Received: 22 August 2023 | Revised: 12 November 2023 | Accepted: 13 December 2023

DOI: 10.1111/1755-0998.13919

MOLECULAR ECOLOGY RESOURCES WILEY

RESOURCE ARTICLE

Chromosome-level genome assembly of the aster leafhopper (*Macrosteles quadrilineatus*) reveals the role of environment and microbial symbiosis in shaping pest insect genome evolution

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Funding information
National Science Foundation Biological Integration Institute, Grant/Award Number: NSF-2214038

Handling Editor: Kin-Ming (Clement) Tsui

Abstract

Leafhoppers comprise over 20,000 plant-sap feeding species, many of which are important agricultural pests. Most species rely on two ancestral bacterial symbionts, *Sulcia* and *Nasuia*, for essential nutrition lacking in their phloem and xylem plant sap diets. To understand how pest leafhopper genomes evolve and are shaped by microbial symbioses, we completed a chromosomal-level assembly of the aster leafhopper's genome (ALF; *Macrosteles quadrilineatus*). We compared ALF's genome to three other pest leafhoppers, *Nephotettix cincticeps*, *Homalodisca vitripennis*, and *Empoasca onukii*, which have distinct ecologies and symbiotic relationships. Despite diverging ~155 million years ago, leafhoppers have high levels of chromosomal synteny and gene family conservation. Conserved genes include those involved in plant chemical detoxification, resistance to various insecticides, and defence against environmental stress. Positive selection acting upon these genes further points to ongoing adaptive evolution in response to agricultural environments. In relation to leafhoppers' general dependence on symbionts, species that retain the ancestral symbiont, *Sulcia*, displayed gene enrichment of metabolic processes in their genomes. Leafhoppers with both *Sulcia* and its ancient partner, *Nasuia*, showed genomic enrichment in genes related to microbial population regulation and immune responses. Finally, horizontally transferred genes (HTGs) associated with symbiont support of *Sulcia* and *Nasuia* are only observed in leafhoppers that maintain symbionts. In contrast, HTGs involved in non-symbiotic functions are conserved across all species. The high-quality ALF genome provides deep insights into how host ecology and symbioses shape genome evolution and a wealth of genetic resources for pest control targets.

KEYWORDS
Auchenorrhyncha, genome, leafhopper, pest insect, symbiosis

1 | INTRODUCTION

Leafhoppers (Hemiptera: Auchennorrhyncha: Cicadellidae) are one of the largest Hemipteran families, encompassing >20,000 described species (Brambila & Hodges, 2008; Dietrich, 2005; Grimaldi et al., 2005). They rely on plants for food and reproduction, with most species exclusively feeding on phloem or xylem plant sap. The feeding range of leafhoppers can vary considerably, with some species exhibiting a high degree of polyphagy, while others specialize exclusively on only one or a few plant species (Weintraub & Beanland, 2006; Wilson & Weintraub, 2007). Leafhoppers are also primary vectors for many viral (e.g., plant viruses) and bacterial (e.g., phytoplasmas) plant pathogens, causing enormous economic losses in agricultural and horticultural industries (Banttari & Zeyen, 1979; Chasen et al., 2015; Greenway, 2022; Hogenhout, Ammar, et al., 2008; Nielson, 1979; Tsai, 1979; Weintraub & Beanland, 2006). Remarkably, however, leafhoppers depend on non-pathogenic, beneficial bacteria to feed on plants in the first place (Buchner, 1965). Due to the essential nutritional deficiencies in their primary diet (xylem and phloem), most leafhopper species have evolved ancient and complex nutritional relationships with bacteria. The abilities of leafhoppers to feed on a wide range of plants in both agricultural and natural ecosystems and their dependence on beneficial bacteria likely exert significant evolutionary pressures across their genomes (Després et al., 2007; Francis et al., 2005; Hogenhout et al., 2009; Wang et al., 2018; Zhang et al., 2022). Yet, we have a limited understanding of how leafhopper genomes evolve and how these specific evolutionary pressures influence that process. We further lack effective comparative tools to investigate such questions fully.

Here, we present the complete genome of the aster leafhopper (hereafter known as ALF), *Macrosteles quadrilineatus* (Hemiptera: Cicadellidae: Deltocephalinae). ALF is a widespread pest that feeds on over 300 agriculturally important plants, including carrots, celery, wheat, barley, flax, and lettuce (Wallis, 1962). It is the primary vector of the Aster Yellows phytoplasma, a bacterium that causes crop stunting, deformation, and ultimately loss (Kunkel, 1926). Given ALF's ability to feed and reproduce on multiple plant species and its dependence on beneficial microbial symbionts, ALF is an emerging model system for understanding vector biology and beneficial symbioses (Bennett & Moran, 2013; Hogenhout, Ammar, et al., 2008; Hogenhout, Oshima, et al., 2008; Mao et al., 2018).

ALF's ability to feed on such a diverse array of plants is owed in part to its dependency on two intracellular bacteria, "*Candidatus* Sulcia muelleri" (*Bacteroidetes*; hereafter *Sulcia*) and "*Candidatus* Nasuia deltocephalinicola" (*Betaproteobacteria*; hereafter *Nasuia*). Both bacteria complement each other to provide ALF with the 10 essential amino acids (EAAs) that are depauperate in its phloem diet and that no animal can make (Bennett & Moran, 2013; Douglas, 2017; McCutcheon & Moran, 2012; Moran et al., 2008). ALF, and many other related auchenorrhynchan insects, house symbionts within specialized cells (bacteriocytes) and organs (bacteriomes) and exclusively transmit bacteria transovarially (Baumann, 2005; Buchner, 1965; Fronk & Sachs, 2022). As a result, *Sulcia* and *Nasuia* are ancient, having been vertically transmitted within lineages and across generations for >300 million years (Bell-Roberts et al., 2019; Moran et al., 2005). These conditions have led to the streamlining and severe reduction of their genomes to <10% of those of their free-living ancestors (190 kilo base-pairs [Kbp] and 112 Kbp in *Sulcia* and *Nasuia*, respectively; McCutcheon & Moran, 2012; McCutcheon et al., 2019). To maintain these highly degraded symbionts, the host or partner symbionts must compensate for incomplete genomic functions (Ankrah et al., 2020; Douglas, 2016; Hansen & Moran, 2011; Mao et al., 2018). To accomplish this, ALF has acquired 100s to 1000s of support genes that differentially support *Sulcia* or *Nasuia*. These support mechanisms evolved from the reassignment of mitochondrial support genes, ancient horizontal gene acquisitions from other infecting bacteria, and widespread gene duplications (Mao et al., 2018). However, how these evolutionary processes have structured the ALF genome, as well as genomes of other leafhoppers with different symbiotic relationships (i.e., losses and replacements), remains unclear.

While ALF and related species, such as the treehoppers from the family Membracidae and leafhoppers from the subfamily Deltocephalinae, generally retain both ancient symbionts, other leafhopper groups have replaced or lost *Sulcia*, *Nasuia*, or both (Bell-Roberts et al., 2019; Bennett & Mao, 2018; Mao, Yang, Poff, et al., 2017; Michalik et al., 2021; Sudakaran et al., 2017). For example, the sharpshooter leafhopper family appears to have replaced the more ancient symbiont, *Nasuia*, with "*Candidatus* Baumannia cicadellinicola" (hereafter *Baumannia*) >60 million years ago (MYA) in a transition between phloem and xylem feeding (Moran et al., 2003). Similar to *Nasuia*, *Baumannia* convergently evolved to provide the same two EAAs as *Nasuia* and a few other nutritional resources (Wu et al., 2006). However, because *Baumannia* is relatively young, its genome encodes more functional capabilities requiring much less support from its partner symbionts (Bennett et al., 2014; Mao & Bennett, 2020; Wu et al., 2006). In other early evolutionary events, some leafhopper lineages, such as the subfamily Typhlocybinae, have lost all obligate symbionts as a consequence of shifting to a more nutrient-rich plant parenchyma diet (Günthardt & Wanner, 1981). Species in this group no longer retain *Sulcia* and *Nasuia*, nor the organs that house and support them (Buchner, 1965; Cao & Dietrich, 2022). In contrast to symbiont replacement in sharpshooter leafhoppers, we have a more limited understanding of how the dramatic symbiotic transitions of symbiont loss influence the evolution of host genomes.

To better understand how pest ecology and obligate symbioses shape ALF and other leafhopper genomes, we used PacBio HiFi long-read sequencing and Omni-C long-range proximity ligation to generate a chromosome-level genome assembly of ALF. In an evolutionary framework, we compared ALF's genome to those of all other existing chromosome-level pest leafhopper genomes. These species come from different leafhopper subfamilies that

have overlapping and distinct symbiotic relationships. Our questions focus on understanding: (i) how leafhopper genomes evolve in a global sense, (ii) what evolutionary pressures pest species biology and agricultural ecology place on leafhopper genomes, and (iii) how major transitions in symbioses shape leafhopper genome evolution. Our results show remarkable conservation of leafhopper genomes but distinct signatures of pest ecology and symbioses in the expansion and retention of genes and molecular evolution of specific genes.

2 | MATERIALS AND METHODS

2.1 | Insect rearing and material preparation

Lab-reared *Macrosteles quadrilineatus* (ALF) insect lines for this analysis were originally field-collected from Yale West Campus, West Haven, Connecticut (USA) in 2013. Specimens were identified according to Kwon (1988) and confirmed with mitochondrial locus Cytochrome Oxidase 1 barcoding (following Bennett & Moran, 2013; Le Roux & Rubinoff, 2009) and whole mitochondria genome sequencing (Mao, Yang, & Bennett, 2017). A single mated female and male of ALF was used to establish an inbred line to reduce genetic heterozygosity. This line was maintained on barley for four generations at 25°C with 12L:12D light/dark. Inbred female and male adults were starved for 6 h, immediately flash-frozen in liquid nitrogen, and stored at −80°C. Pinned representatives of ALF have been deposited in the University of Hawaii Mānoa Insect Museum (UHIM2017.00001 - .00003) and at the UC Berkeley Essig Museum of Entomology (EMEC1749040 - EMEC1749049).

2.2 | Genome sequencing and assembly

A chromosome-level genome assembly was generated with the Omni-C proximity ligation technique developed by Dovetail Genomics (Santa Cruz, CA, USA). The assembly process involved scaffolding assembled genome contigs into chromosomes using a combination of long-read (Pacific Biosciences: PacBio) and short-read (Illumina HiSeqX) sequences. Briefly, a draft genome was assembled using 58.2 giga base-pairs (Gbp) of PacBio HiFi circular consensus sequencing reads and the de novo assembler Hifiasm v0.15.4-r347 with default parameters. Scaffolds identified as possible contamination by blobtools v1.1.1 were removed (Laetsch & Blaxter, 2017). Haplotigs and contig overlaps were removed using purge_dups v1.2.5 (Guan et al., 2020). The dovetail Omni-C library was sequenced on an Illumina HiSeqX platform for ~30× coverage. The de novo PacBio assembly and Dovetail OmniC library reads were used as the inputs for HiRise proximity ligation assembly (Putnam et al., 2016). To evaluate genome completeness, BUSCO v4.0.5 was used on the chromosome-level assembly using the eukaryote_odb10 lineage dataset (Simão et al., 2015; Zdobnov et al., 2017).

2.3 | Genome annotation

The NCBI Eukaryotic Genome Annotation Pipeline was used for genome annotation (NCBI, 2017). Repeat families were identified and masked using WindowMasker (Morgulis et al., 2006). For gene predictions using Gnomon, RNA-seq data from five previously sequenced ALF samples and high-quality protein coding sequence alignments from six closely related insects were aligned to the genome with STAR v2.7.10b and ProSplign v3.8.2 (Dobin et al., 2013; Kiryutin et al., 2007; Mao et al., 2018; Porter et al., 2019). The final annotation quality of ALF's genome was assessed with BUSCO v4.1.4 (Manni et al., 2021; Simão et al., 2015; Zdobnov et al., 2017).

2.4 | Chromosome number confirmation by karyotyping

Three ALF adult male individuals from the same lab culture were dissected in 1× PBS solution. The testicular follicles were separated from the rest of the abdominal contents and transferred to 1.5 mL tubes. The testicular follicles were immersed in 100 μL of 0.075 M sodium citrate solution for 10 min, fixed in 100 μL of modified Carnoy's solution (3:1 absolute ethanol: glacial acetic acid) for 1 h, and treated with 100 μL of 50% acetic acid. The acetic acid solution containing testicular follicle tissue was gently mixed, spotted onto slides preheated to 60°C, and allowed to air-dry at room temperature. Following complete drying of the solution, the spot was stained with 15 μL of 5% Giemsa stain for 30 min. Finally, the slides were thoroughly rinsed and mounted in deionized water. The slides were viewed under a Nikon Eclipse te2000-u inverted fluorescence microscope. Cells with clear chromosome segregation were recorded and photographed with a Nikon DS-Ri2 Microscope Camera to determine chromosome number.

2.5 | Comparative genomics and orthologue analysis

Chromosomal conservation and shared gene families across leafhoppers were inferred using comparative genomic analysis with the green rice leafhopper (Deltocephalinae: *Nephotettix cincticeps*; hereafter known as GRLH; Yan et al., 2021), glassy-winged sharpshooter (Cicadellinae: *Homalodisca vitripennis*; hereafter known as GWSS; Li et al., 2022), and the tea green leafhopper (Typhlocybinae: *Empoasca onukii*; hereafter known as TGLH; Zhao et al., 2022) (Table 1). Prior to all comparative analyses, annotated gene isoforms and transcript duplicates were consolidated into the single longest gene using a custom script. Total orthogroups and single-copy orthologues between all species were identified using OrthoFinder v2.5.4 (Emms & Kelly, 2019). For further clustering of orthologous groups and gene enrichment analyses across the four leafhopper species, we used OrthoVenn2 (settings: *e*-value = 1e-5, inflation value = 1.5) (Xu et al., 2019). Within OrthonVenn2, we identified Gene Ontology

TABLE 1 Four publicly available leafhopper chromosomal-level genomes used for comparative genomics analysis.

Species name	Shorthand naming	Genome size	Subfamily	Feeding	Symbionts
Macrosteles quadrilineatus	ALF	1.3 Gbp	Deltocephalinae	Polyphagous	*Sulcia*, *Nasuia*
Nephotettix cincticeps	GRLH	746 Mbp	Deltocephalinae	Polyphagous	*Sulcia*, *Nasuia*
Homalodisca vitripennis	GWSS	2.3 Gbp	Cicadellinae	Polyphagous	*Sulcia*, *Baumannia*
Empoasca onukii	TGLH	599.3 Mbp	Typhlocybinae	Monophagous	None

Abbreviations: Gbp, giga base-pairs; Mbp, mega base-pairs.

(GO) categories enriched in conserved gene clusters across leafhopper lineages and those exclusive to ALF. Functional gene enrichment analyses were conducted across three levels: (i) shared core genes across all species, (ii) between different symbiotic modalities or relationships (leafhoppers with *Sulcia*, and leafhoppers with both *Sulcia* and *Nasuia*), and (iii) unique gene clusters to ALF. Among all levels of functional enrichment analyses, we identified gene clusters that contribute to pest ecology, as well as symbiosis, in order to understand how these pressures may be shaping leafhopper genomes. We assessed chromosomal synteny among the leafhopper species using BLASTP (settings: *e*-value = 1e-5) and MCScanX with default parameters (Altschul et al., 1990; Wang et al., 2012). Finally, SynVisio was used for the visualization of synteny across the genomes (Bandi & Gutwin, 2020).

2.6 | Gene family expansion and contraction analysis in ALF

To investigate genome-wide family expansions and contractions within the ALF genome, we analysed protein-coding sequences from 20 publicly available insect genomes (Table S1). This dataset comprised 18 species from the Hemipteran order, as well as two from the Hemipteran sister order, Thysanoptera (Johnson et al., 2018). Annotated gene isoforms and transcript duplicates were consolidated into the single longest gene in all 20 species. OrthoFinder v2.5.4 was used to infer gene orthology between species. The resulting single-copy orthologues were aligned with MAFFT v7.52 (settings: -m L-INS-I model) (Emms & Kelly, 2019; Katoh & Standley, 2013). Ambiguously aligned regions were trimmed using BMGE v1.12 (settings: -m BLOSUM90, -h 0.4) (Criscuolo & Gribaldo, 2010). The concatenated alignments were then used to construct a maximum likelihood (ML) phylogenetic tree with IQ-TREE v2.2.03 using the best-fit partition model for each gene set (settings: -m MFP+MERGE, -B 1000) (Minh et al., 2020). The resulting ML tree was used to estimate divergence times between insect species. MCMCTree (PAML v4.10.6) was used to place 95% confidence intervals for the six node calibrations as soft bounds between Thysanoptera and Hemiptera (373.3, 451.2 million years ago [MYA]), Sternorrhyncha and Auchennorrhyncha/Heteroptera (353.8, 427.3 MYA), Psylloidea and Aleyrodidae (322.4, 396.6 MYA), Aphidomorpha and Psylloidea (303.9, 377.2 MYA), Fulgoromorpha and Cicadomorpha (275.3, 348.6 MYA), and finally between the Deltocephalinae tribes Macrostelini and Chiasmini (45.0, 95.0 MYA) (Cao et al., 2022; Johnson et al., 2018; Yang, 2007).

Gene family evolution (e.g., expansions and contractions) was inferred using CAFÉ v5 (setting: *p*-value .01) (Han et al., 2013). Gene families that were identified as significant expansion events in ALF were functionally annotated using eggNOG-mapper (Cantalapiedra et al., 2021). To perform enrichment at the KEGG functional level for significantly expanded families, we used the R package "clusterProfiler" v4.6.2 (settings: pvalueCutoff = 0.01, pAdjustMethod = "BH", qvalueCutoff = 0.01, minGSSize = 10) (Yu et al., 2012).

2.7 | Selection analysis of leafhopper genes

Evolutionary selection operating on leafhopper genes was estimated using the ratio of nonsynonymous to synonymous substitutions ($\omega = dN/dS$) across sites in genes. This analysis was first conducted on genes from ALF, GWSS, TGLH, and GRLH to provide a broader perspective on evolutionary patterns in leafhoppers. A narrower analysis was then performed between only the Deltocephalinae leafhoppers (ALF and GRLH) to reduce the evolutionary divergence and potential for substitution saturation, and to determine the influence of both *Sulcia* and *Nasuia* that are retained in ALF and GLRH.

For estimated rates of evolution, we first identified single-copy orthologues between species using OrthoFinder v2.5.4 (Emms & Kelly, 2019). To ensure accurate alignment and avoid out-of-frame indels, we used PAL2NAL v14 and TranslatorX v1.1 (Abascal et al., 2010; Suyama et al., 2006). Estimated rates of ω among gene sites were conducted using two nested site-specific models (M1a-M2a and M7-M8) from CODEML in PAML v4.10.6 (Nielsen & Yang, 1998; Wong et al., 2004; Yang et al., 2000, 2005). These nested models test for selection at any site in the gene with differences in the number of site classes (M1a: 2; M2a: 3; M7: 10; M8: 11). The M2a and M8 models include an additional class of sites under positive selection. We performed nested likelihood ratio and chi-squared tests ($p < .05$) based on likelihood scores from each model. Specifically, we compared null models (M1a and M7), which do not allow for any sites with $\omega > 1$, against the alternative models (M2a and M8), which permit positive selection. Genes identified as undergoing positive selection were clustered into euKaryotic Orthologous Groups (KOG) and used to perform enrichment of positive selection at the GO functional level using the R package "clusterProfiler" v4.6.2 (settings: pvalueCutoff = 0.01, pAdjustMethod = "BH",

qvalueCutoff = 0.01, minGSSize = 10) (Huerta-Cepas et al., 2019; Tatusov et al., 2003; Yu et al., 2012).

2.8 | Localization of symbiosis support genes

To identify the prevalence of putative symbiosis-related genes (Table S2) in our leafhopper genome evolution analyses, we used genes identified in Mao et al. (2018). Mao et al. (2018) identified 118 genes that are involved in nutrition synthesis, information processing, population regulation, and metabolite transport in *Nasuia* and *Sulcia* bacteriocytes. We mapped these genes to the shared gene clusters identified between leafhoppers (see Sections 2.5 and 3.2) and to genes under positive selection (see Section 2.7) using NCBI-tBLASTX (setting: *e*-value = 0.001).

Finally, one major evolutionary modality to support ancient obligate symbionts is the acquisition of horizontally transferred genes (HTGs) from various bacterial families. To investigate evolutionary conservation of HTGs across all four leafhopper species, we used an NCBI-tBLASTX search (setting: *e*-value = 0.001) of ALF HTG transcripts to each of the leafhopper chromosomal-level sequences to find homologous genes (Table S3). We identified conserved genes across the four species as matches with >40% identity and >100-bit score. We also identified possible HTG remnants as matches with >40% identity and a bit score between 90 and 100.

3 | RESULTS

3.1 | Assembly and annotation of ALF's genome

PacBio sequencing of ALF resulted in 6,333,492 reads and 58.2 Gbp of PacBio HiFi circular consensus sequencing reads at 45× coverage. After scaffolding with the PacBio assembly and OmniC reads, the final assembly included 1164 scaffolds (total size = 1.3 Gbp) with an N50 score of 116.5 mega base-pairs (Mbp) (Table 2). All 1164 scaffolds were larger than 1 KB and consisted of nine scaffolds larger than 78.3 MB (total = 1.12 Gbp). These nine scaffolds in ALF suggested a haploid chromosome count of $n = 9$ (Figure S1).

We performed karyotyping on male ALFs to determine chromosome number. We found that ALF has a haploid chromosome number of $n = 9$ (Figures S1 and S2), which is consistent with the nine large scaffolds from our genome assembly. We observed that two chromosomes were physically longer than the rest, which is consistent with the two larger scaffolds in the genome assembly (Figures S1 and S2).

The final BUSCO score for the ALF assembly was 96.08% (83.92% single, 12.16% duplicated, 1.57% fragmented, and 2.35% missing). Using the NCBI RefSeq annotation pipeline, 43.54% of the genome was masked due to repetitive elements by WindowMasker. A total of 24,178 genes (21,979 protein-coding genes and 103 pseudogenes) were annotated across all 1164 scaffolds. Among the nine chromosome-level scaffolds, 19,395 protein-coding genes were identified, 88.24% of all annotated protein-coding genes.

TABLE 2 Genome statistics of the *Macrosteles quadrilineatus* genome.

Feature	Value
Assembly size	1,317,891,973 bp
Assembly size of chromosomes	1,127,407,133 bp
Scaffold N50	116,548,684 bp
Scaffold L50	5
Number of scaffolds	1164
Number of total genes	24,178
Number of protein-coding genes	21,979
Number of pseudogenes	103

Abbreviation: bp, base-pairs.

3.2 | Comparative genomics and orthologue analysis

To understand general patterns of leafhopper genome evolution, we investigated genomic synteny and gene content. Synteny of leafhoppers is largely conserved, with some large-scale chromosomal rearrangements (Figure 1). For example, there are two notable chromosomal rearrangements between the Deltocephalinae leafhoppers (ALF and GRLH). First, there is the large-scale chromosomal rearrangement between two ALF chromosomes (ALF chr1 and ALF chr2) and two GRLH chromosomes (GRLH chr6 and GRLH chr8). Second, ALF chr6, ALF chr8, and GRLH chr3 have had a chromosomal fusion/fission event. There is also evidence of a chromosomal fusion/fission event between TGLH and GWSS, as well as chromosomal rearrangements between the two species, among other shuffling of smaller chromosomal segments.

At a gene level, ALF, GRLH, TGLH, and GWSS have 14,567 orthogroups comprising 68,553 genes (89.1%). All four leafhoppers share 3450 single-copy gene (1:1:1) orthologues. ALF exhibited the highest number of species-specific genes (2675) and the lowest number of unassigned genes (1129) compared to TGLH (1848 species-specific genes and 4024 unassigned genes), GWSS (2300 species-specific genes and 1895 unassigned genes), and GRLH (812 species-specific genes and 1301 unassigned genes).

Shared gene analysis across the four leafhoppers species was extended to consider gene clusters and includes gene duplications. Using OrthoVenn, we found 7723 gene clusters shared between all four leafhoppers (Figure 2). These shared gene clusters showed enrichment for 10 Gene Ontology (GO) processes (peptidoglycan catabolic process, GO:0009253; DNA integration, GO:0015074; transposition DNA-mediated, GO:0006313; oxidoreductase activity, GO:0016705; trehalose transport, GO:0015771; RNA-directed DNA polymerase activity, GO:0003964; translation, GO:0006412; response to bacterium, GO:0009617; proteolysis,

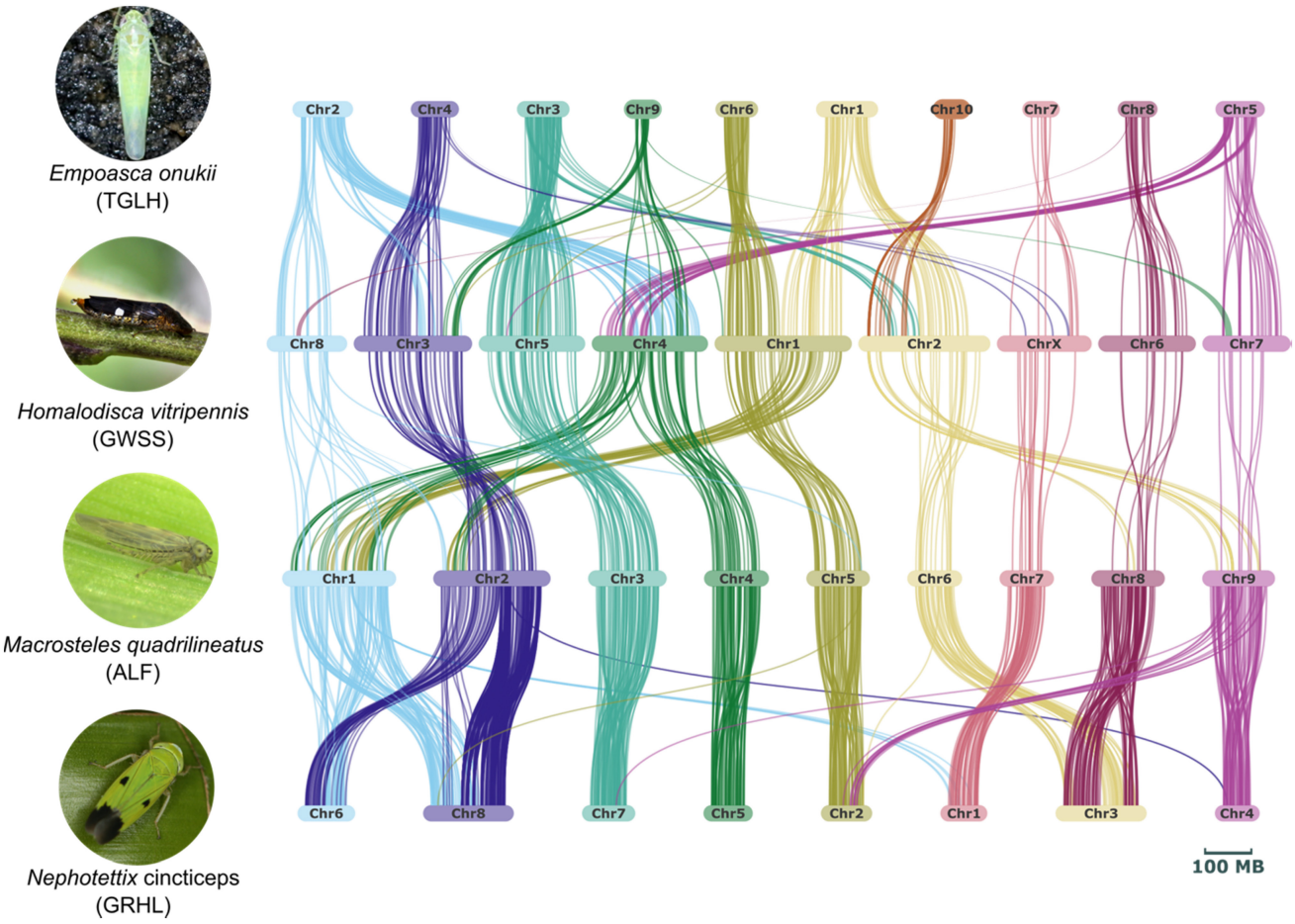

FIGURE 1 Chromosomal synteny between the four leafhopper species. Chromosome numbering in ALF is in scaffold order. Other leafhopper genomes are ordered in relation to ALF chromosomes. Images of *H. vitripennis* and *M. quadrilineatus* are from Dr. Zheng Li and Dr. Xiushuai Yang, respectively. Images of *E. onukii* and *N. cincticeps* are from www.inaturalist.org (from the following users: straybird726 and bob15noble, respectively. All photos have CCBY-NC licence). MB, mega base-pairs.

GO:0006508; and, telomere maintenance, GO:0000723) (Figure 3a; Table S4). Interestingly, the oxidoreductase activity category consisted of cytochrome P450 enzyme duplications (103 genes). Similarly, the trehalose transport category exhibited multiple duplications across species of the facilitated trehalose transporter Tret-1.

TGLH (Subfamily: Typhlocybinae) is the only sequenced leafhopper species in this analysis that does not have known obligate symbiotic associations. Therefore, we identified the shared gene clusters between the three leafhopper species with *Sulcia*: ALF, GWSS, and GRLH. In the three *Sulcia*-associated leafhopper species, we found 986 unique gene clusters enriched for four GO categories (nucleoside triphosphate biosynthetic process, GO:0009142; carboxylic ester hydrolase activity, GO:0052689; tRNA metabolic process, GO:0006399; and succinate metabolic process, GO:0006105) (Figure 3b; Table S4). We further identified shared gene clusters between the two Deltocephalinae leafhoppers, ALF and GRLH, that have a symbiotic association with *Sulcia*'s ancestral symbiont partner, *Nasuia*. In these two host species, we found 789 unique gene clusters enriched for nine GO categories (peptidoglycan catabolic process, GO:0009253; positive regulation of cytolysis in other organisms, GO:0051714; response to bacterium, GO:0009617; regulation of circadian sleep/wake cycle, sleep, GO:0045187; peptidoglycan metabolic process, GO:0000270; Toll signalling pathway, GO:0008063; regulation of inflammatory response, GO:0050727; microtubule-based process, GO:0007017; and apoptotic DNA fragmentation, GO:0006309) (Figure 3c; Table S4). Finally, we identified 598 unique gene clusters specific to ALF that are significantly enriched for four GO categories (telomere maintenance, GO:0000723; receptor-mediated endocytosis, GO:0006898; DNA integration, GO:0015074; response to peptide hormone, GO:0043434) (Figure 3d; Table S4).

3.3 | Gene family expansion and contraction in ALF's genome

We investigated gene family expansions and contractions across evolutionary time using a time-calibrated phylogenetic tree generated from 469 shared single-copy orthologues between 20 insect species. Briefly, the estimated divergence time for the common ancestor of all four leafhopper species is ~150 million years (MY)

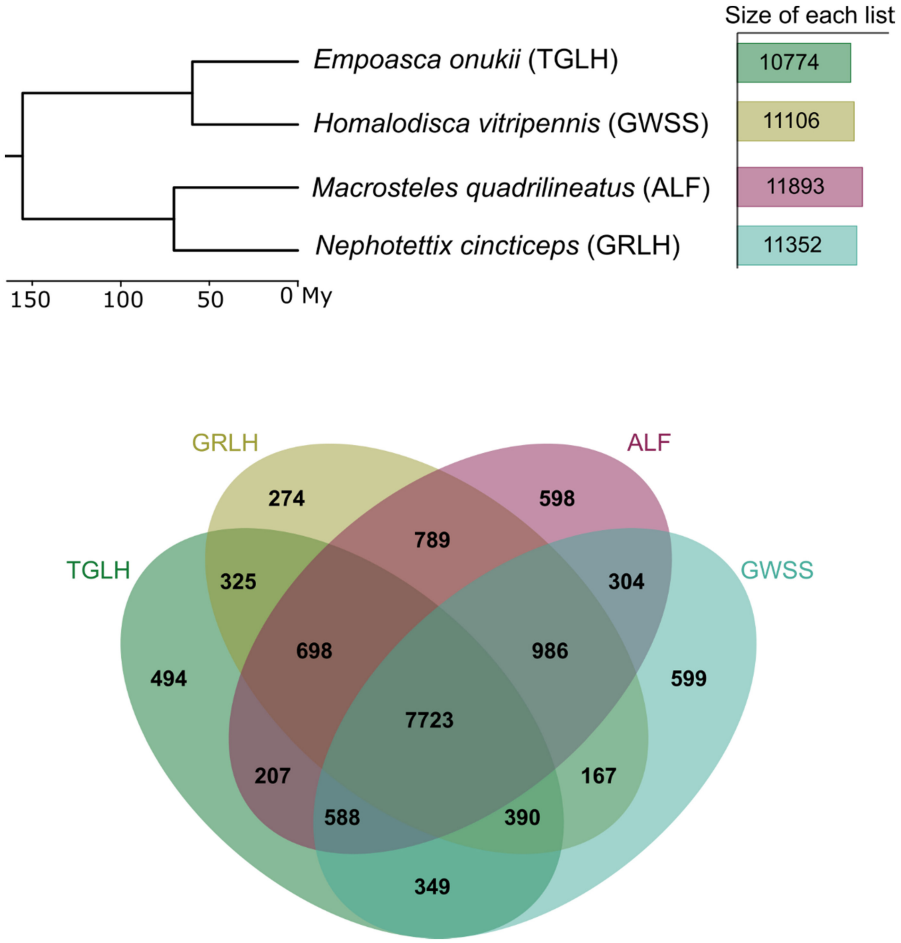

FIGURE 2 Shared gene clusters between leafhopper species. Venn diagram of shared gene clusters, that is clusters that include duplications of genes, among the four species. My, million years.

(Confidence Interval [CI]: 44–282 MY). Between species, the estimated divergence time for ALF and GRLH was ~67 MY (CI: 39–95 MY), and between GWSS and TGLH was ~57 MY (CI: 0–155 MY) (Figure S3). These divergence times are similar to estimated times in previous studies (Cao et al., 2022; Johnson et al., 2018; Moran et al., 2005).

Gene family evolution analyses identified a total of 1834 and 663 gene families that underwent expansions and contractions, respectively, in the ALF genome (Figure S3). When compared to the other 19 insect species, 67 gene families in ALF were significantly expanded (p-value < .05), and 32 gene families were significantly contracted (p-value < .05) (Figure S3). ALF exhibited the highest number of significant gene family expansions and the lowest number of contractions compared to GRLH (25 expansions, 82 contractions), GWSS (64 expansions, 53 contractions), and TGLH (52 expansions, 60 contractions) (Figure S4). Significantly expanded families in ALF were enriched for genes involved in the KEGG categories: G-quadruplex DNA unwinding, major facilitator superfamily sugar transporter family, DDE superfamily endonuclease, cathepsin propeptide inhibitor domain, E3 ubiquitin-protein ligase, K02A2.6-like, and baculoviral inhibition of apoptosis protein repeat (Figure S5; Table S5).

3.4 | Leafhopper genome-wide selection analysis

To understand how selection is broadly shaping leafhopper genome evolution, we investigated site-specific selection across shared single-copy orthologues. Estimated rates of ω among gene sites were conducted using two neutral models (M1a and M7) and two models with an additional class of sites under positive selection (M2a and M8). The stringent M1a-M2a nested model approach identified seven orthologues under positive selection (chi p-value < .05; Table S6). These genes include two uncharacterized genes, an IDLSRF-like protein, inhibitory POU protein (POU4F2), synaptotagmin-7 (SYT7), voltage-dependent calcium channel type A subunit alpha-1 (cac), and homeobox protein SI-like (SIX6). In contrast, the M7-M8 analysis identified 1103 orthologues undergoing positive selection (Table S6). The M7-M8 approach identified all seven genes found in the M1a-M2a models.

Genes undergoing positive selection in the M7-M8 models were binned by euKaryotic Orthologous Groups (KOG) to infer function. Genes with "unknown function" (KOG Category S) showed the highest number of genes under positive selection (Figure S6). Of genes with known functions, signal transduction mechanisms (KOG Category T) and post-translational modification, protein turnover,

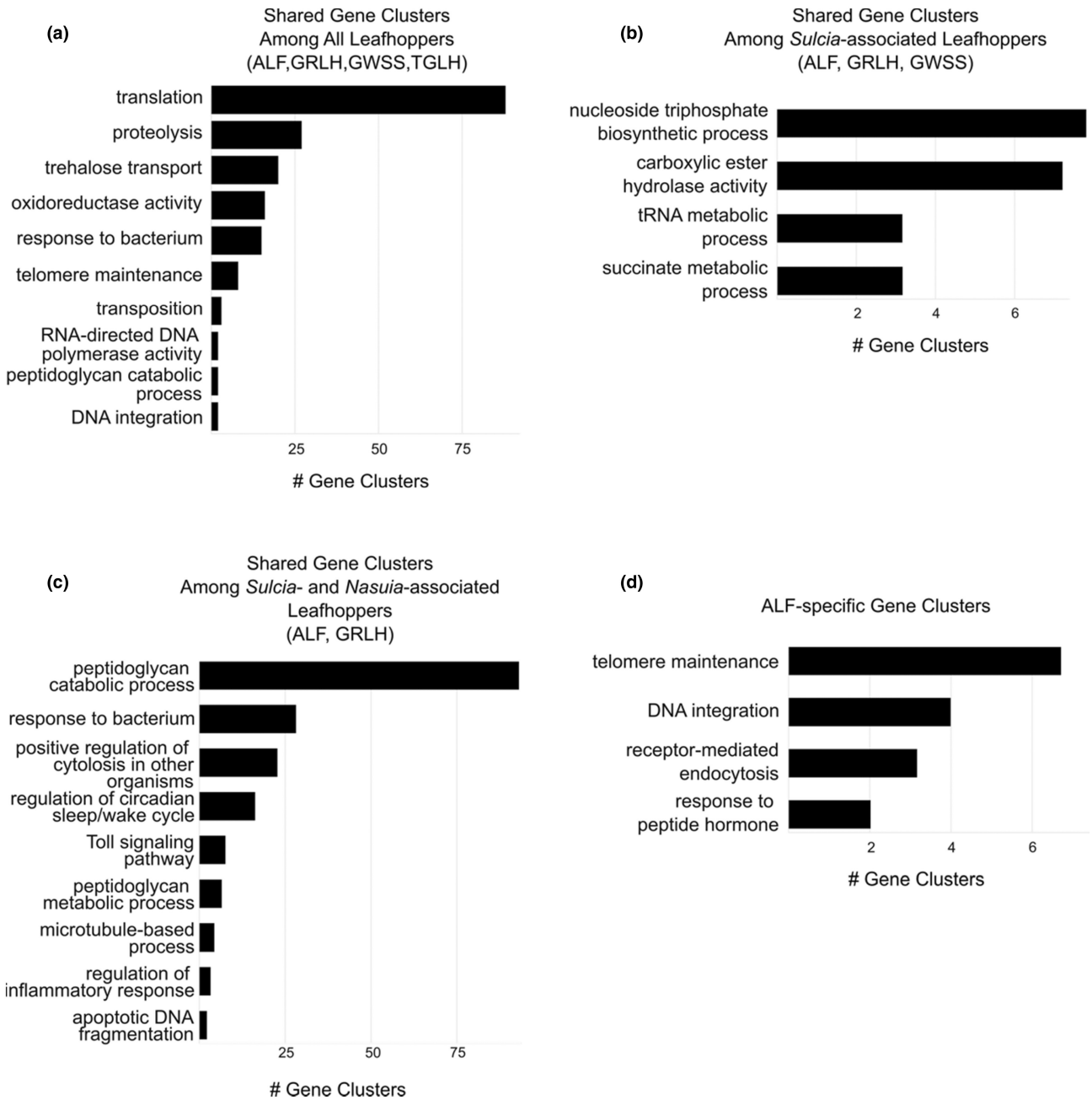

FIGURE 3 Functional enrichment among shared gene clusters. (a) Enrichment among gene clusters shared between all leafhopper species in this study. (b) Enrichment among gene clusters shared between leafhoppers that have an association with the symbiont, *Sulcia*. (c) Enrichment among gene clusters shared between Deltocephalinae leafhoppers, which have an association with both ancient symbionts *Sulcia* and *Nasuia*. (d) Enrichment in gene clusters unique to ALF. GO categories and number of unique gene clusters can be found in Table S4. ALF, *Macrosteles quadrilineatus*; GRLH, *Nephotettix cincticeps*; GWSS, *Homalodisca vitripennis*; TGLH, *Empoasca onukii*.

and chaperones (KOG Category O) had the highest number of genes under positive selection. In gene enrichment analysis of genes with sites under positive selection, generation of neurons (GO:0048699) exhibited the highest gene ratio (204/1103) of genes undergoing positive selection (Figure 4).

Finally, we investigated recent signatures of positive selection between the 6198 single-copy orthologues shared between ALF and GRLH, the two leafhopper species that retain *Sulcia* and *Nasuia*. A total of 131 and 2048 genes show signatures of positive selection with the M1a-M2a and M7-M8, respectively (Table S7). In both nested model analyses, 129 genes were jointly identified as exhibiting sites undergoing positive selection. Gene function binning identified unknown function (KOG Category S), signal transduction mechanisms (KOG Category T), and post-translational modification, protein turnover, and chaperones (KOG Category O) as the three most enriched categories (Figure S7). Gene enrichment analysis at the GO level identified cell morphogenesis (GO:0000902) as exhibiting the highest gene ratio (259/2048) of genes undergoing positive selection (Figure 4).

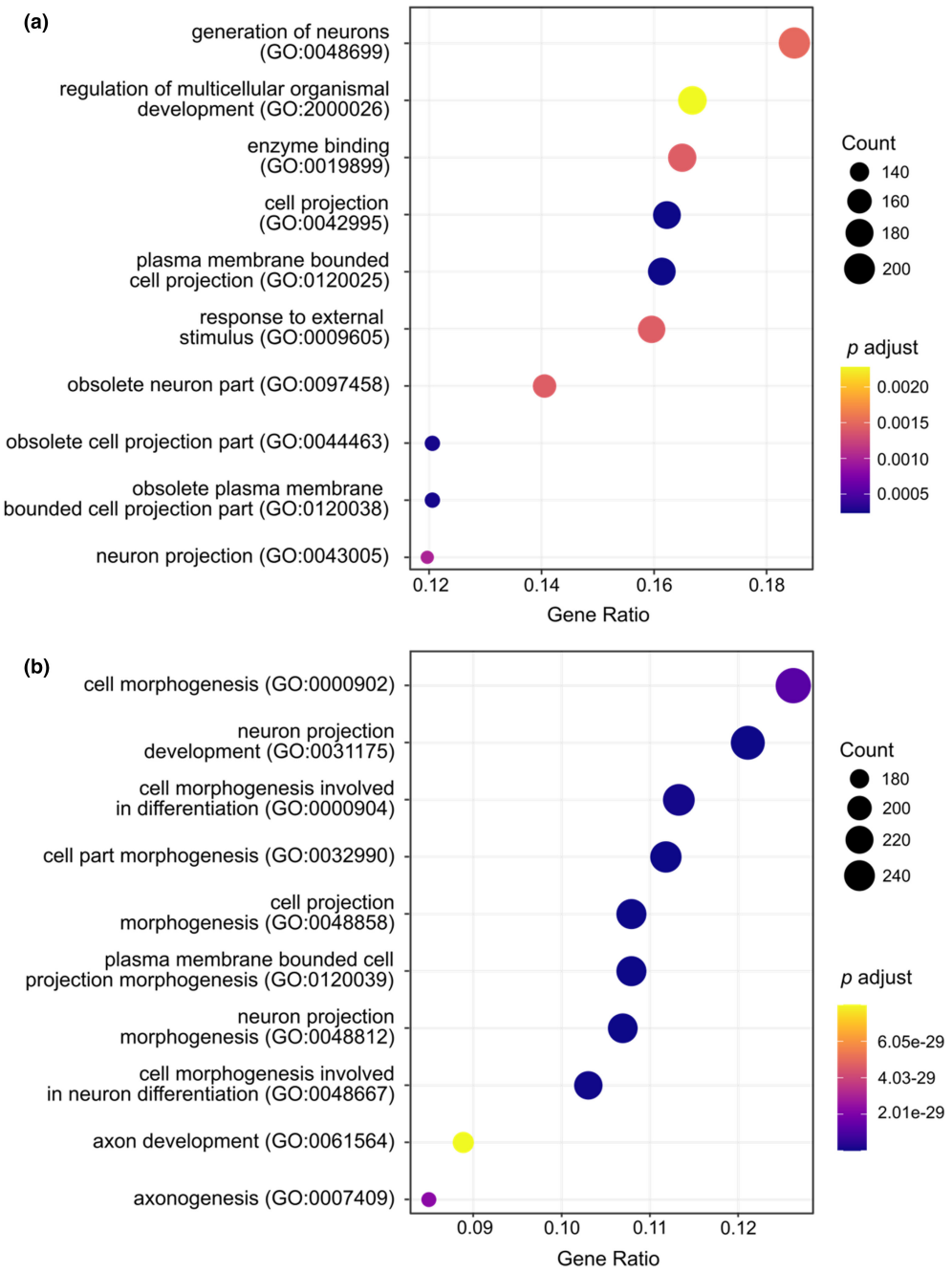

FIGURE 4 Top 10 GO categories enriched for positively selected genes. (a) Enrichment in genes undergoing positive selection among the four leafhopper species. (b) Enrichment in genes undergoing positive selection in the two Deltocephalinae leafhoppers (ALF and GRLH). ALF, *Macrosteles quadrilineatus*; GRLH, *Nephotettix cincticeps*.

3.5 | Genome-wide evolution of symbiosis support genes

A major question concerning symbiotic insects is how their genomes evolve to support obligate symbionts living in their bodies. We mapped the 118 putative symbiosis-related genes identified by Mao et al. (2018) (Table S2) to gene clusters shared between all leafhopper species and found 53 gene clusters containing symbiosis-related genes (Table S8). In ALF, these genes are upregulated in either *Sulcia* or *Nasuia* tissues, as well as in body tissues. Generally, their function falls within the categories of DNA replication & repair and aminoacyl-tRNA formation. Symbiosis-related genes involved in COA synthesis, NH3 recycling, and PEP synthesis are only found in shared gene clusters from all four species. Within shared gene clusters between *Sulcia*-associated leafhopper species (ALF, GRLH, and GWSS), we found 12 gene clusters containing symbiosis-related genes (Table S8). As expected, these genes are upregulated in *Sulcia* tissues in ALF. In the gene clusters shared by Deltocephalinae leafhoppers (ALF and GRLH), we found 18 gene clusters containing symbiosis-related genes (Table S8).

Most of these 18 genes are upregulated in *Nasuia* tissues and include population regulation genes. In the analysis of selection across the four leafhoppers, we found nine genes involved in symbiosis to have support for positive selection (Table S9). In the two Deltocephalinae leafhoppers, 20 symbiosis-related genes are undergoing positive selection (Table S10). These genes occur across multiple categories, with eight genes occurring in amino acid transport and aminoacyl-tRNA formation.

Finally, an important evolutionary mechanism in symbiosis support is the incorporation of horizontally transferred genes (HTGs), which is widely used in leafhopper systems. Here, we investigated where HTGs found in the ALF genome by Mao et al. (2018) occurred and whether they are conserved across all four leafhopper species (Table S3). All HTGs are exclusively located on autosomes, with none observed on the sex chromosome. Among duplicated HTGs, we found no discernable pattern of placement among chromosomes. For instance, the five duplications of peptide deformylase (*def*) occur on three chromosomes with some duplications occurring on the same chromosome. In our analysis of HTGs among our four leafhopper species, we found only three conserved HTGs (*cel-1*, *cel-2*, *pel*) (Figure 5). In ALF, *cel-1* and *cel-2* are upregulated in body tissues, and the *pel* gene is upregulated in both bacteriocytes (Mao et al., 2018). Between the Deltocephalinae species that retain *Sulcia* and *Nasuia*, GRLH retains a total of 27 of 30 HTGs found in ALF (Figure 5). GRLH is missing two *Nasuia* symbiont support genes and one non-symbiotic gene. The sharpshooter genome, GWSS that has replaced *Nasuia* with *Baumannia*, retains 11 *Sulcia* and non-symbiotic support HTGs, as was found previously (Li et al., 2022; Mao et al., 2018). Finally, in the TGLH genome, which has lost all ancestral symbionts, we identified remnants of three genes: *gh25-2* (40% identity, 85 amino acids), *ileS* (60% identity, 60 amino acids), and *yebC-1* (43% identity, 94 amino acids). We additionally found one gene remnant in GWSS: *tmk* (59% identity, 67 amino acids).

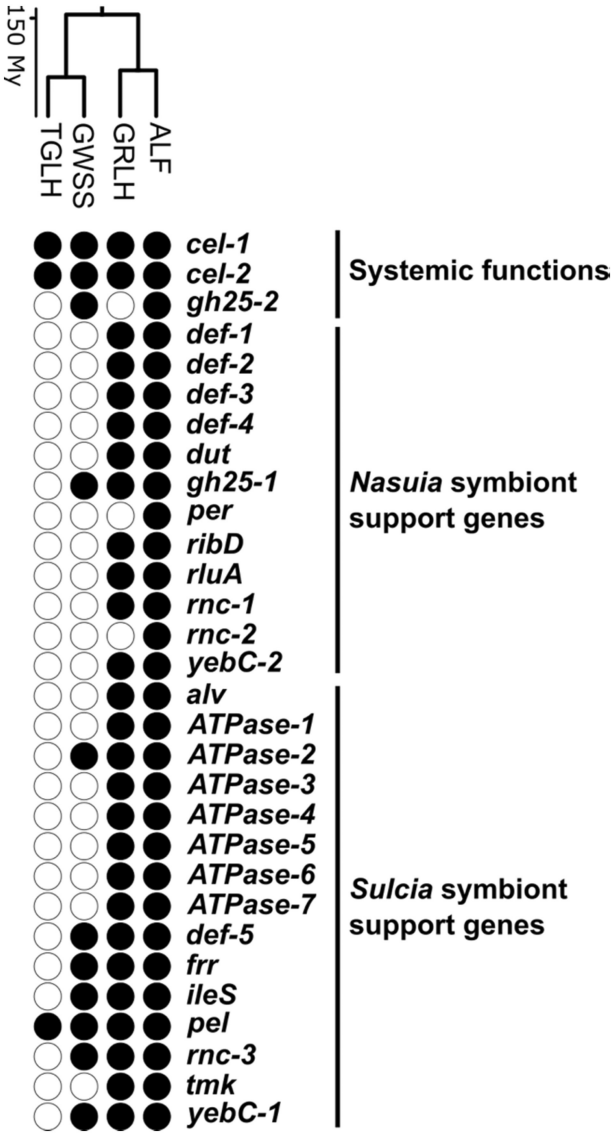

FIGURE 5 Conserved horizontally transferred genes (HTG) among leafhoppers. HTGs are grouped by their role in the symbiosis between ALF, *Sulcia*, and *Nasuia*. Empty circles indicate missing genes, while enclosed circles indicate presence of genes. Gene products can be found in Table S3. ALF, *Macrosteles quadrilineatus*; GRLH, *Nephotettix cincticeps*; GWSS, *Homalodisca vitripennis*; TGLH, *Empoasca onukii*.

4 | DISCUSSION

We present the first chromosome-level genome assembly of the aster leafhopper (ALF), *Macrosteles quadrilineatus*. To better understand how leafhopper genomes evolve, particularly in relation to evolutionary pressures from their pest ecology and symbiotic biology, we compared the genomes of all available leafhopper species in an evolutionary framework. To date, only three other leafhopper chromosomal-level genomes exist: *Nephotettix cincticeps* (GRLH), *Homalodisca vitripennis* (GWSS), and *Empoasca onukii* (TGLH) (Li et al., 2022; Yan et al., 2021; Zhao et al., 2022). These species are all plant pests, spanning over 150 million years of leafhopper evolution. They further have distinct symbiotic modalities, ranging from retention of leafhoppers' ancestral symbionts *Sulcia* and *Nasuia* (ALF and GRLH), symbiont replacement of *Nasuia* (GWSS), and complete loss of both obligate symbionts (TGLH) (Table 1) (Bennett & Moran, 2013; Cao & Dietrich, 2022; Moran et al., 2003; Noda et al., 2012). This taxon sampling uniquely places an understanding of ALF genome evolution in a framework spanning deep divergences among leafhoppers, symbiotic interactions, and parallel pest species biology and ecology.

4.1 | Genome evolution of leafhoppers

The inferred size of the ALF genome is 1.3 Gbp with a BUSCO score of 96.08%, suggesting a complete high-quality assembly (Table 2) (Feron & Waterhouse, 2022; Li et al., 2022; Yan et al., 2021). ALF's genome encodes 21,979 protein-coding genes, which is in alignment with previous leafhopper genomes (e.g., GWSS has 19,904 genes,

and TGLH has 19,642 genes; Li et al., 2022; Zhao et al., 2022). This genome further contains a relatively high number of repetitive elements (e.g., tandem repeats and interspersed repeats) in the genome (43.54%), aligning with the range observed in other hemipteran insects (leafhoppers: 39.4%–46.1%; psyllids: 43.3%) (Kwak et al., 2023; Li et al., 2022; Yan et al., 2021).

At the chromosomal level, ALF and the other sequenced leafhoppers exhibit a relatively high level of synteny conservation (Figure 1). This pattern differs substantially from observations in aphids, which exhibit extensive autosomal rearrangements despite shorter divergence times (<33 MY) (Li et al., 2020; Mathers et al., 2021). Nevertheless, leafhoppers, like sequenced planthoppers and aphids, demonstrate large-scale conservation of the X chromosome that has little to no rearrangements with autosomes (Li et al., 2020; Ma et al., 2021; Mathers et al., 2021). Strong selection pressures against sex chromosome rearrangements may arise from constraints to maintain dosage compensation mechanisms and avoid X chromosome elimination errors in XO male determination (Pal & Vicoso, 2015; Sharp et al., 2002). In the context of leafhoppers that display this XO sex determination, these selection pressures may lead to a lack of sex chromosome rearrangement (Halkka, 1960; Hu et al., 2022). Despite distinctive genomic conservation in leafhoppers, their chromosomes still exhibit some level of architectural plasticity. For example, the relatively closely related ALF and GRLH species exhibit large-chromosomal fusion or fission events. Comparisons in chromosome structure among the other species similarly show large chromosome organization changes.

At the gene level, leafhopper genomes exhibit widespread functional conservation, likely due to fundamentally shared biological and ecological leafhopper traits (Figure 2). For instance, among shared Gene Ontology (GO) categories, translation is most enriched in unique gene clusters, or gene orthologues that include duplications (88 gene clusters; 385 genes; Table S4), indicating a highly conserved biological need for these cellular functions. Leafhopper genomes also exhibit enrichment for functional categories involved in pest ecology, including the detoxification of insecticides (e.g., cytochrome P450 monooxygenases and carboxylesterases), as well as in categories that have a role in maintaining symbionts (Figure 3). The conserved enrichment of these categories points to the ways in which shared evolutionary pressures, the agricultural environment, and dependence on obligate symbioses have the potential to influence gene content in genomes.

Leafhopper genomes have also experienced extensive proliferation of selfish genetic elements. Several categories associated with the movement and integration of genetic elements within genomes, such as transposition (23 genes), DNA integration (191 genes), and RNA-directed DNA polymerase activity (56 genes), are enriched. Genetic elements can contribute to gene duplications and the general expansion of other gene families (Finnegan, 1989). Thus, they can enable duplication of advantageous genes, including those that respond to environmental changes such as host plant chemical defences and climate variation, particularly insecticide resistance (Aminetzach et al., 2005; González et al., 2008, 2010; Gupta & Nair, 2022; Rech et al., 2019; Rostant et al., 2012; Schrader et al., 2014; Stapley et al., 2015).

At the molecular level, positive selection is acting upon multiple single-copy orthologues across various functional categories (Table S6; Figure S6). One such category is signal transduction mechanisms (KOG Category T), with many genes undergoing positive selection (170 out of 401 analysed signal transduction genes). Signal transduction mechanisms include functions such as odorant receptors, gustatory receptors, and Toll-like receptors (Benton et al., 2007; Engsontia et al., 2014; Leulier & Lemaitre, 2008). These genes are likely evolving in response to environmental pressures, possibly in association with leafhopper food-plant finding and oviposition (Smadja et al., 2009).

4.2 | The potential influence of agricultural pest ecology on leafhopper genome evolution

The lifestyle of plant pests, such as leafhoppers, exposes them to strong selective pressures, including various insecticides, fluctuating environmental conditions, and changes in plant chemical defences (Després et al., 2007; Dumas et al., 2019; Khaliq et al., 2014; Nauen & Denholm, 2005; Spencer & Hughson, 2023). One evolutionary strategy to adapt to these pressures is the expansion of genes involved in stress responses (Rostant et al., 2012; Schrader et al., 2014; Stark & Wahl, 1984; Tabashnik, 1990). Genes involved in stress responses can often play dual roles as insect detoxification enzymes that break down plant toxins and insecticides (Després et al., 2007). Several specific genes and gene family expansions, discussed below, have clear roles in selective pressures placed on leafhoppers from their agricultural pest ecology.

Among the leafhopper species examined in this study, their shared genes showed enrichment for functions involved in oxidoreductase activity (103 genes) and trehalose transport (101 genes), both of which enable adaptation to environmental stressors (Heidel-Fischer & Vogel, 2015; Kikawada et al., 2007) (Figure 3a). For example, genes involved with oxidoreductase activity (e.g., cytochrome P450) contribute to the detoxification of xenobiotics, such as insecticides, environmental pollutants, and plant secondary compounds (Peng et al., 2017). Specifically, these detoxification genes play dual roles in aiding the insect's response to plant defence compounds and resistance to insecticides like pyrethroids and neonicotinoids (Heidel-Fischer & Vogel, 2015; Lu et al., 2021; Schuler, 1996). Leafhoppers further show enrichment for genes associated with trehalose transport (e.g., facilitated trehalose transporter *Tret1*) to different insect tissues (Kanamori et al., 2010). Trehalose is the primary sugar in insect hemolymph and is highly regulated in order to maintain sugar levels (Becker et al., 1996; Wyatt & Kalf, 1957). It is involved in energy production and insect growth and is an important bioprotectant under various stressors, including heat, oxidation, cold, dryness, and hypoxia (Elbein et al., 2003; Kanamori et al., 2010; Kikawada et al., 2007; Liu et al., 2013; Tellis et al., 2023).

At the molecular level, >1000 shared single-copy genes show evidence of positive selection (Figure 4a). For example, the enriched GO category undergoing positive selection, generation of neurons includes genes in various important insect functions that include insect chemoreception (e.g., *EonulR25a*), immunity (e.g., toll-like receptor Tollo and toll-like receptor 6), reproduction and development (e.g., homeobox proteins Six4 and Six6), and metabolism and energy regulation (e.g., valine—tRNA ligase and bifunctional coenzyme A synthase) (Mao et al., 2018; Zhang et al., 2016, 2023; Zhao et al., 2020). Some genes in these neural processes, such as those involved in metabolism and energy regulation, may be associated with neurological regulation and response of the bacteriome organ, while others are targets for various insecticidal chemicals. For example, the generation of neurons category includes two voltage-dependent sodium channel genes and four voltage-dependent calcium channel subunit genes. These ion channels are targeted by various insecticide classes like dichlorodiphenyltrichloroethane (DDT), diamides (e.g., chlorantraniliprole), and pyrethroids (e.g., permethrin) (Ffrench-Constant et al., 2016; Silver et al., 2014). While DDT has been banned in the United States since 1972, diamide insecticides (e.g., chlorantraniliprole and flubendiamide) and pyrethroid insecticides (e.g., permethrin, cypermethrin, deltamethrin) are still approved for use in the United States (US EPA, 2014). Moreover, detoxification gene families involved in insecticide resistance, such as cytochrome P450 enzymes, esterases, and carboxylesterases, are also under positive selection (Cui et al., 2015; Montella et al., 2012). Beyond agriculture-specific stressors, leafhopper genes show adaptation to broader environmental pressures. Protectants against thermal and oxidative extremes, such as heat shock proteins (i.e., *HSPA12A* and *HSPA5*) and a peroxiredoxin gene (i.e., *PRDX6*), are undergoing positive selection (Feder & Hofmann, 1999; Radyuk et al., 2001). Taken together, these results suggest that pest leafhopper genomes adapt to agricultural and environmental challenges across multiple scales.

4.3 | The potential influence of microbial symbiosis on leafhopper genome evolution

Symbioses with obligate, ancient symbionts has had significant impacts on leafhopper and related insect genomes (Couret et al., 2019; Hansen & Moran, 2011; Husnik et al., 2013; Kim et al., 2011; Mao et al., 2018; McCutcheon & von Dohlen, 2011; Nikoh et al., 2010; Nygaard et al., 2011; Price et al., 2011; Ratzka et al., 2013). The impact of symbioses on host genomes depends on the interaction modality, symbiont identity, and genetic capabilities of partner microbes. Leafhoppers have relied on two ancient obligate partners, but these relationships have changed within and between lineages over evolutionary time (Bennett, 2020; Koga et al., 2013; Łukasik et al., 2018; Moran et al., 2005). As such, we identified 53 shared gene clusters among all four leafhopper species that contain genes associated with symbiont support and integration (Table S8). Among the leafhopper species compared in this study, however, there have been significant changes in symbiont relationships outlined below.

Two leafhopper species in this study, ALF and GLRH, share the ancestral symbionts, *Sulcia* and *Nasuia*, the latter which may have been present with *Sulcia* since the origin of leafhoppers and possibly the Cicadomorpha and Auchenorrhyncha (Bennett & Mao, 2018; Koga et al., 2013). Due to their age, *Sulcia* and *Nasuia* genomes are among the smallest known from any system, requiring extensive host support (Bennett & Moran, 2013). To provide this support, ALF distinctly expresses thousands of previously identified host genes in symbiont-containing bacteriocytes and bacteriome organs (Mao et al., 2018). ALF and GRLH genomes are enriched for GO categories involved in peptidoglycan catabolic process (208 genes), positive regulation of cytolysis in another organism (47 genes), and response to bacterium (59 genes). Concurrently, there is enrichment in the functions peptidoglycan metabolic process (12 genes), apoptotic DNA fragmentation (four genes), and Toll signalling pathway immune response (15 genes) for defence against pathogenic microbes (Bao et al., 2014; Hoffmann et al., 1999; Schauvliege et al., 2007). Furthermore, among these ALF and GLRH genes, some show evidence of recent positive selection, including enrichment of multiple morphogenesis-related functions (Figure 4b). We identified 20 symbiont-support genes undergoing positive selection, which may be an underestimate due to the evolutionary distance and saturation among evolving genes in these species (Table 3; Table S10). Nevertheless, the highest count of positively selected symbiosis-related genes fell within the amino acid transport (five genes) and aminoacyl-tRNA formation categories (three genes).

The 20 genes identified in our tests of selection are involved in the direct interaction between the insect host and bacterial symbionts, such as the exchange of resources (e.g., nutrition and energy) (Table 3; Table S10). For example, genes involved in essential metabolites synthesis (coenzyme A) and recycling (glutamate NH3 synthesis) are among those undergoing positive selection. These precursor metabolites (e.g., glutamine, glutamate, coenzyme A) are required by symbionts to synthesize essential amino acids for their hosts, as well as their exchange (Duncan et al., 2014; Price et al., 2011). In the case of *Sulcia* and *Nasuia*, two of the most ancient symbionts known from any insect system, extensive genome reduction has led to the loss of most independent cellular functions and metabolisms (Koga & Moran, 2014; McCutcheon & Moran, 2007; Moran & Bennett, 2014). As a result, insect genes involved in supporting symbionts play essential roles in the provisioning and exchange of resources between host and symbiont cells. Thus, positive selection acting on these insect genes is likely due to their intimate and co-evolving interactions in prokaryotic cellular processes. In contrast, recent symbiont replacements like *Baumannia* in sharpshooter leafhoppers (discussed below) have larger genomes that encode more independent capabilities (Wu et al., 2006). In these systems, selection acting upon symbiont-associated host genes is likely to differ as there are fewer of them, and they may be less dependent on other insect-derived cellular resources and metabolites (Mao, Yang, & Bennett, 2017). Exploring patterns of selection in systems where symbiont losses and replacements have occurred can provide further information about how hosts and their symbionts co-evolve on

TABLE 3 Putative symbiont support genes undergoing positive selection in ALF and GRLH.

Gene description	Gene	EC number	Function
DNA mismatch repair protein Msh2	MSH2	N/A	DNA repair & replication
Glycine-tRNA ligase	GLYS	6.1.1.14	Aminoacyl-tRNA formation
b(0,+)-type amino acid transporter 1-like	BAT-1	N/A	Amino acid transport
b(0,+)-type amino acid transporter 1-like	BAT-2	N/A	Amino acid transport
DNA polymerase theta-like	POLQ	2.7.7.7	DNA repair & replication
Facilitated trehalose transporter Tret1-2 homologue	TRET-6	N/A	Sugar transport
Bifunctional coenzyme A synthase	COASY	2.7.7.3 & 2.7.1.24	CoA synthesis
Y+L amino acid transporter 2	YLAT-1	N/A	Amino acid transport
Glutamine synthetase 2 cytoplasmic	GS	6.3.1.2	NH3 recycling
Translation factor GUF1 homologue, mitochondrial-like	GUF-1	N/A	Translation
Uncharacterized protein LOC128983935	GOGAT	1.4.1.13	NH3 recycling
Proton-coupled amino acid transporter-like protein CG1139	PAT-4	N/A	Amino acid transport
Threonylcarbamoyl-AMP synthase	RPC10	2.7.7.6	Transcription
Phosphopantothenate—cysteine ligase	PPCS	6.3.2.5	CoA synthesis
Bifunctional 3′-phosphoadenosine 5′-phosphosulfate synthase-like	PAPSS	2.7.1.25 & 2.7.7.4	Sulphur metabolism
Peptidoglycan recognition protein 3-like	PGRP-SC	N/A	Population regulation
Excitatory amino acid transporter-like	EAAT	N/A	Amino acid transport
Bifunctional glutamate/proline—tRNA ligase	EPRS	6.1.1.15 & 6.1.1.17	Aminoacyl-tRNA formation
Facilitated trehalose transporter Tret1-2 homologue	TRET-2	N/A	Sugar transport
Probable proline—tRNA ligase, mitochondrial	PROS	6.1.1.15	Aminoacyl-tRNA formation

Note: For more information, including FPKM values in each host-specific tissue, see Table S10.

a gene-by-gene level. However, such assays require a more comprehensive sampling of insect species and across more recent divergence times.

Similarly to the deltocephaline species (i.e., ALF and GLRH), the GWSS leafhopper shares the ancestral *Sulcia* symbiont (Johnson et al., 2018; Moran et al., 2005). However, this lineage has completely replaced *Nasuia* with *Baumannia* >60 MYA (Moran et al., 2003; Wu et al., 2006). Several recent projects have identified thousands of host support genes expressed among the bacteriocytes and bacteriomes that house *Sulcia* and *Baumannia* (Li et al., 2022; Mao et al., 2018). Given *Baumannia*'s relative youth, it requires far fewer support genes than *Sulcia* and *Nasuia* (reviewed in Li et al., 2022). *Sulcia* symbionts, on the other hand, generally have among the smallest, most dependent genomes (Chang et al., 2015; Łukasik et al., 2018; McCutcheon & Moran, 2010; Moran et al., 2005; Shih et al., 2019; Takiya et al., 2006). Among *Sulcia*-associated leafhopper species, we confirmed global enrichment of GO categories linked to multiple metabolic processes, such as nucleoside triphosphate biosynthetic process (31 genes), tRNA metabolic process (10 genes), and succinate metabolic process (10 genes) (Table S4). Enrichment in these genes highlights the shared dependence of *Sulcia* symbionts on their hosts. The same mechanisms of support may be required to sustain this ancient and indispensable association among leafhoppers and likely also the Auchenorrhyncha (Campbell et al., 2018; Gossett et al., 2023; Michalik et al., 2021).

Finally, species in the Typhlocybinae leafhopper lineage have gone further and purged all obligate bacterial symbionts (Buchner, 1965; Cao & Dietrich, 2022). It has remained an open question as to what happens to host genomes when they lose symbionts and whether genomic palaeontology can reveal remnants of these associations. Remarkably, despite having lost its obligate symbionts, the TGLH species still indeed retains remnants of genes inferred to support *Sulcia* and *Nasuia* in other leafhoppers (e.g., Li et al., 2022; Mao et al., 2018; Mao & Bennett, 2020). For example, symbiosis-related genes involved in aminoacyl-tRNA formation (13 genes), amino acid transport (12 genes), and DNA repair and replication (nine genes) are found in gene clusters retained in all four leafhoppers (Table S8). The possible retention of symbiont-related genes in TGLH might indicate the retention of vestigial genes or the functional adaptation and reassignment of these genes for alternate host functions. Their retention further provides support for the early loss of *Sulcia* and *Nasuia* from the Typhlocybinae lineage when it transitioned to a more nutrient-rich food source (Bennett, 2020; Buchner, 1965; Moran et al., 2005; Sudakaran et al., 2017).

4.4 | Horizontally transferred genes in leafhoppers

Horizontally transferred genes (HTGs) present the opportunity for an organism to gain novel traits (Husnik & McCutcheon, 2018). This appears to be a common mechanism to support symbioses in insects, particularly leafhoppers and other hemipterans (Husnik et al., 2013; Luan et al., 2015; Nikoh et al., 2010; Sloan et al., 2014). ALF has 30 HTGs from various bacterial origins, most of which were inferred to support either *Sulcia* or *Nasuia* (Mao et al., 2018). Given the likely age of these HTGs (10s to 100s of millions of years old), pinpointing their origins, such as whether they resulted from tandem duplication or repeated horizontal transfer, is difficult to determine. Additionally, some paralogues exhibit low expression in ALF, suggesting potential non-functionality. Nevertheless, GRLH retains most of the HTGs present in ALF, with notable differences (Figure 5). By contrast, GWSS retains only 11 HTGs that are also present in ALF, most of which are predicted to support *Sulcia* (Li et al., 2022; Mao & Bennett, 2020).

A major focus of HTG analysis was the fate of these genes in species that have lost (TGLH) and replaced more ancient symbionts (GWSS). TGLH and GWSS retain orthologous HTGs involved in other host functions, including cell wall degradation (*cel-2*, *cel-1*, and *pel*). However, while GWSS has lost those related to *Nasuia*, TGLH has lost all full-length symbiosis support genes found in the other leafhopper species. To investigate whether remnants of lost ancestral HTGs in GWSS and TGLH, we scanned their genomes for gene fragments (Queffelec et al., 2022). Remarkably, we found remnants of three genes in TGLH used to support *Sulcia* (*ileS* and *yebC-1*) and *Nasuia* (*gh25-1*) in ALF (Mao et al., 2018). We further found a remnant of the cell growth gene, thymidylate kinase (*tmk*), in GWSS, which also supports *Sulcia* in ALF (Chaperon, 2006). We note that the specific function of these HTGs in leafhoppers remains untested and is inferred from their identities and specific expression patterns in ALF.

4.5 | ALF's distinct genomic traits highlight its polyphagous ecology

Species-specific gene groups and gene family expansions in ALF can help to identify adaptations to its unique biology and ecology. As an illustrative point, ALF's genome has uniquely experienced gene family expansion involved in sugar transport and protease genes (Price et al., 2010; Rispe et al., 2008). Enrichment in these specific genes likely underlies the differences between ALF's host plant associations and the other species investigated here. ALF is a massively polyphagous pest that feeds on a sugar-rich phloem diet across hundreds of plants. GRLH feeds narrowly on some monocot species (i.e., rice and other grasses), while GWSS is restricted to the sugar-depauperate xylem saps (Pathak, 1968; Turner & Pollard, 1959). TGLH is a monophagous pest that feeds on leaf parenchyma cell contents in tea plants (Günthardt & Wanner, 1981; Kawai, 1997; Qin et al., 2015). We speculate that the feeding habit of ALF as a polyphagous pest on eudicots plants may lead to an expansion of these sugar-related genes.

5 | CONCLUSION

The completion and analysis of the ALF genome assembly has unveiled new insights into leafhopper genomics and the broad evolutionary history of this group. Our study underscores the intricate balance between genomes, symbionts, and the environment in the field of insect pest evolution and adaptability. Comparisons among the genomes of four pest species highlight the influence of environmental pressures, including from insecticide treatments, farm cropping strategies (e.g., mono-cropping seasonality and cover crops), climate and its changes, and a range of plant secondary defensive compounds (Bai et al., 2022; Després et al., 2007; Dumas et al., 2019; Khaliq et al., 2014; Nauen & Denholm, 2005; Spencer & Hughson, 2023; Trenbath, 1993). The ability of these species to persist in these environments further depends on their obligate associations with a diversity of bacterial symbionts. These relationships similarly place significant evolutionary pressures on host genomes and are likely to influence the environmental interactions of insects (Bennett & Moran, 2015; Brodbeck et al., 1990, 2014). Major transitions in symbiotic relationships among leafhoppers have left strong signatures of evolution, affecting the expansion and contraction of gene families, as well as the fate of ancient HTGs.

Taken together, the addition of the ALF genome to the insect genome resources contributes to our broader understanding of insect genomics, their evolutionary adaptations, and potential impacts on their ecology and symbiotic relationships. Since ALF is a major pest of North American agriculture (Beanland et al., 2005; Wallis, 1962), these results can be used to inform and develop modern pest management strategies such as RNAi gene expression interference (Baum & Roberts, 2014; Jain et al., 2021). ALF's shared and unique genomic features identified in this study, including HTGs, selection events, and symbiosis-related genes, can potentially be targeted for disrupting the survival of these insects.

AUTHOR CONTRIBUTIONS

This study was conceived by G.M.B. and Y.M.V., karyotyping was performed by Z.L. and A.Z.X., Z.L. provided insightful feedback to results and contributed to the manuscript, genome analyses were performed by Y.M.V., the manuscript was written by Y.M.V. and G.M.B.

ACKNOWLEDGEMENTS

We would like to thank Dovetail Genomics for guidance and advice in the building of this genome. We thank Dr. Nancy Moran, Kim Hammond, and other members of her lab for supporting this work and providing resources for some molecular and microscopy work, as well as helping to sustain insect stocks. Additionally, we would like to thank DiemQuynh Nguyen for leafhopper support and Dr. Oscar Davalos for help with scripts for analyses. We thank Dr. Emily Jane

McTavish, Dr. Carolin Frank, and the members of the Bennett Lab for edits, constructive criticism, and feedback on the manuscript. We also thank the anonymous reviewers for their valuable comments and suggestions that helped to improve this work. This project was supported by the National Science Foundation Biological Integration Institute, INSITE- The Institute for Symbiotic Interactions, Training, and Education in the Face of a Changing Climate (NSF-2214038).

CONFLICT OF INTEREST

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

Macrosteles quadrilineatus genome assembly and sequencing data are available on NCBI under the BioProject ID PRJNA928792. Refseq genome annotation is available at https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/028/750/875/GCF_028750875.1_UCM_ALF_1.0/. Code used in this manuscript can be found at https://github.com/yumaryvasquez/Vasquezetal2023_MoleEcoRes.git.

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How to cite this article: Vasquez, Y. M., Li, Z., Xue, A. Z., & Bennett, G. M. (2024). Chromosome-level genome assembly of the aster leafhopper (*Macrosteles quadrilineatus*) reveals the role of environment and microbial symbiosis in shaping pest insect genome evolution. *Molecular Ecology Resources*, *24*, e13919. https://doi.org/10.1111/1755-0998.13919