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Research

Phylogenomics, population structure, and symbiont diversity refine the systematics of Southeast Asian rice leafhoppers (Hemiptera: Cicadellidae)

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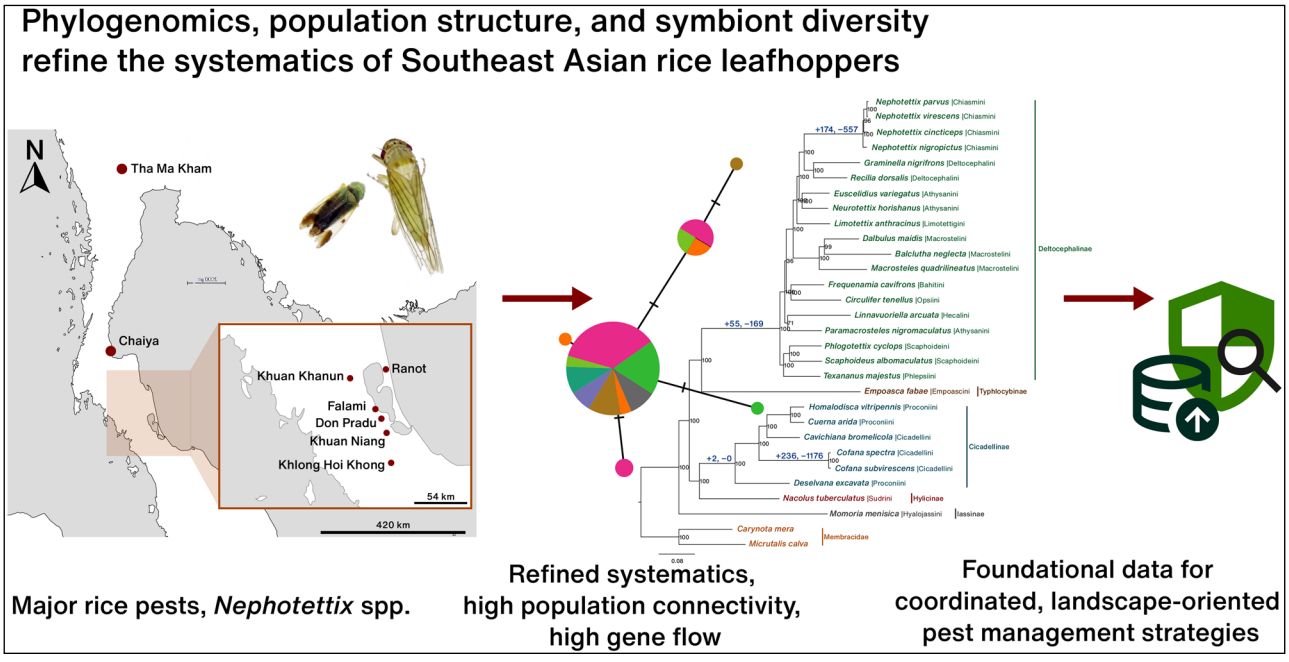
Leafhoppers of the genus *Nephotettix* (Hemiptera: Cicadellidae) are major vectors of rice tungro disease in Southeast Asia. Despite their economic significance, their evolutionary history, genomic adaptations, and associations with microbial symbionts remain poorly understood. Here, we aim to resolve the phylogenomic placement of *Nephotettix*, characterize patterns of gene family evolution, assess population connectivity and document the prevalence of bacterial symbionts. Using transcriptomes from 30 species, we reconstructed a robust phylogeny of Cicadellidae, recovering *Nephotettix* as a strongly supported monophyletic clade in our Cicadellidae phylogeny. Comparative analyses revealed lineage-specific expansions and contractions of gene families, including calcium-binding proteins and membrane-trafficking factors, with several genes showing signatures of positive selection potentially linked to host adaptation. Sequences from individuals sampled across 8 Thai localities indicated shallow haplotype networks and low nucleotide diversity in *Nephotettix virescens* (Distant 1908) and *Nephotettix nigropictus* (Stål 1870), patterns consistent with recent expansion or high dispersal. Screening for symbionts revealed universal infections with the obligate symbionts *Karelsulcia* and *Nasuia*, while facultative symbionts, *Wolbachia* and *Spiroplasma*, occurred at low prevalence across species and sites. This integrative approach refines *Nephotettix* systematics, identifies candidate genes underlying adaptation, and provides insights into dispersal and symbiont associations. These findings expand genomic resources for rice leafhoppers and offer a framework for improved diagnostics and evidence-based management of tungro disease vectors.

Keywords: endosymbiont, green leafhopper, insect, *Nephotettix*, *Cofana*

Graphical abstract



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Introduction

Rice (*Oryza sativa*) is a staple food for more than half of the world's population, with Southeast Asia representing a global center of rice production and consumption (Khush 2005). However, the productivity and sustainability of rice agroecosystems are persistently threatened by insect pests, among which green leafhoppers (Hemiptera: Cicadellidae: *Nephotettix* spp.) rank as some of the most damaging (Fujita et al. 2013). These insects inflict yield losses both directly, through phloem feeding and plant weakening, and indirectly as highly efficient vectors of devastating viral pathogens such as rice tungro virus, rice dwarf virus, and related taxa (Ammar and Nault 2002, Fujita et al. 2013).

Integrated pest management in rice ecosystems remains a major challenge, due in part to the high diversity, rapid adaptation, and cryptic speciation within leafhopper complexes (Sreejith et al. 2015, Manurung et al. 2019, Zhang et al. 2024). In Thailand and much of Southeast Asia, green leafhopper species, *Nephotettix malayanus* (Ishihara and Kawase 1968), *Nephotettix nigropictus* (Stål 1870), *Nephotettix parvus* (Ishihara and Kawase 1968), and *Nephotettix virescens* (Distant 1908), frequently coexist in paddy fields, often displaying overlapping morphological characters that complicate accurate identification (Ghauri 1971, Duan and Zhang 2014). In addition to *Nephotettix*, the white leafhopper, *Cofana spectra* (Distant 1908), is a common rice-associated species in Southeast Asia and was included here as a related lineage to provide phylogenetic context and to contrast patterns of gene family evolution and selection outside the focal genus. For *C. spectra*, a major gap is the limited availability of ecological, genetic, and genomic data, and the underrepresentation of this genus in systematic surveys and molecular analyses (Sreejith and Sebastian 2014). Misidentification or underestimation of cryptic species can lead to ineffective pest management strategies, unanticipated pest outbreaks, and failure to control virus transmission (Manurung et al. 2021, Wang et al. 2025a).

Molecular approaches have revolutionized pest diagnostics and surveillance, enabling identification of evolutionary significant units, hidden diversity, and gene flow in insect populations (Hebert et al. 2003, Junqueira et al. 2024). Studies using population genetics and DNA barcoding have found numerous hidden genetic differences within pest groups, which shows that accurate identification is needed (de Moya et al. 2019, Zhang and Bu 2022). Nevertheless, comprehensive population genetic and phylogenomic analyses of Southeast Asian green leafhoppers remain scarce, particularly for populations in Thailand where ecological conditions and pest management regimes may differ from those elsewhere (Manurung et al. 2019, 2020). At broader phylogenetic scales, morphological and phylogenomic studies have documented paraphyly in several traditionally recognized groups and persistent instability at deep nodes (Zahniser and Dietrich 2013, Dietrich et al. 2017, Hu et al. 2023). Although recent phylogenomic frameworks have improved resolution of the membracoid backbone, relationships within many cicadellid lineages, including speciose and economically important genera such as *Nephotettix*, remain poorly characterized due to limited intrageneric sampling and an emphasis on higher level relationships (Hu et al. 2023).

Sap-feeding leafhoppers rely on complex bacterial symbioses for survival and evolution. These insects harbor obligate endosymbionts such as *Candidatus Karelsulcia muelleri* and *Candidatus Nasuia deltocephalinicola* (Ishii et al. 2013, Gossett et al. 2023). Beyond these obligate partners, leafhoppers host a diverse assemblage of facultative symbionts, including *Wolbachia*, *Rickettsia*, *Spiroplasma*, *Cardinium*, and other Proteobacteria, whose presence, prevalence, and coinfection patterns vary widely among species, populations, and geographic regions. Surveys and experimental studies in rice agroecosystems indicate that *Wolbachia* infections are common but highly heterogeneous in frequency and density, and that interactions among coexisting symbionts can influence host reproduction, fitness, and population structure (Kittayapong et al. 2003, Wiwatanaratanabutr 2015, Yoshida et al. 2019).

High-throughput transcriptome sequencing and comparative genomics have provided unprecedented resolution for reconstructing evolutionary relationships, understanding population structure, and detecting adaptation in nonmodel pest taxa (Lizana et al. 2022). These tools are especially valuable for refining phylogenetic hypotheses, identifying functional genetic variation, and guiding sustainable pest management practices in dynamic agricultural landscapes (Lizana et al. 2022, Wang et al. 2024b). For example, population genomics of the Turnip moth, *Agrotis segetum* (Denis and Schiffermüller 1775), has identified selective sweeps in genes linked to pesticide tolerance and local adaptation (Wang et al. 2024a), while integrative omics studies of insect pests, such as *Tetranychus urticae* (Koch 1836), have revealed the genetic basis of resistance, dispersal, and resilience (Chen et al. 2025). In leafhoppers, adaptive evolution is expected to involve gene families associated with detoxification and sensory perception, reflecting their feeding biology (Zhao et al. 2022, Wang et al. 2025b). Yet, because most genomic work to date is either single-species or coarse between-family comparisons, robust genus-level inference remains limited.

Recent transcriptome-based phylogenomic analyses have clarified higher level relationships within Membracoidea, including the placement of *Nephotettix* (eg Hu et al. 2023). These analyses were designed to resolve backbone relationships across the superfamily and included a single representative of *Nephotettix*, providing an important phylogenetic framework while leaving evolutionary relationships within the genus itself largely unexplored. In this study, we investigate the population genetics and phylogenomics of rice-associated *Nephotettix* leafhoppers from paddy fields in southern Thailand, and we include *C. spectra* to provide phylogenetic context and to enable comparison of branch-level gene family turnover and selection patterns outside the focal genus. By integrating mitochondrial cytochrome oxidase I (COI) haplotype analysis, endosymbiont screening, and transcriptome-based phylogenomics, we aim to (i) clarify species boundaries and genetic diversity among these key pest species, (ii) resolve their evolutionary relationships, and (iii) provide a molecular framework to inform integrated pest management in Southeast Asian rice agroecosystems. Our findings contribute essential genomic resources and insights into the development of targeted, evidence-based strategies for sustainable pest control.

Methods

Sample Collection and Species Identification

Adult green leafhoppers were collected from paddy fields in 2023–2024 (Table 1, Supplementary Fig. S1). At each locality,

we surveyed standardized transects within actively cultivated paddies and collected leafhoppers by sweep net. Sampling coincided with the rice vegetative to early reproductive stages, when *Nephotettix* abundance is high. This minimized phenological heterogeneity among sites. Immediately following collection, specimens were transferred into 90% ethanol and transported to the laboratory, where they were stored at −20 °C until further processing. Taxonomic identification was performed based on morphology according to Ghauri (1971).

DNA Extraction and Species Confirmation

Genomic DNA was extracted from whole individual leafhoppers (Table 1) using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA quality was assessed by agarose gel electrophoresis, and DNA concentration was determined spectrophotometrically using a spectrophotometer (DeNovix Inc., Wilmington, Delaware, United States). Species identity was confirmed by amplification of COI gene using the LCO, 1490; and HCO, 2198; primers (Folmer et al. 1994). PCR was performed in a 20 µL reaction containing 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 0.5 U AllTaq polymerase (Qiagen, Hilden, Germany), and 1 µL of genomic DNA. Thermal cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 52 °C for 15 s, and 72 °C for 10 s. PCR products were visualized on 1.5% agarose gel, purified with ExoSAP-IT (Thermo Fisher Scientific, Massachusetts, United States), and Sanger sequenced (ATGC, Pathum Thani, Thailand). The COI sequences were BLASTed against the core nucleotide database for species confirmation.

RNA Extraction and Sequencing

For RNA extraction, adult males and females of each species were randomly selected across all sampling sites, yielding 5 individuals per species, except for *N. parvus*, for which 4 individuals were included, all collected from Chaiya. These individuals were distinct from those used in DNA-based population analyses and were preserved immediately after collection in DNA/RNA Shield reagent (Zymo Research, California, United States) to maintain RNA integrity. For transcriptome-designated individuals, species identity was confirmed by DNA barcoding using genomic DNA extracted from leg tissue, while whole-body DNA extractions were used for population genetic and symbiont screening analyses. PCR amplification was performed as previously described. After species confirmation, total RNA was extracted individually from each leafhopper using the GENEzol TriRNA Pure Kit (Geneaid, New Taipei City, Taiwan), following the manufacturer's protocol, with an additional DNase treatment step. RNA quality and quantity were verified by agarose gel electrophoresis and Qubit4 fluorometer with the RNA High Sensitivity Kit (Thermo Fisher Scientific, Massachusetts, United States), respectively. For each species, RNA samples from 5 individuals were pooled in equimolar amounts to obtain sufficient material for library preparation. This resulted in 1 pooled transcriptome library per species, yielding a total of 4 libraries corresponding to the 4 species analyzed. Library construction and paired-end sequencing (2 × 150 bp) were performed at BGI Genomics (Hong Kong, China) on the DNBSEQ platform. Each library yielded approximately 160 million clean paired-end reads. Ribosomal RNA removal and library preparation were performed by the sequencing provider following their standard short-insert library protocols.

RNA-seq Data Processing and Assembly

In addition to sequencing RNA from collected specimens, publicly available leafhopper RNA-seq datasets were downloaded from the NCBI Sequence Read Archive (SRA; Supplementary Table S1). All RNA-seq datasets, both newly generated and downloaded, were processed identically. Raw reads were quality-filtered and adapter-trimmed using fastp v0.23.4 (Chen 2023) with default parameters, followed by de novo transcriptome assembly using Trinity v2.15.1 (Grabherr et al. 2011). Protein-coding sequences were predicted using TransDecoder v5.5.0 (https://github.com/TransDecoder/TransDecoder) with the universal genetic code. Transcriptome completeness after isoform removal was assessed using BUSCO v5.8.0 (Simão et al. 2015) with the insecta_odb10 lineage dataset. Only transcriptomes with BUSCO completeness scores greater than 85% were retained for downstream orthology inference and phylogenetic reconstruction.

Phylogenomic Analysis

Predicted protein sequences from all transcriptomes passing the BUSCO completeness threshold were analyzed with OrthoFinder v2.5.5 (Emms and Kelly 2019) using DIAMOND for all-versus-all protein searches, multiple sequence alignment

Table 1. Collection localities, geographic coordinates, and specimen counts for molecular analyses

Location	Date	Coordinates	*N. virescens*	*N. nigropictus*	*N. malayanus*	*C. spectra*
Don Pradu	2 December 2023	7°17′01.2″N 100°18′31.3″E	7 (5)			
Khuan Niang	3 December 2023	7°09′14.3″N 100°21′22.5″E	6 (4)	6 (5)		
Khlong Hoi Khong	4 December 2023	6°52′47.7″N 100°23′52.3″E	5 (4)	4 (4)		
Khuan Khanun	16 December 2023	7°39′10.6″N 100°01′20.7″E	39 (25, 2)	27 (22, 2)		
Ranot	24 December 2023	7°44′03.0″N 100°20′59.6″E	12 (11, 1)	14 (10, 1)		
Falami	30 December 2023	7°20′14.8″N 100°16′40.1″E	7 (5)	12 (10)		
Chaiya	25 September 2024	9°23′20.7″N 99°12′32.9″E	10 (5, 2)	12 (10, 2)	3 (1)	10 (2, 5)
Tha Ma Kham	13 October 2024	14°01′47.5″N 99°29′33.7″E	13 (7)	4		

Values outside parentheses indicate the total number of individuals collected per species at each locality. Numbers in parentheses indicate the number of individuals processed for molecular analyses, with the first value corresponding to DNA-based analyses and, where applicable, the second value corresponding to RNA extraction for transcriptome sequencing. Where only a single value is given in parentheses, individuals were used exclusively for DNA-based analyses. Four *Nephotettix parvus* individuals collected from Chaiya were processed exclusively for RNA extraction.

with MAFFT v7.526 (Katoh and Standley 2013), and phylogenetic tree construction with IQ-TREE v2.3.6 (Minh et al. 2020). Gene family expansions and contractions were inferred using CAFE v5.1 (Mendes et al. 2021) with default parameters by comparing observed gene family sizes at the tips to maximum-likelihood estimates of ancestral gene family sizes reconstructed along the species tree. Inferences of "expansion," "contraction," or "stability" therefore refer to statistically supported changes along specific branches relative to their inferred ancestral states, rather than absolute differences among species. Enrichment of gene families at particular nodes reflects lineages showing significantly elevated rates of gain or loss compared to the genome-wide background under the fitted birth-death model. To detect episodic positive selection, we used the adaptive Branch-Site Random Effects Likelihood (aBSREL) model implemented in HyPhy v2.5.74 (Smith et al. 2015). This analysis was conducted on 73 one-to-one orthogroups shared across all taxa, which were identified by filtering the OrthoFinder Orthogroups.tsv output to retain groups with strictly single-copy representation for every species. For each orthogroup, protein sequences were aligned with MAFFT v7.526 (Katoh and Standley 2013), and codon alignments were generated with PAL2NAL v14.1 (Suyama et al. 2006). Phylogenetic tree was inferred from codon alignments using IQ-TREE v2.3.6 (Minh et al. 2020). Genes were considered to be under positive selection if at least one branch showed a significance signal (adjusted $P < 0.05$) and further filtered to retain only those with $\omega > 1$ affecting at least 5% of codon sites. Functional annotation of the predicted protein sequences was performed using EggNOG-mapper v2.1.8 (Cantalapiedra et al. 2021) against the EggNOG v5.0.2 database (Huerta-Cepas et al. 2019). Homology searches were conducted using DIAMOND (Buchfink et al. 2021) in sensitive mode with an E-value threshold of 0.001. Gene Ontology annotations were filtered to retain only terms supported by non-electronic evidence codes.

Detection of Symbionts

To determine the prevalence of facultative endosymbionts, we screened genomic DNA extracted from whole individual leafhoppers (see DNA Extraction and Species Confirmation) to ensure reliable detection of systemic infections. The presence of *Wolbachia* was detected by PCR targeting the *wsp* gene using the wsp81F and wsp691R primers (Braig et al. 1998) with an annealing temperature at 55 °C. Detection of *Spiroplasma* was performed using primers targeting the *p18* gene (Jaenike et al. 2007) with an annealing temperature of 50 °C. Individual PCR screening was preferred over transcriptome mining for these symbionts because the RNA-seq libraries were pooled, which precludes prevalence estimation, and because RNA-seq screening provided only low read support for facultative symbionts. Amplicons were checked on 1.5% agarose gel and purified with ExoSAP-IT prior to bidirectional Sanger sequencing.

To provide an independent check of facultative symbiont signal in the pooled RNA-seq data, we also screened the transcriptomes for *Wolbachia* and *Spiroplasma* by read mapping to reference genomes. The reference genomes were selected to represent well-annotated facultative symbionts from insect hosts, prioritizing genome completeness and breadth of gene content to maximize sensitivity of read mapping. For *Wolbachia*, we used the endosymbiont of *Diaphorina citri* (Kuwayama 1908), a sap-feeding hemipteran, while for *Spiroplasma* we used the genome from *Colias croceus* (Geoffroy 1785), which is among the larger *Spiroplasma* genomes available, reducing the risk of false negatives due to missing gene regions. Paired-end RNA-seq reads were mapped against genomes of *Wolbachia* (NZ_CP051608.1) and *Spiroplasma* (NZ_OZ026536.1) with minimap2 v2.28-r1209 (Li 2018) using the short-read preset (-ax sr). Alignment files were processed with SAMtools v1.22.1 (Danecek et al. 2021), and read support was quantified as mapped read pairs, counted once per pair by retaining only read1 alignments (flag 64) and excluding secondary and supplementary alignments. For conservative detection, we required a minimum mapping quality of MAPQ ≥ 20 for read counting. Read support was summarized as reads per million total read pairs (RPM), calculated as (mapped read1 count/total read1 count) × 10^6.

In contrast, obligate primary endosymbionts of leafhoppers, *Karelsulcia* and *Nasuia*, were detected from the transcriptome data. First, reads were mapped to the reference genomes (GCF_003215265.1 *Karelsulcia muelleri* and GCF_024820155.1 *Nasuia deltocephalincola*) using minimap2 with default options. Then mapped reads were extracted and assembled using metaSPAdes v4.2.0 (Prjibelski et al. 2020). The assembled contigs were annotated with Prokka v1.14.6 (Seemann 2014). Because obligate primary endosymbionts possess highly reduced and conserved genomes and are vertically transmitted, 16S rRNA has been widely used to confirm symbiont identity and broad phylogenetic placement, rather than to resolve fine-scale evolutionary relationships (eg Ishii et al. 2013, Kobiałka et al. 2018, Wu et al. 2023). In our pooled RNA-seq data, symbiont representation was low and genome recovery was fragmented, precluding genome-scale phylogenetic analyses. Therefore, 16S-based reconstruction was applied conservatively to validate symbiont presence and higher level relationships only. This approach is consistent with previous studies of leafhopper and planthopper symbioses where bacteriocyte-restricted endosymbionts are detected at low abundance in whole-body transcriptomes. Due to fragmented recovery of genome sequences that yielded only partial gene coverage, we used 16S rRNA gene sequences to construct phylogenetic analysis by aligning them with Clustal W (Thompson et al. 1994), and reconstructing phylogenetic tree using IQ-TREE v2.3.6 (Minh et al. 2020) with 10,000 ultrafast bootstraps.

Haplotype Analysis

Because mitochondrial COI represents a single, maternally inherited locus, inferences are interpreted conservatively and are used to evaluate broad-scale population connectivity and demographic patterns rather than fine-scale population subdivision (Zhang and Bu 2022). The mitochondrial COI gene, previously demonstrated to effectively differentiate population structures and assess genetic diversity in *Nephotettix* species (Sreejith et al. 2015, Yuniawati et al. 2019), was used to analyze haplotype diversity and relationships in *N. nigropictus* and *N. virescens*. Network analysis was performed using PopART v1.7 (Leigh and Bryant 2015), using the TCS network algorithm. We used DnaSP v6.12.03 (Rozas et al. 2017) to calculate nucleotide diversity (π), number of segregating sites, Tajima's *D* for each species population, and fixation index (F_{ST}) between populations. To further investigate population structure, we performed a discriminant analysis of principal components (DAPC) using the adegenet v2.1.11 (Jombart and Ahmed 2011) in R. Aligned sequences were converted into

genind objects, and individuals were assigned to populations based on sampling locality. The optimal number of clusters and number of principal components to retain were determined by cross-validation, and discriminant functions were then used to visualize genetic clustering among populations.

Results

Phylogenomic Relationships and Comparative Genomics of Leafhoppers

Prior to phylogenomic inference, we assessed the quality of the 30 transcriptome assemblies using BUSCO. The assemblies exhibited high completeness, with recovery of conserved single-copy orthologs ranging from 85.0% to 98.0% across species (Supplementary Table S1). Overall, high BUSCO completeness supports the suitability of these assemblies for comparative analyses. Comparative genomics analysis across 30 leafhopper transcriptomes reveals largely consistent gene content, with annotated gene numbers ranging from approximately 15,000 to over 59,000 per species (Supplementary Table S2). On average, 94.4% of all predicted genes are assigned to orthogroups, with individual species values exceeding 90% in most cases. The proportion of unassigned genes is generally low (mean 5.6%), though a few species, such as *Balclutha neglecta* (DeLong and Davidson 1933) and *Empoasca fabae* (Harris 1841), show elevated percentages.

Phylogenomic inference using 73 single-copy orthogroups resulted in a species tree for Cicadellidae and Membracidae with strong support at most internal nodes (Fig. 1). Within Cicadellidae, the subfamily Deltocephalinae forms a major clade. The tribe Chiasmini, including four *Nephotettix* species, is recovered as a strongly supported monophyletic group. The tribe Scaphoideini is monophyletic, with *Phlogotettix cyclops* (Mulsant and Rey 1855) and *Scaphoideus albomaculatus* (Li 1990) clustering together. In contrast, the tribes Cicadellini, Proconiini, and Athysanini each form paraphyletic assemblages, with representatives scattered among related lineages. For example, Cicadellini and Proconiini do not form monophyletic clades within Cicadellinae.

Having established the species tree, we next examined gene family dynamics along key branches (Supplementary Table S3). At the ancestral Cicadellidae node, gene content remained relatively stable, with statistically supported expansions inferred along this branch relative to the reconstructed ancestral gene family repertoire, particularly among metabolic and detoxification families, including Cytochrome P450s (eg *Cyp6a9*) and proteases. The *Cofana* clade shows statistically supported expansion of gene families containing reverse transcriptase (RVT_1) and endonuclease (Exo_endo_phos) domains, and the contraction of phosphatase families. Because our inferences are based on predicted proteins derived from transcriptome assemblies, expansions of retro-element-associated domains are interpreted as increased transcript evidence for these elements, rather than direct estimates of genomic transposable element content.

In the *Nephotettix* ancestor, gene family turnover inferred along this branch involved expansions and contractions in families linked to host-associated traits. Expansions inferred along the *Nephotettix* ancestral branch were disproportionately represented among signaling and sensory functions, including Zinc finger-related proteins (eg *ZNF711* and *KMT2D*), EF-hand calcium-binding proteins, and the conserved fructose sensor Gustatory Receptor 43a (*Gr43a*). Concurrently, this lineage showed pronounced contraction of multiple gene families, including multiple zinc finger proteins (eg *sptf-3*), ubiquitin ligases (eg *BMI1*), diverse peptidases, and reverse transcriptase and integrase domains associated with transposable elements. Across *Nephotettix*, lineage-associated patterns were inferred at the ancestral branch, including enrichment of retrotransposon-associated domains (RVT_1/RVT_2 and Gag-polypeptide families) and regulatory candidates such as *AGAP3* and *PHF12*.

To investigate lineage-specific adaptive evolution on one-to-one orthogroups, we identified orthogroups exhibiting signatures of diversifying selection. At the subfamily level, 8 orthogroups showed evidence of diversifying selection within Deltocephalinae, including genes encoding transcriptional regulators (eg *MYC*), vesicle transport proteins (eg *SEC24C*), and signaling proteins (eg *SDCBP*). Signals of selection were unevenly distributed and were most frequent within *Nephotettix*, with 81 orthogroups in *Nephotettix cincticeps* Uhler, 1896, 59 in *N. nigropictus*, 41 in *N. parvus*, and 33 in *N. virescens* (Supplementary Table S4). Positively selected genes spanned diverse functional categories, including kinases and signaling regulators, BTB/POZ domain proteins, chromatin and transcription-associated domains (including KIX, bromodomain, ZZ-type zinc finger, and zf-BED), ubiquitin-related proteins (eg *UBE2B* and *UBXN1*), and metabolic enzymes (eg class II aldolase and cytidylyltransferases). Additional candidates included proteins implicated in membrane transport and vesicle trafficking (DUF2053, v-SNARE N-terminus, SNARE, and WD40 repeat proteins), as well as Ras family proteins, cytochrome b5-like heme or steroid-binding domains, and lysosome-associated membrane glycoproteins.

Sample Collection, Species Identification, and Haplotype Analysis

A total of 191 leafhoppers were collected from eight locations in southern and western Thailand (Table 1). Morphological and COI barcode-based identification classified specimens into three species, *N. virescens* ($n = 99$), *N. nigropictus* ($n = 79$), and *N. malayanus* ($n = 3$). Ten individuals of *C. spectra* were sampled from a single locality and used as a comparative lineage and population genetic analyses were not conducted for this species. Species composition varied by site, *N. virescens* was the most widespread, found at all locations, and dominated collections from Khuan Khanun. *N. nigropictus* was present in 7 sites and was most abundant in Khuan Khanun and Ranot. *N. malayanus* was detected only in Chaiya.

Haplotype network analysis of *N. nigropictus* revealed 3 distinct haplotypes. The largest haplotype group included 51 individuals from many localities. This pattern indicates low overall genetic differentiation and suggests recent common ancestry or extensive gene flow among populations. For *N. virescens*, the haplotype network displayed 2 major haplotypes. The main haplotype group consists of 53 individuals from diverse sampling sites. Another major haplotype included 8 samples. Most haplotypes were separated by only a single mutation (Fig. 2). Nucleotide diversity was low in both species ($\pi < 0.001$) and negative Tajima's D (−0.50 for *N. nigropictus* and −1.55 for *N. virescens*).

Pairwise F_{ST} analysis further supported the high connectivity between populations, although some local substructure was

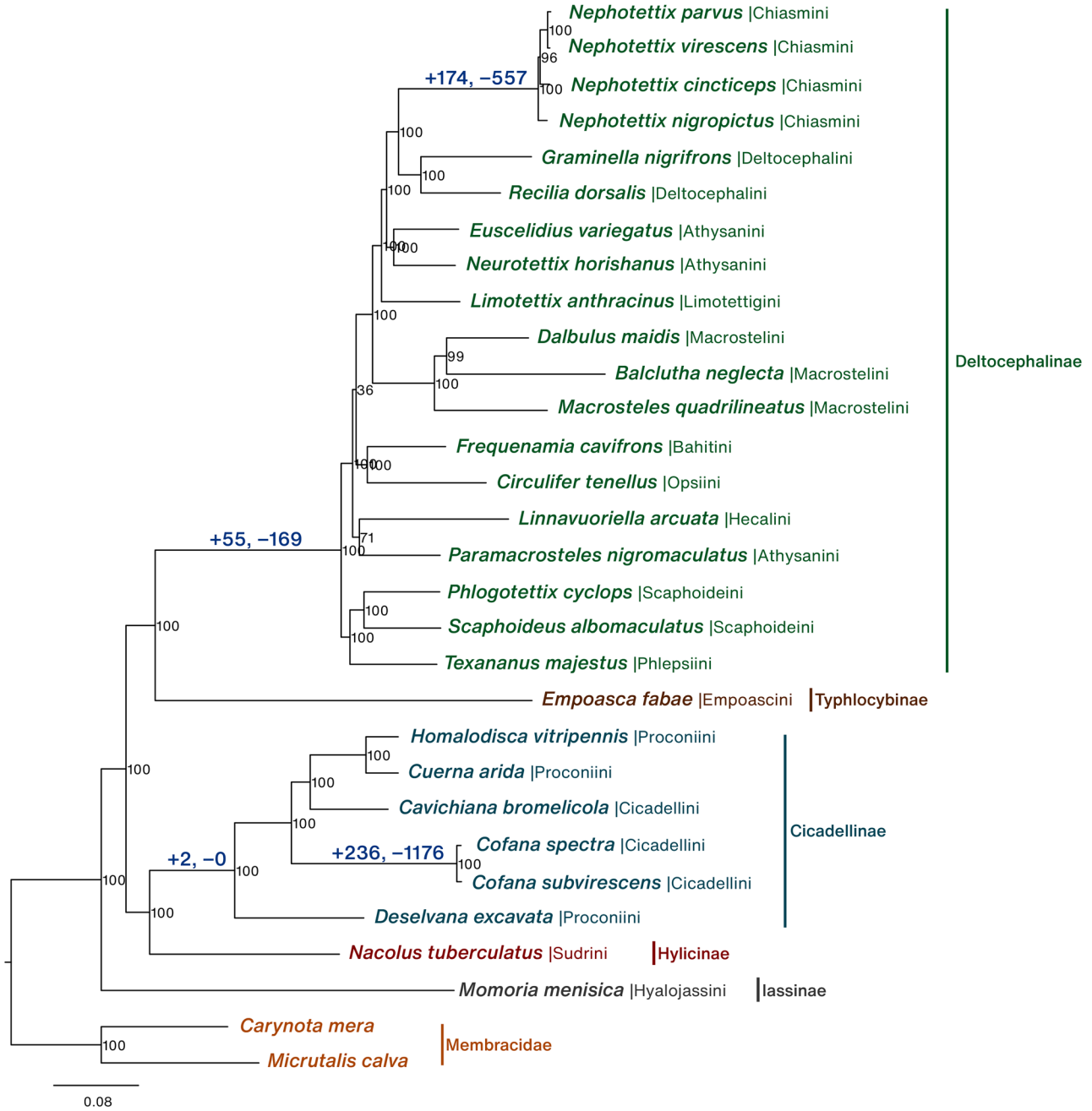

Fig. 1. Maximum-likelihood phylogenetic tree of leafhopper species within Cicadellidae based on transcriptome data. Number at each node indicates Ultrafast Bootstrap value. Tribes are indicated to the right of the species names. *Carynota mera* (Say 1830) and *Micrutalis calva* (Say 1831) of the family Membracidae were used as outgroups.

evident (Supplementary Tables S5 and S6). In *N. nigropictus*, F_{ST} values ranged from 0.00 to 0.29, while populations like Ranot, Chaiya, and Khuan Niang appeared panmictic (F_{ST}=0.00), the Khuan Khanun population showed moderate differentiation from others (eg vs. Ranot, F_{ST}=0.29). Similarly, *N. virescens* exhibited generally low genetic differentiation, with most pairwise comparisons below 0.10, though specific interactions (eg Khuan Niang vs. Khlong Hoi Khong) reached higher values (F_{ST}=0.33). Together with haplotype analysis, DAPC revealed minimal genetic structuring within both *N. virescens* and *N. nigropictus*. Individuals from different collection sites largely clustered together in discriminant space, indicating high gene flow or recent shared ancestry among populations (Fig. 3).

Endosymbiont Detection

Finally, we screen primary and facultative symbionts to evaluate coinfections and phylogenetic affinities across hosts. Screening for endosymbionts across all leafhoppers revealed a heterogeneous prevalence of *Wolbachia* and *Spiroplasma* among individuals and sites (Fig. 4). *Wolbachia* infections were

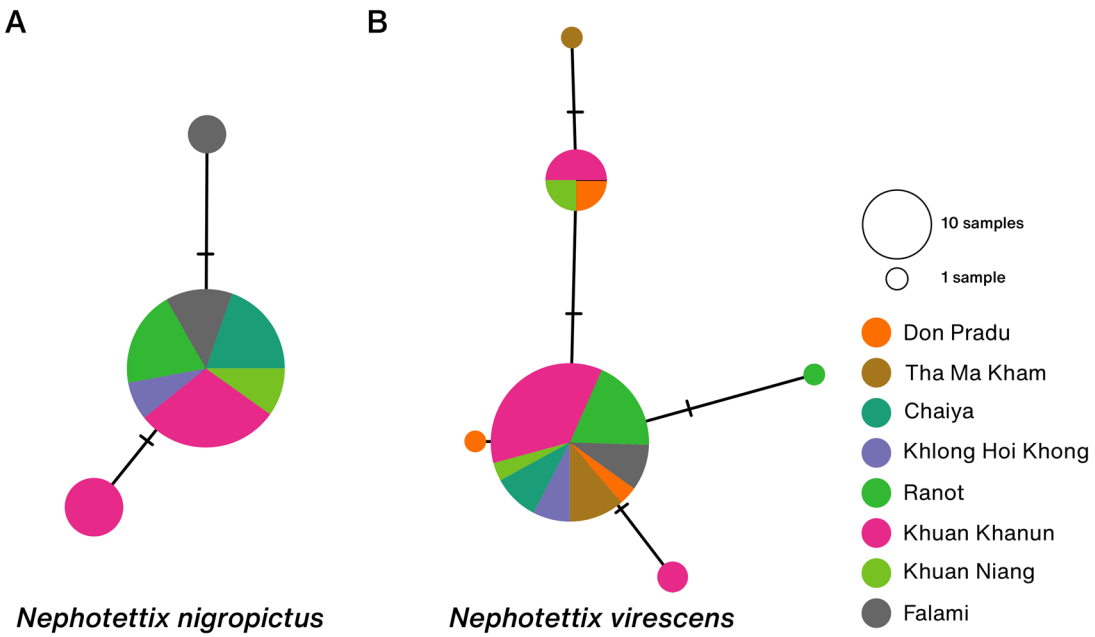

Fig. 2. Haplotype networks of *Nephotettix nigropictus* and *Nephotettix virescens* populations based on COI sequences, constructed using the TCS method. Each circle represents a unique haplotype, with the size of the circle proportional to the number of individuals sharing that haplotype.

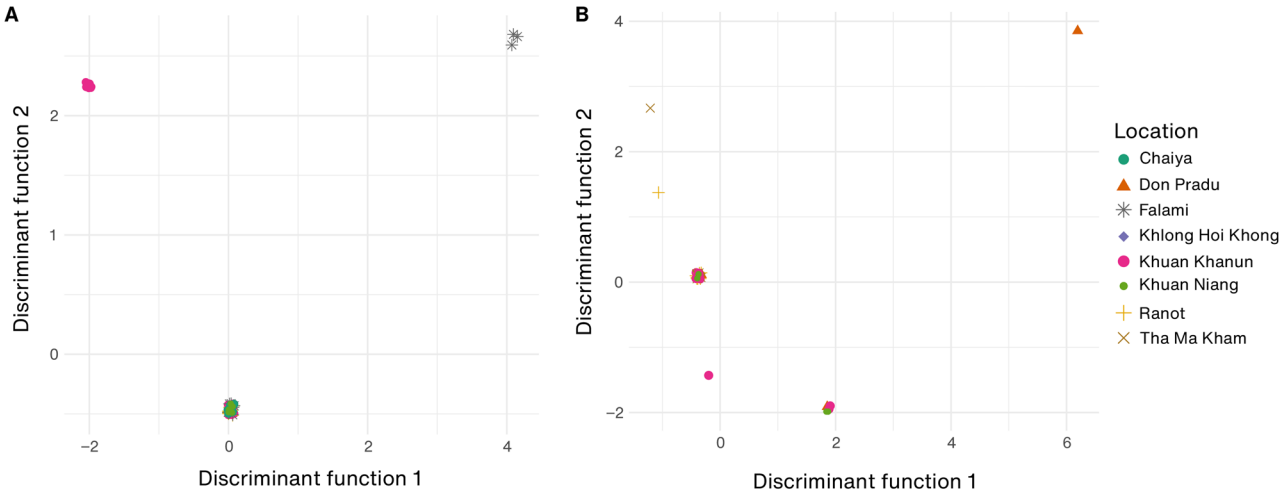

Fig. 3. Discriminant analysis of principal components (DAPC) based on mitochondrial COI haplotypes for *Nephotettix nigropictus* (A) and *Nephotettix virescens* (B). Each point represents an individual, colored by sampling locality.

present in 6 individuals, including 3 *N. nigropictus* (2 from Ranot and 1 from Khuan Khanun), 1 *N. virescens* from Ranot, 1 *N. malayanus*, and 1 *C. spectra* from Chaiya. *Spiroplasma* was rare, identified in only 2 *N. nigropictus* individuals, 1 from Chaiya and 1 from Falami. No coinfections with both *Wolbachia* and *Spiroplasma* were observed. Consistent with these low PCR detection frequencies, pooled RNA-seq screening showed low read support for *Wolbachia* (5.7 to 10.7 RPM across species) and near-zero support for *Spiroplasma* (0 to 0.042 RPM, Supplementary Table S7).

The 16S rRNA gene sequences of both *Karelsulcia* and *Nasuia* formed well-supported, monophyletic clades specific to Deltocephalinae, with the *Nephotettix*-associated strains clustering together (Fig. 5A and B). In contrast, analysis of the *Wolbachia wsp* gene revealed that *Nephotettix* individuals harbored *Wolbachia* strains belonging to both supergroups A and B, with sequences distributed across different clades (Fig. 6A). Similarly, *Spiroplasma p18* gene sequences from *N. nigropictus* were distributed among distinct clades. Sequence analysis confirmed that all detected *Wolbachia* and *Spiroplasma* were closely related to known strains from other cicadellids, with no evidence of novel or highly divergent lineages. Overall, obligate symbiont phylogenies broadly tracked host taxonomy, whereas facultative symbionts showed a patchier distribution across host lineages, consistent with recurrent gain and loss and possible horizontal transfer.

Discussion

Phylogenomic Resolution and Taxonomic Implications

Our comparative analysis of the 30 transcriptomes revealed substantial variation in gene content. While most species possessed 14,595 to 27,614 predicted genes, *E. fabae* and *B. neglecta* exhibited larger gene sets (>40,000). High

Fig. 4. Heatmap summarizing infection prevalence of facultative endosymbionts across sampled leafhopper populations. Values within cells indicate the number of infected individuals over the total number screened at that site (infected/total). For *Wolbachia* detections, the letter in parentheses denotes the inferred supergroup (A or B) based on phylogenetic reconstruction.

orthogroup assignment across taxa (86.1% to 97.6%, mean 94.4%; Supplementary Table S2) indicates that most predicted genes cluster into conserved gene families, although the elevated gene counts and higher unassigned fractions in some assemblies likely reflect differences in assembly redundancy and annotation completeness. Notably, extensive transcript diversity is also biologically plausible in specific tissues, for example, the large salivary transcript catalog reported for *E. fabae* (DeLay et al. 2012).

Recent transcriptome-based phylogenomic analyses have substantially clarified higher level relationships within Membracoidea, providing a robust backbone framework for the superfamily (Hu et al. 2023). In that study, *Nephotettix* was represented by a single species, which was appropriate for resolving deep relationships but not intended to address diversification or evolutionary dynamics within the genus. Our phylogenomic results are fully congruent with this framework, recovering *Nephotettix* as a strongly supported monophyletic lineage within Chiasmini and confirming the broader tribal relationships reported by Hu et al. (2023). Consistent with their findings, we also observe short internal branches and paraphyly in several higher level cicadellid lineages, reinforcing the view that rapid diversification and incomplete lineage sorting continue to limit resolution at deeper nodes. By increasing taxon sampling within *Nephotettix* and including closely related deltocephaline lineages, our study extends this backbone framework to the genus level, enabling species-level phylogenomic inference and comparative analyses that were not feasible in broader backbone studies.

Using 73 single-copy orthogroups, our phylogenomic inference yielded a well-resolved species tree for Cicadellidae and Membracidae. Within Deltocephalinae, the tribe Chiasmini was recovered as a strongly supported monophyletic group that includes all four *Nephotettix* species, consistent with mitogenomic and multilocus studies that also place *Nephotettix* as monophyletic within Deltocephalinae (Zahniser and Dietrich 2013, Gao et al. 2021, Shah et al. 2024). We also recovered Scaphoideini as monophyletic, with *Phlogotettix* and *Scaphoideus* clustering together, aligning with recent mitogenomic evidence supporting monophyly of this tribe (Shah et al. 2024). At the same time, earlier work with denser taxon sampling has suggested that the limits of Scaphoideini may require reevaluation, highlighting that broader sampling remains essential for stable tribal delimitation (Zahniser and Dietrich 2013).

In contrast, we recover extensive paraphyly across the large Cicadellidae tribes (eg Cicadellini and Proconiini) and in parts of Athysanini. This mirrors a long-standing pattern in leafhopper systematics, where morphology-based tribes do not always map cleanly onto evolutionary lineages, mainly because of incomplete sampling and rapid diversification (Dietrich et al. 2017). Our short internal branches connecting the Cicadellinae lineages are consistent with a rapid radiation and the incomplete lineage sorting that often erodes phylogenetic signal at deep nodes. Recent phylogenomic analysis of Membracoidea

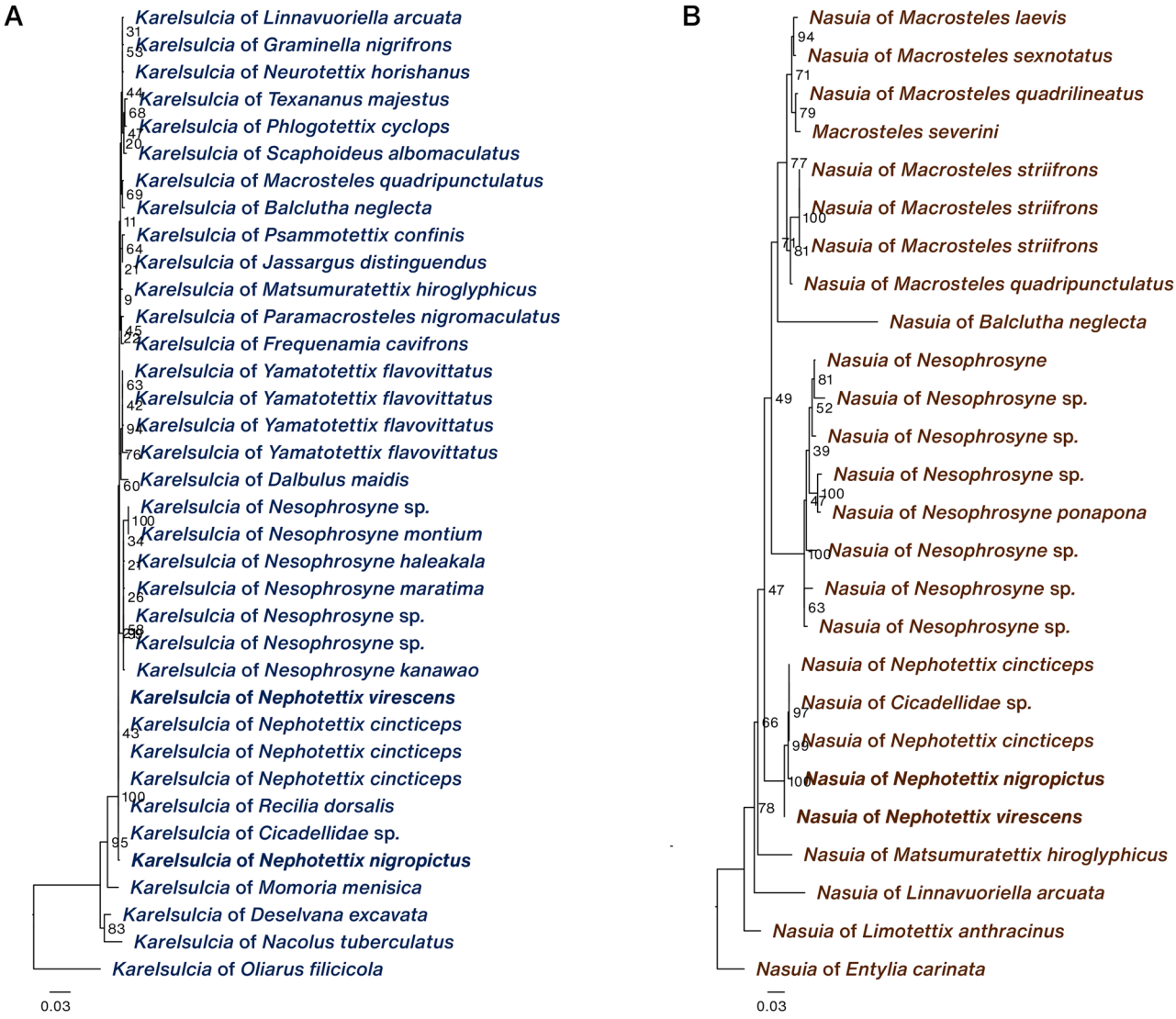

Fig. 5. Maximum-likelihood phylogenetic trees of obligate primary endosymbionts associated with leafhoppers. (A) *Candidatus Karelsulcia muelleri* and (B) *Candidatus Nasuia deltocephalinicola* phylogenies inferred from 16S rRNA gene sequences recovered from pooled transcriptomes (study sequences in bold). Node values indicate Ultrafast Bootstrap support. The trees are interpreted at a higher taxonomic level and are used to confirm symbiont identity and host-associated clustering rather than strain-level relationships.

reached similar conclusions for difficult backbone relationships (Dietrich et al. 2017) that suggest the need for more genome-scale datasets and broader sampling.

Gene Family and Evolution across Major Lineages

Across the species tree, gene family turnover in the *Nephotettix* ancestor was characterized by expansions enriched in signaling and sensory functions, including EF-hand calcium-binding proteins, zinc finger-related proteins, and the conserved fructose sensor Gr43a, together with contractions affecting broad regulatory and metabolic families, and multiple transposable element-associated domains. The expansion of EF-hand proteins is consistent with shifts in calcium-mediated signaling and stress-responsive pathways that can influence diverse physiological traits, including neuromuscular function and cellular homeostasis (Lehne and Bogdan 2023). More broadly, the combination of sensory and signaling expansions with contractions in broadly acting regulatory families is compatible with lineage-specific tuning of host-associated traits in *Nephotettix*, rather than a uniform increase in gene repertoire. As emphasized in recent transcriptome-based phylogenomic studies, including Hu et al. (2023), gene family expansions and contractions inferred from transcriptome data should be interpreted conservatively as relative, branch-level patterns rather than direct estimates of genomic copy number.

In the *Cofana* clade, we observed pronounced retro-element-associated dynamics, with expansions of families annotated with reverse transcriptase (RVT_1) and endonuclease (Exo_endo_phos) domains, alongside contractions in phosphatase-related families. Because these inferences are derived from predicted proteins in transcriptome assemblies, the most conservative interpretation is that these patterns reflect increased transcript evidence for retro-element-associated sequences, not direct genomic estimates of transposable element abundance. Nevertheless, expansions and contractions of transposable elements are common across arthropods, and retrotransposons can contribute substantially to genome size

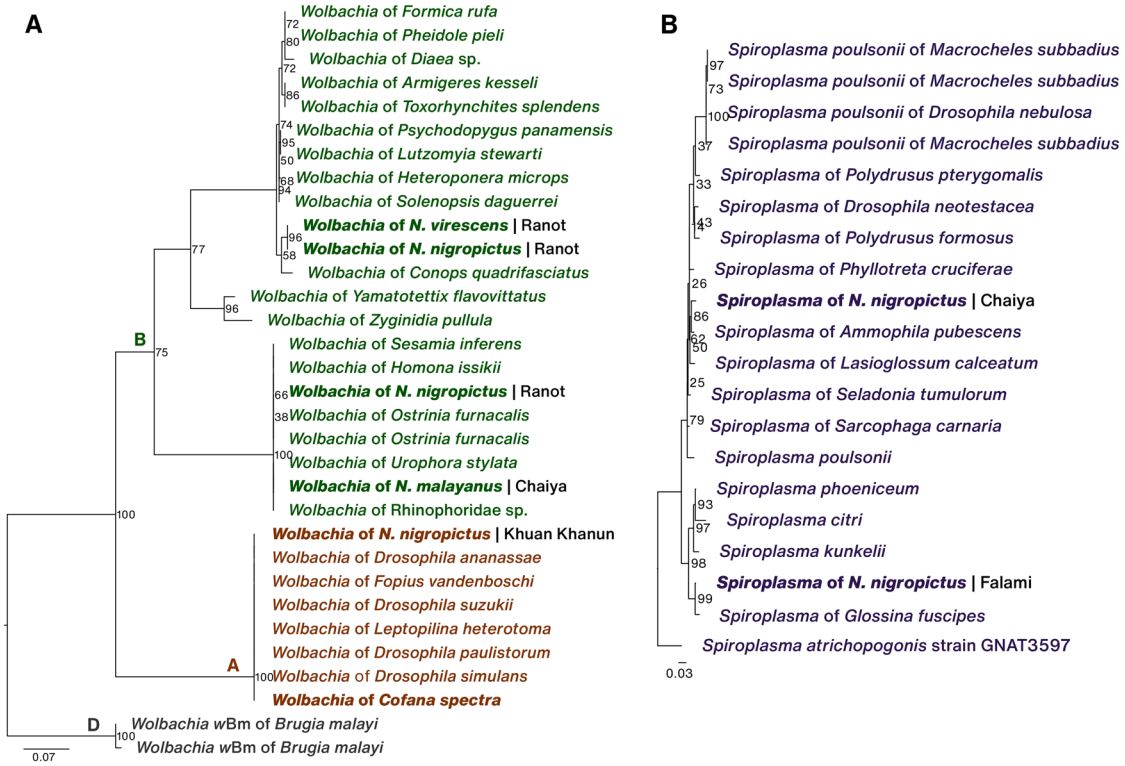

Fig. 6. Maximum-likelihood phylogenetic trees of facultative symbionts detected in leafhoppers. (A) *Wolbachia* phylogeny inferred from *wsp* sequences. Letters A, B, and D denote *Wolbachia* supergroups. (B) *Spiroplasma* phylogeny inferred from *p18* sequences. Study sequences are in bold. Node values indicate Ultrafast Bootstrap support.

variation and divergence over evolutionary time (Petersen et al. 2019, Wu and Lu 2019).

Consistent with the concentration of positive selection signals within *Nephotettix* in our aBSREL analyses, several orthogroups exhibiting diversifying selection encode membrane transport and vesicle-trafficking factors, including domains associated with v-SNARE N-terminus and SNARE machinery, as well as WD40 repeat proteins and other trafficking-related candidates. Selection on trafficking pathways is biologically plausible in phloem-feeding Auchenorrhyncha, where salivary secretion and effector delivery are central to host manipulation and feeding success (Fu et al. 2020). Comparative salivary transcriptome and proteome studies in planthoppers and leafhoppers, including *Sogatella furcifera* and *N. cincticeps*, have repeatedly implicated secreted enzymes and secretion-related pathways in interactions with plant defenses (Hattori et al. 2015, Miao et al. 2018), and SNARE-regulated exocytosis provides a mechanistic link between vesicle trafficking and secretory output (Suzuki and Iwata 2018). In addition, the presence of diversifying selection among transcriptional, chromatin-associated, ubiquitin-related, and metabolic genes is consistent with rapid evolutionary change in regulatory and metabolic architecture, which may facilitate adaptation to host chemistry and management pressures documented in rice pest systems (Matsumura and Sanada-Morimura 2010, Khoa et al. 2018), although functional validation will be needed to connect specific loci to these ecological drivers.

Complex Host-Endosymbiont Relationships

Our symbiont data revealed a microbial mosaic, ancient, obligate partners mirroring host phylogeny alongside facultative symbionts that are frequently replaced through horizontal transmission. Similar to other Auchenorrhyncha, all *Nephotettix* screened carried the coprimary, nutritional endosymbionts *Karelsulcia* and *Nasuia*. These endosymbionts complement amino acid pathways, and codiversification with their hosts is a hallmark of these systems (Bennett and Moran 2013, McCutcheon et al. 2019). Recent comparative genomics further shows that *Karelsulcia* and *Nasuia* evolve under different rates yet retain stable metabolic complementary across leafhopper lineages (Vasquez and Bennett 2022).

In contrast, facultative symbionts such as *Wolbachia* and *Spiroplasma* appeared inconsistently distributed across individuals and species. Their phylogenetic placements were nonmonophyletic relative to host patterns, suggesting horizontal transmission rather than strict cospeciation. Consistent with this, we also detected geographic differentiation among *Wolbachia* strains, with infections structured by locality and supergroup, as infected individuals from Ranot carried Supergroup B, whereas an infected individual from Khuan Khanun carried Supergroup A. This pattern supports site-specific strain turnover and a geographic mosaic most consistent with local, horizontal acquisition rather than a single ancient infection tracking host lineages. *Wolbachia* of supergroups A and B are the dominant infections in insects and have been detected across Hemiptera, including leafhoppers, with well-known reproductive effects that can structure population and potentially modulate gene flow in partially isolated host demes (Kittayapong et al. 2003, Zhang et al. 2021, Cooper et al. 2023). The patchy distribution of *Wolbachia* is typical for leafhoppers (Ishii et al. 2013) and fits the global estimate that approximately 40% of terrestrial arthropod species harbor

the bacterium (Zug and Hammerstein 2012). *Spiroplasma* have also been shown to protect hosts against natural enemies in other insects (Xie et al. 2014, Ballinger and Perlman 2017) that might be worth testing in *Nephotettix* and *Cofana* systems.

Rice fields likely provide a complex arena for horizontal symbiont transfer that could generate the locality-linked structure we observe. Experimental and comparative studies demonstrated such facultative symbionts can be transmitted among insects, sometimes via plants, and can confer parasitoid defense or manipulate reproduction such as cytoplasmic incompatibility (Oliver et al. 2010, Chrostek et al. 2017, Li et al. 2017). Parasitoid wasps have also been proposed to act as dirty needles, vectoring *Wolbachia* between different hosts (Ahmed et al. 2015, Lai et al. 2024). Under this framework, the segregation of *Wolbachia* supergroups A and B between Ranot and Khuan Khanun is consistent with differences in parasitoid communities, plant-mediated transmission routes, or broader ecological interaction networks between these sites, that could create partially isolated local pools of *Wolbachia*.

Population Structure and Connectivity

COI haplotypes in *N. virescens* and *N. nigropictus* are broadly shared across southern Thailand, with negative Tajima's *D*, shallow networks, and low genetic differentiation. These patterns indicate recent demographic expansion or high gene flow across the areas that are commonly observed in rice-inhabiting leafhoppers (Manurung et al. 2019, 2020). For example, *N. nigropictus*, exhibits little differentiation between Indonesian and Indian populations, indicating recent common ancestry or ongoing gene flow across Southeast Asia (Manurung et al. 2019). Similarly, *N. virescens* from Samosir Island in Indonesia showed 98.6% COI sequence identity with populations from Bangladesh, supporting a pattern of limited mitochondrial divergence across broad geographic distances (Manurung et al. 2020). Such low population-level divergence may reflect intensive rice cultivation that promotes habitat connectivity and repeated anthropogenic movement (Yuniawati et al. 2019). Such low population-level divergence could reflects episodic wind-assisted movement combined with multigenerational, stepping-stone dispersal across contiguous rice landscapes that enhance habitat connectivity and are reinforced by repeated anthropogenic movement (Otuka 2013, Yuniawati et al. 2019). This interpretation aligns with the demonstrated dispersal capacity of *Nephotettix*, as tethered-flight experiments show relatively high flight propensity in *N. virescens* (Widiarta et al. 1993). In addition, occasional long-distance dispersal events may occur through human-mediated transport associated with agricultural trade and the movement of infested plant materials (Gippet et al. 2019).

Comparable dynamics have been reported for migratory brown planthopper, *Nilaparvata lugens* (Stål 1854), where continent-scale gene flow rapidly spread adaptive alleles across East and Southeast Asia (Matsumura et al. 2018, Hereward et al. 2020). Given the comparable ecology and cropping calendars, we infer that southern Thai rice paddies serve as pathways for *Nephotettix* dispersal. This inference aligns with migration patterns observed in other leafhoppers species (Wu et al. 2019).

Implications for Pest and Disease Management

Because *Nephotettix* are primary vectors of rice tungro viruses, even modest increases in regional connectivity or salivary efficiency may shift disease risk (Wang et al. 2022). Given the high gene flow we infer, resistance or virulence traits can spread quickly, as observed in *N. lugens* (Khoa et al. 2018). Thus, surveillance and interventions should be coordinated across provinces and tuned to migration windows. At the same time, integrated pest management, reduced prophylactic sprays, and conservation of natural enemies have lowered hopper pressure and pesticide use in Asian rice and provide a framework for embedding molecular diagnostics for early warning and response (Pretty and Bharucha 2015).

Conclusion

In summary, our integrated analysis links deep evolutionary history with contemporary population processes for the principal green leafhopper pest of Asian rice. Whole-transcriptome phylogenomics resolves Chiasmini as monophyletic and exposes paraphyly in several tribes. Comparative genomics reveals broad orthogroup conservation punctuated by lineage-specific shifts and signatures of positive selection in secretion, regulatory, and cytoskeletal pathways that are plausible drivers of host use and pesticide response. COI haplotypes show recent demographic expansion and high regional connectivity that can rapidly propagate adaptive alleles, whereas symbiont screening confirms the universal presence of the obligate symbiont partners *Karelsulcia* and *Nasuia* alongside patchy *Wolbachia* and *Spiroplasma*. Together, these results deliver validated species boundaries, evolutionary context, and candidate molecular targets for practical applications, such as surveillance and diagnostics, and argues for coordinated, landscape-scale integrated pest management that minimizes prophylactic spraying and prioritizes targeted intervention. Future priorities include functional validation of candidate genes, genome-wide single nucleotide polymorphisms population analyses to test fine-scale structure and sex-biased dispersal not captured by mitochondrial COI, and expanded geographic sampling to refine movement pathways and symbiont dynamics across Southeast Asia.

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Author Contributions

Matsapume Detcharoen (Conceptualization [equal], Data curation [lead], Formal analysis [lead], Funding acquisition [equal], Investigation [equal], Methodology [equal], Software [equal], Validation [equal], Visualization [equal], Writing—original draft [lead], Writing—review & editing [equal]), Venus Saksongmuang (Conceptualization [equal], Funding acquisition [equal], Resources [lead], Writing—review & editing [equal]), and Areeruk Nilsai (Conceptualization [equal], Funding acquisition [equal], Resources [lead], Writing—review & editing [equal])

Supplementary Material

Supplementary material is available at *Journal of Insect Science* online.

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Conflicts of Interest

The authors declare no competing interests.

Data Availability

Raw transcriptomes were deposited on NCBI SRA under the project PRJNA1312137. COI, *Wolbachia*, and *Spiroplasma* sequences were deposited on NCBI GenBank under accessions PX242680 to PX242747 and PX277293 to PX277300.

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