distinct *Carsonella* haplotype from the other six analysed here. These six shared the same haplotype of *Trioza* sp. A, an hypothesised species, genetically very close to *T. vitreoradiata.*









1. **Discussion**
   1. ***A philogenetic structure of the New Zealand psyllids.***

The general shape of the species tree, especially at a taxonomic level, matches the most recent phylogenetic works on the Psylloidea (e.g., Percy *et al.* 2018; Cho *et al.* 2019). The families Psyllidae, Triozidae and Calophyidae, together with the species *Diaphorina citri,* form the PTCD clade (Percy *et al.* 2018), at the top of the tree, and were separated from the family Liviidae, represented in New Zealand by the two non-native species of the genus *Psyllopsis*, with maximum posterior probability support (Figure 1). Similarly, the family Homotomidae is represented by a single adventive species, *Mycopsylla fici,* branching with maximum posterior probability (Figure 1). At the base of the tree, the family Aphalaridae appears to be paraphyletic, with the subfamily Rhinocolinae branching earlier than the Spondyliaspidinae, in accordance with the most recent works (Cho *et al.* 2019). Separated from all the other families, a single branch that included the species *Atmetocranium myersi* showed no affinity to any other group.

Therefore, the species tree obtained here appears robust and well supported at both deeper and shallower nodes, despite the use of a relatively small number of markers, supporting the previously defined taxonomic relationships between species, genera and families, as defined by their morphological characteristics (Burckhardt and Ouvrard 2012). Hence, this phylogenetic structure can be used further for comparisons and to determine evolutionary history for a number of New Zealand psyllid groups.

* 1. ***Ancestral arrivals of today’s New Zealand endemic psyllids.***

Many New Zealand insect taxa are known to have been derived by dispersal rather than vicariance (Goldberg *et al.* 2008, Buckley *et al.* 2015). Previous work on the origin and evolution of the New Zealand psyllids (Dale 1985) regarded the presence of genera common to New Zealand, New Caledonia, Australia or South America (i.e. *Ctenarytaina* and *Psylla*) as a possible explanation for a Gondwanan origin for these insects (Dale 1985), as opposed to a dispersal. This interpretation was probably influenced by the outdated view that the New Zealand native biota was primarily a product of long-standing geographical isolation resulting from the Gondwanan split, happened approximately 80 Mya (Goldberg *et al.* 2008). Based on the molecular phylogeny presented here, we could confirm monophyly for a number of New Zealand native psyllid genera, suggesting the present fauna of these groups is the results of separate arrivals into the region.

This study suggests that all New Zealand *Trioza* species (except *T. curta*) form a monophyletic grouping that appears to have come from a single arrival to New Zealand. These New Zealand *Trioza* were not clearly related to the Australian triozids represented here. However, the Australian triozids are also distant from one another, suggesting that they may not be a natural monophyletic group. This could be the result of a previous arrival to Australia by multiple dispersals or it could suggest they are the remnants of a larger Australian group that has lost members over time.In order to understand the origin of the New Zealand *Trioza* a more complete sampling of triozids from the Asia-Pacific is required. In particular, it would be advantageous to study *T. oleariae* Froggatt 1903, from Tasmania which is hosted by *Olearia* (Asteraceae) (Hollis 2004), as are many of the New Zealand *Trioza*. Consequently, T. curta appears to be from a second distinct ancestral arrival or even of Australian origin, considering how it appears to be genetically similar to *T. adventicia.*

Another example of monophyly that suggests a separate arrival is presented by the genus *Psylla,* recording seven species in New Zealand, while being absent in Australia. The identity of New Zealand *Psylla* species has been hypothesised to be different to that of their European counterparts based on morphological characters, possibly placing them in a new genus (Martoni *et al.* 2016). The results obtained here appear to be in agreement with this hypothesis, showing a well-supported separation between the New Zealand species and their European counterparts. The two other *Psylla* species in the analysis, *P. buxi* and *P. alni* from Europe, were relatively distant from the New Zealand taxa. The fact that the European *Psylla* appear more closely related to other genera in the Psyllidae (*Baeopelma* and *Arytainilla* - subfamily Psyllinae; *Heteropsylla* subfamily Ciriacreminae) than they are to the New Zealand *Psylla* (subfamily Psyllinae) is consistent with the hypothesis that the New Zealand clade belong to an entirely different genus. Morphological characters such as 8-segmented antennae and marginal setae on the caudal plate place New Zealand *Psylla* within the Psyllinae but outside *Psylla* (Martoni *et al.* 2016). Nevertheless, the taxa included here are insufficient to determine the closest relatives of the New Zealand *Psylla*; inclusion of those from Fiji and the Asia/Pacific region would be necessary.

A fourth arrival is hypothesised here for the New Zealand species of the genus *Ctenarytaina.* There are five *Ctenarytaina* species in Australia (Ouvrard 2019) and the fact a number of other psyllids species arrived in New Zealand from there in recent times (Martoni *et al.* 2016), an Australian origin of the New Zealand *Ctenarytaina* species could be hypothesised. Nonetheless, a number of *Ctenarytaina* species are also distributed across the Pacific islands, e.g. *C. distincta* (Tuthill, 1943) from Fiji, *C. lulla* Tuthill, 1942 and *C. remota* Tuthill, 1956 from French Polynesia (Ouvrard 2019; Tuthill, 1942, 1943, 1956), which highlights the importance of wider geographic collections in the future, as already stated elsewhere (Martoni and Armstrong 2019a). Possible alternative sources to Australia is also consistent with the fact that there are no *Psylla* in Australia (Hollis 2004; Ouvrard 2019). But their worldwide presence includes *P. compta* Crawford, 1919 in Fiji as the closest location to New Zealand (Ouvrard 2019).

A fifth ancestral arrival is that of the genus *Anomalopsylla.* The phylogenetic position of *Anomalopsylla* here is consistent with recent taxonomic classifications placing this genus in the family Aphalaridae. Polyphyly of this family is consistent with the subfamily division, with the subfamily Rhinocolinae (including *Rhinocola* and *Anomalopsylla* - Burckhardt and Ouvrard 2012) separated from the Spondyliaspidinae (all other genera). While the subfamily Rhinocolinae includes 13 genera distributed worldwide (Burckhardt and Lauterer, 1989), *Anomalopsylla* is the only genus present in the Asia-Pacific area and the only one hosted by Asteraceae. The addition to the analysis of the European species *Rhinocola aceris* highlighted how this species is relatively phylogenetically close to *Anomalopsylla.*

The sixth ancestral arrival is *Atmetocranium myersi,* the only representative of this genus. This species showed no phylogenetic affinity with any other psyllid species or family.In a recent morphological classification of the Psylloidea, *Atmetocranium* was tentatively placed within the Calophyidae, because of its distinctive metatibia (Burckhardt and Ouvrard 2012). The Calophyidae includes at least 118 species (Ouvrard 2019), but none are native to New Zealand. *Atmetocranium* was earlier placed with the Aphalaridae, based on wing morphology (Klimaszewski 1964). Based on the results obtained here, it would appear that it does not belong to any of the Aphalaridae subfamilies included here. Samples from the other three subfamilies (Aphalarinae, Pachypsyllinae and Togepsyllinae) will need to be analysed before a linkage between *Atmetocranium* and Aphalaridae can be dismissed. Furthermore, the superfamily Psylloidea includes another two families not present in New Zealand, Carsidaridae and Phacopteronidae (Burckhardt and Ouvrard 2012), that appear to be genetically close to the family Aphalaridae (Percy *et al.* 2018). However, *A. myersi* has also been noted as having a “highly autapomorphic morphology which makes it difficult to relate to other psylloid groups” (Mifsud and Burckhardt 2002). This detail appears to be in agreement with the results obtained here, showing a clear separation between *Atmetocranium* and all the other psyllid families presented in this study. This, together with its peculiar morphology (Mifsud and Burckhardt 2002), suggest that *Atmetocranium* could belong to an entirely new psyllid family.

* 1. ***Radiation and host plant association of the New Zealand Trioza***

Confirming a trend widely accepted elsewhere for psyllids (e.g., Ouvrard *et al.* 2015), New Zealand *Ctenarytaina* and *Psylla* are associated with only one or a few host plant families (Burckhardt *et al.* 2014). On the other hand, worldwide, *Trioza* shows an unusually large range of plant genera associations, with a recent study listing 346 *Trioza* psyllid species on 154 plant genera in 59 plant families (Ouvrard *et al.* 2015). However, it is unclear if *Trioza* psyllids are more prone to host switching, or if the potentially polyphyletic nature of *Trioza* may distort the breadth of host-plant associated with this genus (Ouvrard *et al.* 2015). The New Zealand species were known to be consistent with the worldwide genus in that they do occupy many different host plant families (Martoni *et al.* 2016), but the lack of a phylogenetic structure could not clarify if the genus was monophyletic.

The results of this phylogenetic analysis suggest the monophyly of the New Zealand *Trioza*, hence demonstrating that *Trioza* species have indeed acquired a large number of new hosts since arriving in New Zealand, and that this is not a case of polyphyly. Therefore, pairing the phylogeny of the New Zealand *Trioza* analysed in this study with their host plants shows for the first time how the radiation of the psyllids developed on multiple plant genera and families after the arrival of the first ancestral *Trioza* psyllid to New Zealand.

For example, the psyllids’ association with the most common host plant family (Asteraceae), hosting 14 *Trioza* species, appears to be the result of multiple colonization events from these insects. With a cluster of 12 closely related psyllid species positioned apically in the tree (Figure 3, left) suggesting a more recent colonization event as compared to the association between *T.* “Massey” or *T.* “Fortrose”, distant from the other Asteraceae feeding psyllids. Similarly, a single psyllid species in the cluster, *T. decurvata*, is found on *Dracophyllum* (Ericaceae) which, although positioned within the same major plant clade as the Asteraceae, is remote from it. Prior to this study, Asteraceae had been thought to be the ancestral host of New Zealand *Trioza* based on morphology and host associations (Martoni *et al.* 2016). However, the host association with Asteraceae was not clear-cut; while one of the earliest diverging species, *T.* “Massey” has an Asteraceae host, most of the remaining Asteraceae inhabiting psyllids appeared to have derived from a more recent host adoption/speciation event.

Another example is the association between the psyllids *T. colorata* and *T. dacrydii* with the Podocarpaceae (*Halocarpus bidwillii*), a conifer lineage. These two psyllid species are branching within the broader clade of *Trioza*, suggesting a shift from an angiosperm host within New Zealand.

These results also suggest that the New Zealand *Trioza* has evolved becoming more speciose than any other genus, including the *Ctenarytaina* and the *Psylla* which branched at the same time. However, survey technique should be considered given more than half of the *Psylla* (4 out of 7 New Zealand species) and 80% of the *Ctenarytaina* (12 out of 15 New Zealand species) is composed of undescribed species, as compared to only 35% for the *Trioza* (11 out of 31 New Zealand species).

* 1. ***The microbial diversity harboured by the New Zealand psyllids***
     1. Symbiotic bacteria

The microbial dataset generated and analysed here included 236 psyllids, belonging to 65 species, 18 genera and six families. This is a significant advance on that of previous such studies that either focused on a smaller taxonomic range of insects, such as the Australian genus *Cardiaspina* (Hall *et al.* 2016) or used different techniques that generated smaller numbers of sequences (Thao *et al.* 2000b, Spaulding and von Dohlen 2001).

As expected, given the obligate status of *Candidatus* Carsonella rudiias primary symbiont in psyllids (Thao *et al.* 2000a, Thao *et al.* 2001, Hall *et al.* 2016), this was recorded in all the samples here, confirming its role as primary symbiont. Alignment of the different sequences and the construction of a Maximum Likelihood tree suggest that the radiation of this bacterium matched the psyllid phylogeny (Figure 3). This supports the long-lasting *Carsonella-*psyllids coevolution hypothesis, possibly originated from a single, ancestral infection (Thao *et al.* 2000a, Thao *et al.* 2001, Hall *et al.* 2016).

Beside *Carsonella,* the family Enterobacteriaceae was the most abundant (Table 5.1). We identified 78 distinct Enterobacteriaceae OTUs, only some of which identified as *Sodalis*. When compared to sequences on GenBank, some of the *Sodalis* sequences obtained here showed a 91%-94% similarity with *Sodalis* sequences. However, other Enterobacteriaceae sequences showed different similarities to other sequences belonging to this family. For example, some were closely associated with the genus *Arsenophonus.* However, they also show a distribution across the different psyllid species which is sometimes limited to a single Enterobacteriaceae per taxa. For example, *Hamiltonella* was present only in *Anomalopsylla,* highlighting a strict psyllid-Enterobacteriaceae relationship. This is in agreement with recent work suggesting coevolution between psyllids and S-symbionts and may indicate an obligate instead of the anticipated facultative symbiosis (Hall *et al.* 2016). A less strict symbiosis than that with the P-symbiont also supports the concept of a dual nature for psyllid S-symbionts, suggesting they could be both vertically and horizontally transmitted as hypothesised elsewhere (Hall *et al.* 2016).

The role of other Enterobacteriaceae genera, such as *Arsenophonus*, *Sodalis, Schneideria* and *Blochmannia,* as a S-symbiont of insects has been widely reported for other insects, such as Glossinidae flies (Diptera) (Aksoy *et al.* 1997), lygaeid stinkbugs (Matsuura *et al.* 2012), carpenters ants (Schroder *et al*. 1996; Sauer *et al.* 2000) and a weevil (Heddi *et al.* 1998). This may explain why, with the exception of *Schneideria*, all Enterobacteriaceae have been previously assumed as S-symbionts of psyllids as well (e.g., Thao *et al.* 2000b; Hall *et al.* 2016). Ultimately, the results obtained here suggest that some Enterobacteriaceae S-symbionts of psyllids have a strong history of coevolution with the members of this insect superfamily. The ~90% sequence similarity with the closer sequences on the database reported here unfortunately does not enable these bacteria to be clearly assigned to any specific genera, for which further analyses using multiple markers will be needed. Such an approach has been taken for a S-symbiont phylogeny but was restricted to only 16 psyllid species (Hall *et al.* 2016).

* + 1. The Psyllids-bacteria relationship: a new case of phylosymbiosis.

Alpha diversity is the diversity of organisms in one environment (Whittaker 1960), and the environment considered in this study is the insect. However, diversity of the microbial composition is a function of the sequencing depth, which is extremely variable where extremes of low sequencing depth may still capture high diversity or high sequencing depth may still result in low apparent diversity (Figure 5). In fact, a variation can be clearly observed with samples recording a number of reads even higher than 80,000 (for more than 100 bacterial OTUs) while others have just a few thousands reads. Diversity has also been demonstrated here to be a function of the individual PCR characteristics (Figure?). For this reason, the number of OTUs recorded and the alpha diversity generated could be biased by the number of specimens analysed for each species. Consequently, the sample with the smallest number of reads has been used to rarefy the plot in Figure 6. While aware of the limitations of rarefaction (McMurdie and Holmes 2014), this allowed the alpha diversity to be compared between the different psyllid species as if every sample had the same number of sequences. The rarefied test shows how the microbiome diversity compares amongst species within the same genus and family (Figure 6).

After confirming that the microbial composition of psyllids was not randomly distributed (Alpha and Beta diversity) and associating this variation with psyllid taxa (ADONIS approach accounting for the 60% of the variation in the microbial composition), the microbial composition was shown to be more similar in closely related psyllid species. Thus, considering the P-symbiont and pool of S-symbionts, the phylogeny of the insect would be expected to be the predominant driver of microbiota structure. In fact, studies elsewhere focusing both on the P-symbiont (Thao et al. 2000a, Spaulding and von Dohlen 2001, Thao et al. 2001, Hall et al. 2016) and on the S-symbionts (Thao et al. 2000b, Hall et al. 2016, Morrow et al. 2017), showed different degrees of association between psyllids and their symbionts. This includes recent studies confirming degrees of vertical transmission for S-symbionts (Hall et al. 2016). Moreover this signal of a strong association of the “group of common OTUs” with the insect phylogeny is in accordance with the recently defined insects-bacteria relationship of “phylosymbiosis” (Brooks et al. 2016). In fact, not only the P-symbiont, but also the group of S-symbionts recorded here appears to be a component of the psyllids microflora composition strongly connected with the insects evolution.

Geography may also influence distribution of the P- and S-symbionts as indicated here with the same psyllid species collected in New Zealand compared to Australia showing different P- and S-symbiont composition. This has similarly been recorded for *D. citri,* which shows genetic variation in its P-symbionts between populations in Asia and in the United States (Wang et al. 2017). However, a Mantel test in this study showed relatively little signal associated with the geographical distribution of the species collected in New Zealand. Possibly, a more extensive sampling across a wider area could report bio-geographic associations as those recently presented for the nettle-psyllid, *Trioza urticae,* in Europe (Wonglersak et al. 2017).

Given the specificity of psyllids to their plant hosts, close association of the microbes to the species of psyllid may alsosuggest that microbial composition could depend on the psyllid host plants. However, the Mantel and Partial Mantel tests confirmed that, while the microbial composition is highly correlated (almost 40%) to the genetic distance between insects after accounting for the host plant variation, inverting the variables does not support plants as a driver; the host plant associations are responsible for just 15% of the microbial composition after accounting for the psyllid genetic distance. Similarly, the different psyllid species collected from the same individual plants, but which gave different Enterobacteriaceae OTUs, also showed different levels of *Wolbachia.* These observations are consistent with the idea that the psyllid microbial composition is influenced by the psyllid species and not the plant.

Together, the analyses here support the strong correlation between the insects’ phylogenetic relationships and the microbiome, with evolutionary changes in the insect -such as speciation and radiation- resulting in changes in the microbiome. Therefore, these results suggest that the insect-bacteria associations can be understood in light of the “phylosymbiosis” theory shown by congruence between the psyllids evolutionary history and the associated microbial communities. Accordingly, as phylosymbiosis is inferred as the major driver of bacterial composition, then host plant specificity of the psyllids does not appear to be driven by symbionts as was proposed by Hansen and Moran (2014). In fact, the current study showed at least two instances where closely related species feeding on the same host plant show different symbiotic bacteria.

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**Figure SM1: Maximum Likelihood COI gene tree (10000 replicates, K2P).**

**Figure SM2: Maximum Likelihood 18S gene tree (10000 replicates, K2P).**

**Figure SM3: Psyllid species host plants associations** for the New Zealand native *Trioza* species. The psyllid phylogeny (left), from Figure 2, is associated with the host plant phylogeny (right), represented at the higher taxonomic level only, to order (green) and family (various colours).

**Table SM1: Psyllid samples used in this study.** The table lists the species analysed in this study and the family they belong to. Information on the country of origin of the samples are provided together with the number of samples and populations. Number of DNA sequences used is reported together with accession numbers for the COI, EF-1α and 18S genes. Accession numbers in bold are for the sequences generated in this study.