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Low *Wolbachia* incidence in *Bactrocera* and *Zeugodacus* species from Thailand and genome analysis of *Wolbachia* associated with *Zeugodacus apicalis*

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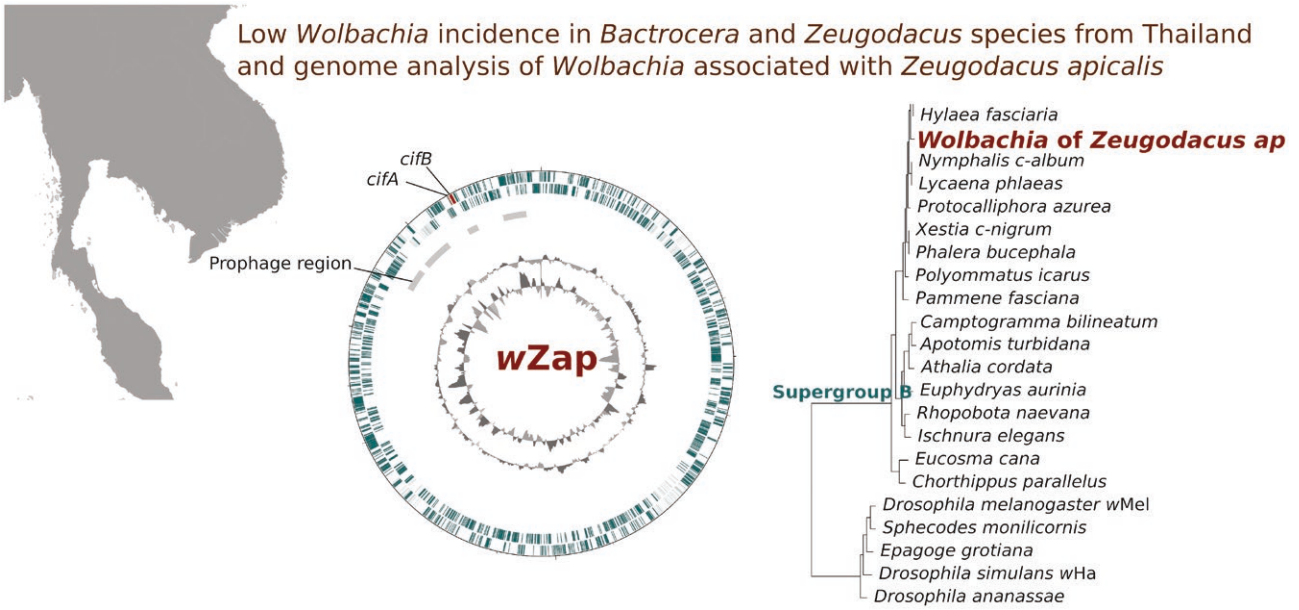
Wolbachia are bacterial endosymbionts found widely in arthropods and filarial nematodes. Infecting about half of all arthropod species, *Wolbachia* manipulate their hosts in various ways, including cytoplasmic incompatibility. Here, we investigated *Wolbachia* diversity in *Bactrocera* and *Zeugodacus*, two prevalent tephritid fruit fly genera, using molecular methods. *Wolbachia* was only detected in *Zeugodacus apicalis* (de Meijere) (Diptera: Tephritidae) and not in the other 7 studied species. This newly discovered strain, named *w*Zap, belongs to supergroup B with a 1.3 Mb genome containing 1,248 genes. Phylogenetic analysis of its cytoplasmic incompatibility factor genes *cifA* and *cifB* revealed their placement within the Type I clade. Given the presence of *cif* genes in the *w*Zap genome, further research into their roles in fruit flies could be crucial for developing pest control strategies that exploit CI mechanisms.

Keywords: Bactrocera, Diptera, endosymbionts, Tephritidae, *Zeugodacus*

Graphical Abstract



Introduction

Insects harbor numerous bacterial endosymbionts that can be beneficial (eg, by providing essential vitamins and nutrients) or parasitic. These endosymbionts are important in the reproduction and evolution of insects as they affect their hosts to ensure maximum transmission to the next generation (Feldhaar 2011, Perlmutter and Bordenstein 2020). Although they are typically passed from mother to offspring, horizontal transmission between different insect species has also been observed (Engelstädter and Hurst 2006, Stahlhut et al. 2010, Schuler et al. 2013, Wallau et al. 2016). Despite the vast diversity of insect endosymbionts, our understanding of them remains limited (Sepúlveda et al. 2017, Detcharoen et al. 2019, Kanakala and Ghanim 2019).

Wolbachia, Alphaproteobacteria of the order Rickettsiales, are the most prevalent endosymbionts worldwide, with approximately 100,000 strains expected to exist in nature (Detcharoen et al. 2019). *Wolbachia* can be classified into supergroups based on their molecular phylogeny, of which the ones that infect insects mainly belong to supergroups A and B (Lo et al. 2007). *Wolbachia* exhibit 4 major phenotypes in hosts, feminization, male killing, cytoplasmic incompatibility (CI), and parthenogenesis (Werren et al. 2008), all of which benefit *Wolbachia* as they increase the production of infected females. They can also influence the morphology and behavior of their hosts, such as wing and larval size (Dutra et al. 2016, Detcharoen et al. 2020). However, hosts can also benefit from *Wolbachia* infections (ie, the active invasion and replication of *Wolbachia* within host cells, signifying an established association), such as higher fecundity and increased mating rate (De Crespigny et al. 2006, Guo et al. 2020).

Tephritid fruit flies feed on hundreds of plant species, with some destroying a wide range of crops (Clarke et al. 2005, Vargas et al. 2015). Recent studies revealed variations in the prevalence of *Wolbachia* across different regions and species. For example, *Rhagoletis cerasi* (Linnaeus) showed variable detection rates (ie, identifying *Wolbachia* DNA through molecular methods, which does not necessarily imply active infection) with the *Wolbachia* strain *w*Cer2 (Schebeck et al. 2019), while in South America, all *Anastrepha fraterculus* Wiedemann flies were tested positive for *Wolbachia* (Conte et al. 2019). In contrast, only 3% of *Bactrocera dorsalis* (Hendel) from African countries tested positive (Gichuhi et al. 2019). The effects of *Wolbachia* in tephritid hosts are diverse, from CI, eg, *w*Cer2 in *R. cerasi* (Riegler and Stauffer 2002), *Ceratitis capitata* (Wiedemann) (Zabalou et al. 2004), and *Bactrocera oleae* (Rossi) (Apostolaki et al. 2011), to male-killing, eg, *w*AfraCast1_A in *A. fraterculus* (Conte et al. 2019).

Previous studies on *Wolbachia* genomes in tephritid flies revealed their evolutionary history with hosts, including *R. cerasi*, *Rhagoletis cingulata* (Loew), and *C. capitata* (Morrow et al. 2020, Morrow and Riegler 2021, Wolfe et al. 2021). Comparative genomic analyses discovered genetic differences among closely related strains, such as variations in CI factor (*cif*) gene copy number, genome synteny, and single nucleotide polymorphisms (Morrow et al. 2020, Morrow and Riegler 2021). In addition, genomic data proved to be more effective than traditional typing scheme in distinguishing between the closely related *Wolbachia* strains *w*Cer2 and *w*Cin2 strains (Wolfe et al. 2021).

Two closely related genera of tephritid flies, *Bactrocera* and *Zeugodacus*, are commonly found in Thailand (Boontop et al. 2017, Kunprom and Pramual 2018, Kitthawee and Julsirikul 2019). This study aimed to determine *Wolbachia* diversity among *Bactrocera* and *Zeugodacus* in Thailand. Through PCR and Nanopore sequencing, we discovered the presence of a new *Wolbachia* strain of supergroup B in *Zeugodacus apicalis* (de Meijere) (Diptera: Tephritidae), expanding our understanding of the evolutionary history and *Wolbachia* diversity among tephritid flies in this region.

Methods

Fly Collection, DNA Extraction, and *Wolbachia* Screening

Adult tephritid fruit flies were collected from fresh fruits and vegetables using traps placed in 13 orchards and vegetable plantations in Thailand between August 2021 and November 2023 (Table 1, Supplementary Fig. S1). The distance between each site

Table 1. Collection sites, land use, collection method, number of flies collected (*n*), and detection of *Wolbachia* of the tephritid flies

Species	Location	Coordinates	Date	Land use	Collection method	*n*	*Wolbachia*
B. albistrigata	Pasemat, Narathiwas	6°03′02.1″N, 101°59′07.2″E	8 August 2021	Jackfruit trees	Infested fruit	4	-
B. cucurbitae	Kho Hong, Songkhla	7°00′14.8″N, 100°30′14.2″E	13 November 2022	Vegetable farm	Field collection	8	-
B. dorsalis	Tha Sao, Kanchanaburi	14°17′36.8″N, 99°00′10.8″E	10 November 2023	Mixed agriculture fruit trees, mainly mangos and guava	Field collection	4	-
	Sahakon Nikhom, Kanchanaburi	14°40′55.0″N, 98°43′48.4″E	11 November 2023	Mango trees	Field collection	18	-
	Sukhirin, Narathiwas	5°56′31.5″N, 101°46′08.7″E	10 August 2021	Langsat orchard	Field collection	2	-
	Su-ngai Kolok, Narathiwas	6°03′31.3″N, 101°59′10.2″E	8 August 2021	Jackfruit trees and rubber plantations	Field collection	12	-
	Tanyongmat, Narathiwas	6°17′26.3″N, 101°42′54.6″E	8 August 2021	Langsat orchard	Infested fruit	6	-
	Kuanjong, Songkhla	6°58′29.7″N, 100°31′10.3″E	12 November 2021	Mixed agriculture fruit trees	Field collection	11	-
	Na Mom, Songkhla	6°59′20.3″N, 100°35′01.7″E	6 March 2022	Vegetable farm	Field collection	14	-
	Kho Hong, Songkhla	7°00′14.8″N, 100°30′14.2″E	13 November 2022	Vegetable farm	Field collection	26	-
	Thung Yai, Songkhla	7°01′19.5″N, 100°33′16.8″E	19 November 2022	Mixed agriculture fruit trees	Field collection	5	-
	Kamphaeng Phet, Songkhla	7°08′10.6″N, 100°15′36.7″E	20 November 2022	Guava orchard	Field collection	2	-
	Thalang, Phuket	7°58′04.4″N, 98°21′27.0″E	24 May 2023	Rose apple orchard	Field collection	3	-
B. latifrons	Su-ngai Kolok, Narathiwas	6°03′31.3″N, 101°59′10.2″E	8 August 2021	Mixed agriculture fruit trees, Langsats and jackfruits	Field collection	6	-
B. umbrosa	Na Mom, Songkhla	6°59′20.3″N, 100°35′01.7″E	6 March 2022	Vegetable farm	Field collection	67	-
	Kho Hong, Songkhla	7°00′14.8″N, 100°30′14.2″E	13 November 2022	Vegetable farm	Field collection	12	-
Z. apicalis	Hin Tung, Nakhon Nayok	14°19′14.5″N, 101°18′43.5″E	19 October 2023	Mango trees	Field collection	4	+ (4/4)
Z. diversus	Kuanjong, Songkhla	6°58′29.7″N, 100°31′10.3″E	12 November 2021	Mangosteen trees	Field collection	9	-
Z. tau	Kho Hong, Songkhla	7°00′14.8″N, 100°30′14.2″E	13 November 2022	Vegetable farm	Field collection	11	-

ranged from one to several hundred kilometers, covering various environments, including tropical fruit orchards, mixed agricultural areas, and vegetable farms. Occasionally, fruits and vegetables infested by larvae were collected and reared in the laboratory until they reached adulthood. All the flies were stored in 95% ethanol and kept at –20 °C. All samples were identified under a stereomicroscope using the keys of Drew and Romig (2016 2013) and Plant Health Australia (2018).

DNA from the whole fly was extracted individually from all collected flies using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Each sample was homogenized with a sterile plastic pestle and incubated at 56 °C for 16 h before proceeding with the rest of the extraction steps. The DNA was eluted in 50 µl of sterile water, and its quality and quantity were assessed using a spectrophotometer. Fly species were confirmed via PCR targeting the *COI* gene using the primers LCO1490 and HCO2198 (Folmer et al. 1994). The PCR conditions were as follows, 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 52 °C for 15 s, and 72 °C for 10 s, and the final extension at 72 °C for 5 min. All PCRs were done using AllTaq DNA polymerase (Qiagen, Hilden, Germany). The *COI* gene was successfully used to delimit the tephritid fly species, as reported previously (Kunprom and Pramual, 2017, 2019). To detect *Wolbachia*, we used wsp81F and wsp691R primers (Braig et al. 1998) targeting the *Wolbachia* surface protein gene. The PCR conditions were the same as for the *COI* gene, except for the annealing temperature of 55 °C. The *Wolbachia* strains were identified using multilocus sequence typing (MLST) loci, following the primers reported by Baldo et al. (2006). The annealing temperatures were 54 °C for *gatB*, *coxA*, *hcpA*, *ftsZ*, and 59 °C for *fbpA*. All PCR products were cleaned using ExoSAP-IT (Thermo Fisher Scientific, MA, USA) and sent for sequencing. The MLST sequences of each *Wolbachia* positive sample were compared to the PubMLST database (Jolley et al. 2018) to determine the sequence type. All data were deposited on NCBI under the project number PRJNA1053509.

Wolbachia Genome Sequencing, Assembly, and Annotation

To better understand the *Wolbachia* endosymbiont of *Z. apicalis*, we used Nanopore sequencing to study its genome. The genomic DNA of the 4 *Z. apicalis* samples was pooled. Approximately 1,000 ng of pooled DNA was used to construct a Nanopore sequencing library without a selective enrichment process. The DNA was repaired and end-prepped using the NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing Kit (E7180L, NEB, MA, USA), followed by purification with AMPure XP Beads (Beckman Coulter, IN, USA). Sequencing adaptors were ligated to the end-prepped DNA using the Ligation Sequencing Kit V14 (SQK-LSK114, Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's protocol. The library was purified using the Short Fragment Buffer and AMPure XP Beads provided with the kit and eluted in 15 µl of Elution Buffer. The library quantity was accessed using the Qubit dsDNA HS assay (Thermo Fisher Scientific, MA, USA). The library was sequenced on a Nanopore MinION device with R10.4.1 flow cells for 24 h.

Base calling was performed using Dorado v0.5.0 (Oxford Nanopore Technologies) with the dna_r10.4.1_e8.2_400bps_hac@v4.2.0 model. Reads were quality-checked using fastp v0.23.3 (Chen et al. 2018) with default parameters and then assembled using the metagenome option in Flye v2.9.1-b1780 (Kolmogorov et al. 2019). The circular *Wolbachia* genome was polished using Medaka v1.7.2 (Oxford Nanopore Technologies) with a consensus option. The start position was determined using Circlator v1.5.5 (Hunt et al. 2015). The original reads were mapped against the assembled genome using minimap2 v2.22-r1101 (Li 2018) to obtain sequencing coverage. The genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (NCBI PGAP) v2023-10-03.build7061 (Li et al. 2021). Genome completeness was checked using Benchmarking Universal Single-Copy Orthologs (BUSCO) v5.5.0 (Simão et al. 2015) with the Rickettsiales odb10 dataset. MLST profile was determined using PubMLST (Jolley et al. 2018) via MLST v2.22.0 (https://github.com/tseemann/mlst, last accessed December 2023). Prophage sequences were identified using PHASTER (Arndt et al. 2016). A circular genome plot was created using DNAPlotter v18.2.0 (Carver et al. 2009). Genome alignment was performed using Mauve v2.4.0 (Darling et al. 2004), and the results were visualized using the R package GenoPlotR v0.8.11 (Guy et al. 2011). We used the NCBI PGAP for genome annotation and MLST to identify *Wolbachia* strains in the genomes of *Wolbachia* from *Celastrina argiolus* (Linnaeus) (Lepidoptera: Lycaenidae) (GCF_947251805) and *Hylaea fasciaria* (Linnaeus) (Lepidoptera: Geometridae) (GCF_947251975), using the same parameters as for the *w*Zap genome.

Detection of Insect Parasitoids

A previous study reported that the detection of *Wolbachia* in some Australian tephritid species was actually due to the presence of the endoparasite *Dipterophagus daci* Drew & Allwood (Strepsiptera, Halictophagidae) (Towett-Kirui et al. 2021). To check for other insect parasites that are not visually detectable, we first checked the chromatograms of the *COI* amplicons amplified using the LCO1490/HCO2198 primers from the *Z. apicalis* samples for any suspicious peaks. Secondly, we mapped our Nanopore reads to the 2,608,366 *COI* sequences downloaded from the NCBI nucleotide database (accessed on 12 August 2024) using the search term 'Insecta[ORGN] AND COI[Gene]' with KMA v1.4.15 (Clausen et al. 2018) using the default parameters.

Phylogenetic Analysis

We used OrthoFinder v2.5.4 (Emms and Kelly 2019) to identify orthologous genes in 25 other *Wolbachia* genomes (Supplementary Table S1). Single-copy orthologous genes were aligned using MAFFT v7.520 (Katoh and Standley 2013) via OrthoFinder using the options -M msa -A mafft. The resulting alignment was used to construct a maximum likelihood phylogenetic tree using IQ-TREE v2.1.4 (Minh et al. 2020) using Q.bird + F + R4 substitution model based on the Bayesian information criterion (BIC) and accessing branch support with 1,000 ultrafast bootstraps and 1,000 Shimodaira–Hasegawa approximate likelihood-ratio tests (SH-aLRT). For the tree based on 5 MLST genes, *Wolbachia* genomes were aligned to each MLST gene using MegaBLAST, and the resulting sequences were further aligned with ClustalW in MEGA v11.0.13 (Kumar et al. 2018). The *Wolbachia w*Bm of the nematode *Brugia malayi* (AE017321.1) was used as an outgroup. The maximum likelihood phylogenetic tree was constructed using IQ-TREE with 1,000 bootstraps. Substitution models were selected based on BIC as follows, TPM3u + F + R2 for *coxA*, HKY + F + G4 for *fbpA*, TN + F + R2 for *ftsZ*, K3Pu + F + I + G4 for *gatB*, and K3Pu + F + I for *hcpA*. Additionally, the CI factor genes *cifA* and *cifB* were individually aligned against other sequences reported by Martinez et al. (2021) using TranslatorX (Abascal et al. 2010) with MAFFT (Katoh and Standley 2013) and GBlocks (Talavera and

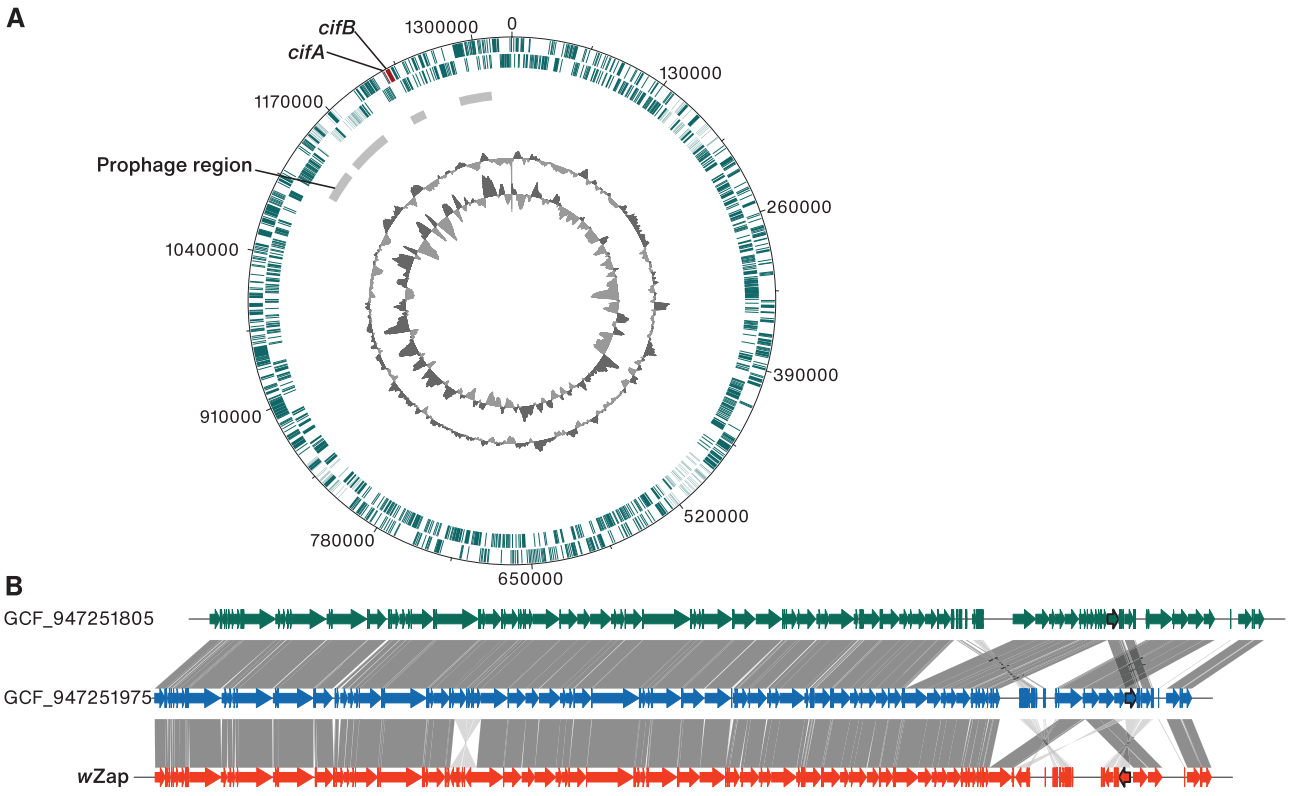


Fig. 1. Genome of *Wolbachia* of *Zeugodacus apicalis, w*Zap. A) Circular genome plot of *w*Zap. The outer circles represent the coding sequences on the forward (first circle) and reverse (second circle) strands, and the third and fourth circles show the GC skew and GC content, respectively. The gray bars indicate prophage regions. B) Genome alignment between *Wolbachia* of *Celastrina argiolus* (GCF_947251805, green), *Hylaea fasciaria* (GCF_947251975, blue), and *w*Zap (orange). The black arrows show regions containing the *cifA* and *cifB* genes.

Castresana 2007). Maximum likelihood trees were produced using IQ-TREE with 1,000 ultrafast bootstraps and 1,000 SH-aLRT with the TPM3u + F + I + G4 and TIM3 + F + R2 substitution models for the *cifA* and *cifB* trees, respectively. All trees were visualized using FigTree v1.4.4 (Rambaut 2014).

Results

Fly Collection and *Wolbachia* Detection

A total of 176 flies were collected from 13 locations, including 5 *Bactrocera* and 3 *Zeugodacus* species, namely *Bactrocera albistrigata* (de Meijere), *Bactrocera cucurbitae* (Coquillett), *B. dorsalis*, *Bactrocera latifrons* (Hendel), *Bactrocera umbrosa* (Fabricius), *Z. apicalis*, *Zeugodacus diversus* (Coquillett), and *Zeugodacus tau* (Walker) (Diptera: Tephritidae) (Table 1). The number of the collected flies varied across species and locations, with *B. dorsalis* and *B. umbrosa* being the most prevalent. Using wsp primers, *Wolbachia* sequences were detected in all *Z. apicalis* samples and none of the other species (Table 1). The MLST sequences revealed that all *Z. apicalis* samples belonged to sequence type 125, similar to those of *Wolbachia* endosymbionts of the lepidopterans *Hypolimnas bolina* (Linnaeus) (Lepidoptera: Nymphalidae) and *Talicada nyseus* (Geurin) (Lepidoptera: Lycaenidae).

Wolbachia Genome of *Z. apicalis*

A total of 1,143,971 reads were obtained from Nanopore MinION sequencing with a mean read length of 2,257 bp. After the quality check, 985,246 reads remained with a mean read length of 2,271 bp. There was no evidence of insect parasites in the *COI* chromatogram, and the mapping of the Nanopore reads to the *COI* database also showed no signs of insect parasites. Most reads were correctly assigned to *Z. apicalis* (Supplementary Table S1).

The final assembly revealed a 1.3-Mbp circular genome with 99.7% BUSCO completeness (Fig. 1A, Supplementary Table S2). We named this strain of *Wolbachia w*Zap. The genome contains 1,149 protein-coding genes, 58 pseudogenes, one intact prophage region (43.3 kb), and 3 incomplete prophage regions (Supplementary Table S3). In the *w*Zap genome, the lengths of *cifA* and *cifB* were 1,476 and 3,525 bp, respectively, and they are coded on the same strand with *cifA* upstream of *cifB*. Phylogenetic analyses revealed that both *cif* genes of *w*Zap belong to Type I (Fig. 2).

Further analyses of *Wolbachia w*Zap and its relationship with other genomes confirmed its classification within supergroup B. An analysis using MLST genes identified *Wolbachia* from the bird blowfly, *Protocalliphora azurea* (Fallén), as its closest related species (Supplementary Fig. S2). Additionally, orthologous gene analysis of *w*Zap with 25 other *Wolbachia* genomes from supergroups A, B, and D classified 30,696 genes into 1,400 orthogroups (Supplementary Table S4). Of these, 650 orthogroups contained genes from all the genomes. Phylogenomic analysis using 615 single-copy orthologous genes shared with the other 25 genomes placed *Wolbachia w*Zap in supergroup B, in the same clade as *Wolbachia* of *H. fasciaria* (GCF_947251975) and *C. argiolus* (GCF_947251805) (Fig. 3). Comparative analysis between these genomes revealed similarities in gene number, GC content, and genome size (Supplementary Table S5), with some differences in regions predominantly containing prophage genes, which correspond to the opposite patterns observed in the other two genomes (Fig. 1B).

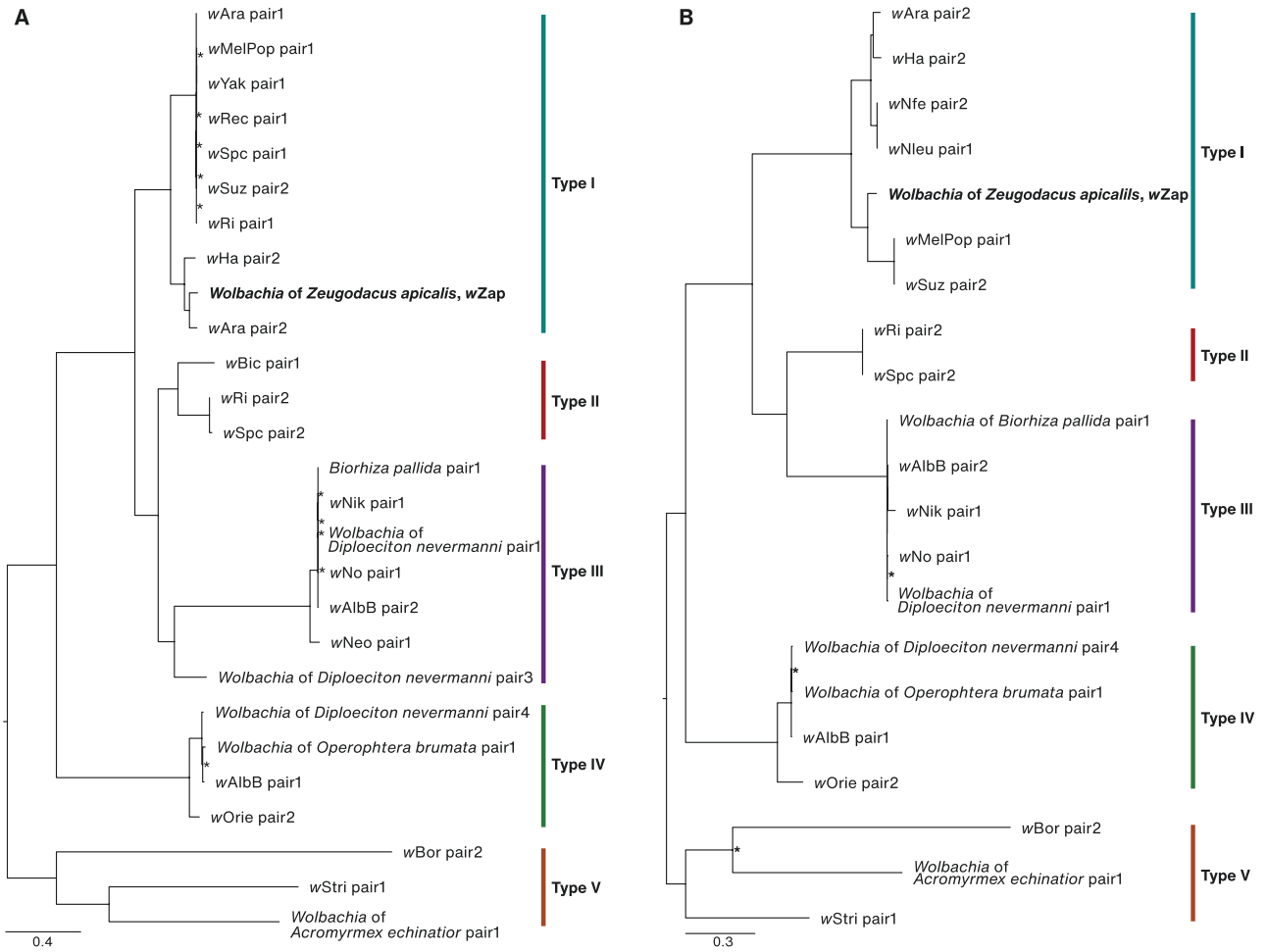


Fig. 2. Rooted maximum likelihood phylogenetic trees of the *cifA* (A) and *cifB* genes (B). *Wolbachia* *w*Zap is in bold. An asterisk shows a node with less than 80% support of SH-aLRT and ultrafast bootstraps.

Discussion

Diversity of Tephritid Fruit Flies and their Endosymbionts

In this study, 5 *Bactrocera* and 3 *Zeugodacus* species were collected from 13 locations, with *B. dorsalis* as the most prevalent. Found in several areas, this species feeds on various host plants (Kunprom and Pramual 2019). Interestingly, despite previous reports of co-existence in Thailand's orchards (Danjuma et al. 2013), neither *Bactrocera zonata* (Saunders) nor *Bactrocera correcta* (Bezzi) (Diptera: Tephritidae) was observed in our study, likely due to their low abundances. The second most collected species was *B. umbrosa*, exclusively found in vegetable crops. This species is prevalent in the Thai–Malay Peninsula and is known to feed on various plants (Clarke et al. 2001, Danjuma et al. 2013). Given our aim to collect as many flies as possible to check for *Wolbachia*, the number of flies we were able to collect depended heavily on the specific conditions of each collection site. For instance, we collected a relatively high number of *B. umbrosa* from a vegetable farm in Na Mom, Songkhla, primarily because it is a large vegetable farm, providing an ideal habitat for this species. In addition, *B. dorsalis* was the most dominant species in Kho Hong, whereas *B. cucurbitae* was the least dominant. The uneven distribution of species across different sites posed a challenge in achieving a large sample size for each species, likely due to their low abundance and a sampling bias in our collection effort. To address this limitation, future studies will aim to expand the sample size and geographic scope to provide a more comprehensive understanding of *Wolbachia* prevalence and diversity in *Z. apicalis*. Such efforts will help mitigate sampling bias and enhance the robustness of our findings regarding the distribution and ecological impacts of *Wolbachia* in tephritid flies.

We observed a low frequency of *Wolbachia* in the tephritid flies, as only the nonpest *Z. apicalis* showed *Wolbachia* detection. This report is the first to detect *Wolbachia* in this species. Interestingly, *Z. apicalis* was collected near an orchard, unlike the other species found directly within fruits and vegetables. Despite being the most common species, *B. dorsalis* showed no evidence of *Wolbachia* across 11 populations. Similar to other reports from India, Bangladesh, and Thailand (Kittayapong et al. 2000b, Asimakis et al. 2019), our findings revealed no *Wolbachia* found in *B. umbrosa* and *Z. tau*. Moreover, while *Wolbachia* supergroup B has been documented in 2 adult *B. latifrons* in Malaysia (Yong et al. 2017), it was not observed in this study.

This study found no evidence of insect parasitoids in the *COI* sequences and through mapping Nanopore reads to the *COI* database, suggesting the absence of such parasites in the *Z. apicalis*. However, considering previous findings of *Wolbachia* in tephritids and some parasitoids, such as *Fopius arisanus* (Sonan) (Hymenoptera,

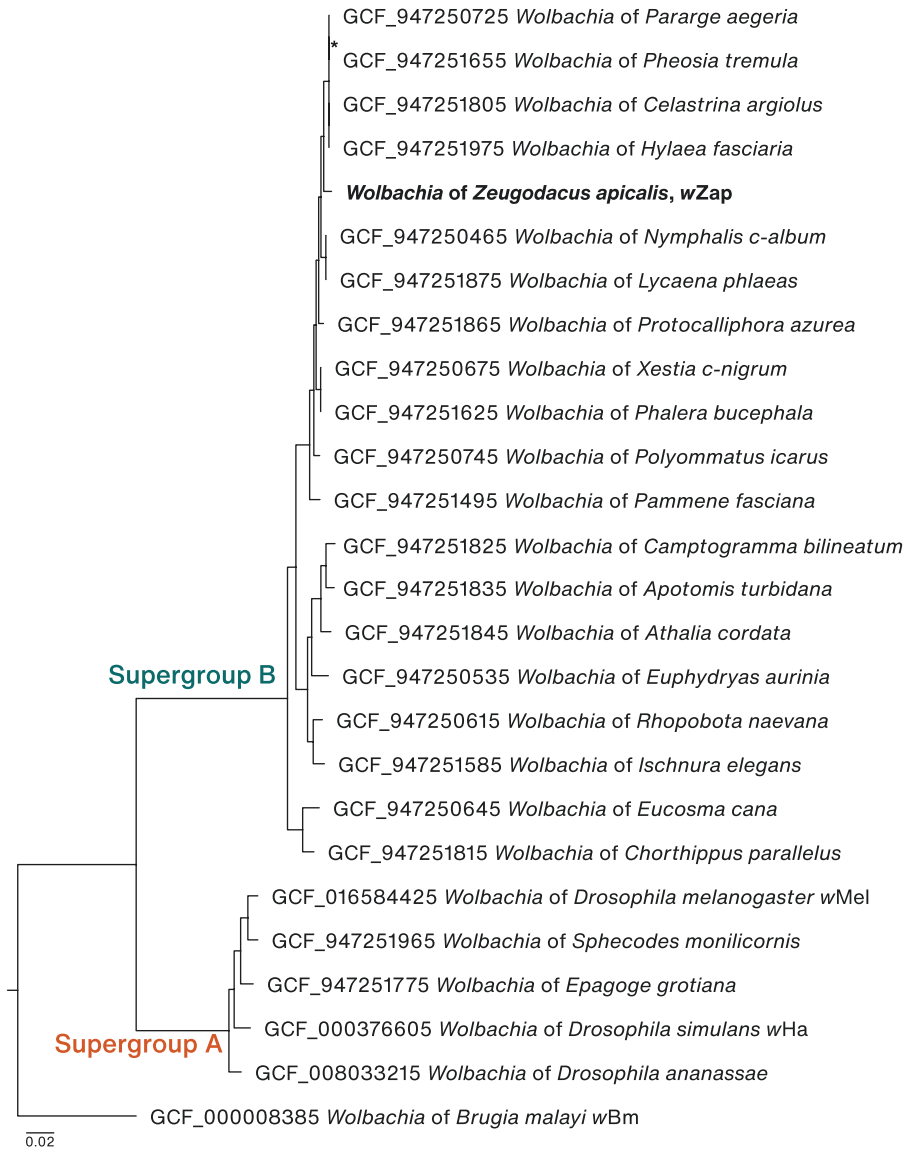


Fig. 3. Rooted phylogenetic tree based on single-copy orthologous genes among *Wolbachia* genomes ($n = 26$). An asterisk shows a node with less than 80% support of SH-aLRT and ultrafast bootstraps.

Braconidae) and *D. daci* (Morrow et al. 2014 2015, Mohammed et al. 2017, Towett-Kirui et al. 2021), we cannot completely exclude their presence. It is possible that endoparasites were present at low titers, which was undetectable due to the limited sequencing depth. Additionally, Morrow et al. (2015) reported pseudogenes in the genomes of *Bactrocera peninsularis* (Drew & Hancock) and *Bactrocera perkinsi* (Drew & Hancock), whereas low *Wolbachia* prevalence in other species was later found due to the infections by endoparasites (Towett-Kirui et al. 2021).

The prevalence of *Wolbachia* among tephritids largely depends on the fly population and geographical location. Previous reports found that wild individuals of *B. dorsalis* in Thailand and China harbored *Wolbachia* but at a very low prevalence (2 out of 222 larvae and 19 out of 1,500 individuals, respectively) (Kittayapong et al. 2000b, Sun et al. 2007). However, not all tephritid fly populations collected in Thailand tested positive for *Wolbachia* (Kittayapong et al. 2000b). In contrast, no *Wolbachia* were detected in laboratory populations of *B. dorsalis* collected in Thailand (Augustinos et al. 2015). Notably, 53% of *B. dorsalis* collected from 2 populations in India harbored *Wolbachia*, whereas none of the 4 populations from Bangladesh showed any presence of the bacteria (Asimakis et al. 2019). Similarly, a study on *B. dorsalis* in Africa reported a low detection of natural *Wolbachia* (Gichuhi et al. 2019). Additionally, variable and often low infection rates of *Wolbachia* have been documented among mosquito populations (Kittayapong et al. 2000a). This low prevalence of *Wolbachia* is similar to that observed among arthropods around this study area, including *Drosophila* species (Detcharoen and Nilsai 2023).

Several factors influence *Wolbachia* infection in insects, including host specificity, temperature, and genetic background. Some *Wolbachia* strains can be host-specific and co-evolve with certain species, limiting their ability to infect a wide range of hosts. For example, *Drosophila nigrosparsa* transinfected with the *w*Mel strain from *Drosophila melanogaster* can survive, whereas those with the *w*MelCS and *w*MelPop strains did not (Detcharoen et al. 2020). This indicates that certain *Wolbachia* strains may be lethal to specific

host species, thereby removing them from the population. Cool and warm temperatures also influence *Wolbachia* prevalence and their hosts' life-history traits (Hague et al. 2020, Lau et al. 2020, Chrostek et al. 2021). In addition, the host's genetic background is essential in determining compatibility with different *Wolbachia* strains, leading to variability in *Wolbachia* density and distribution within host populations (Dean 2006, Capobianco et al. 2018).

The Genome of *Wolbachia* from *Z. apicalis*

The 1.3 Mb-long *w*Zap genome has features such as GC content and prophage regions similar to those of other genomes within *Wolbachia* supergroup B (Vancaester and Blaxter 2023). Phylogenomic analysis placed *w*Zap at the base of a clade comprising several *Wolbachia* strains in lepidopterans belonging to supergroup B, such as *C. argiolus* and *H. fasciaria*. Other members of this clade also include *Wolbachia* from some mosquitoes such as *Culex quinquefasciatus* Say and *Culex molestus* Forskal (Diptera: Culicidae) (Vancaester and Blaxter 2023). Furthermore, MLST analysis revealed the same sequence type as observed in other lepidopterans, *H. bolina* and *T. nyseus*. The similarity of *w*Zap genome to other *Wolbachia* genomes within supergroup B genomes suggests horizontal transmission from lepidopterans to *Z. apicalis* via host plants, as reported previously in other *Wolbachia* strains (Sintupachee et al. 2006, Li et al. 2017).

Wolbachia are usually infected by a bacteriophage known as phage WO, which plays a significant role in their genome evolution and host interactions (Bordenstein and Bordenstein 2022). Prophage fragments are prevalent in *Wolbachia* genomes within arthropod hosts and correlate with genome size (Gavotte et al. 2007, Bordenstein and Bordenstein 2022, Vancaester and Blaxter 2023). Usually, at least 1 intact prophage region is found in the genome (Kent et al. 2011). In our study, *w*Zap harbored 3 prophage regions, 1 of which was intact, similar to those of *Wolbachia* in *C. argiolus* and *H. fasciaria*, but these regions were arranged differently. Such differences in gene arrangement may indicate unique evolutionary events like recombination or horizontal gene transfer (Wang et al. 2020). Prophage regions vary greatly among *Wolbachia* genomes of arthropod hosts, as some regions might be fully intact, whereas others are degraded with pseudogenized genes (Bordenstein and Bordenstein 2022). The presence of an intact prophage in *w*Zap is noteworthy, as intact prophages can carry genes involved in host manipulation (Lindsey et al. 2018).

The *cifA* and *cifB* genes, typically located in a prophage region, are important for inducing CI in arthropods (Beckmann et al. 2017, LePage et al. 2017). During spermatogenesis, the ribonuclease CifA depletes long noncoding RNA needed for the histone-to-protamine transition, while both CifA and CifB act as deoxyribonucleases, inducing DNA damage in the late spermatogenesis. These modifications result in defective sperms, leading to CI. Rescue occurs when females harbor the same *Wolbachia* strain, with CifA expressed in the ovary counteracting the sperm modifications caused by both Cif proteins, preventing embryonic lethality (Beckmann et al. 2019, Shropshire and Bordenstein 2019, Kaur et al. 2024).

Potential Applications of *Wolbachia* and Incompatible Insect Techniques

Wolbachia-mediated CI and the Incompatible Insect Technique (IIT) have several potential applications. In our study, *cifA* was located upstream of *cifB*, similar to most *Wolbachia* genomes (LePage et al. 2017, Martinez et al. 2021). Both *cif* genes belong to Type I, the most common variant and are experimentally linked to the induction and rescue of CI phenotypes in several *Wolbachia* strains (LePage et al. 2017, Martinez et al. 2021). The presence of these genes in *w*Zap indicates potential for CI, although functional CI and its direct effects were not assessed in this study. This finding is particularly promising, as CI forms the basis of IIT, which could serve as a tool in the biological control of pest tephritid species.

The successful application of *Wolbachia*-induced CI for pest suppression has been demonstrated in various insect systems. In *Aedes* mosquitoes, mass-reared males infected with *Wolbachia* strains that induce strong CI, such as *w*AlbB, have been released into the field to suppress populations by preventing the production of viable offspring (Crawford et al. 2020, Beebe et al. 2021). Similarly, IIT has been explored in agricultural pests such as the spotted wing drosophila, *Drosophila suzukii*, a pest of soft-skinned fruits that causes significant crop losses. Recent studies have shown that integrating Sterile Insect Technique (SIT) with IIT can maintain reproductive competitiveness while still inducing sterility under variable field conditions (Nikolouli et al. 2018, 2020). Moreover, mass-rearing protocols for *D. suzukii* have been developed to support large-scale releases, making it a strong candidate for *Wolbachia*-based population suppression (Krüger et al. 2021). In addition, IIT has also been tested in other dipteran pests, including *C. capitata* and *B. oleae*. In *C. capitata*, *Wolbachia* has been successfully integrated into genetic sexing strains without affecting mating compatibility, whereas in *B. oleae*, the infection with the *Wolbachia* strain *w*Cer2 has been shown to reduce male performance (Zabalou et al. 2009, Kyritsis et al. 2022).

Several factors must be considered when applying IIT to tephritid fruit flies. First, *Wolbachia* strain must be maintained at high frequencies in mass-reared colonies (Mateos et al. 2020). Second, effective sex separation is important to prevent the accidental release of females that could counteract suppression efforts (Lombardi et al. 2024). Advanced sex-sorting techniques already used in SIT programs for tephritids can be adapted for IIT (Zabalou et al. 2009, Mateos et al. 2020, Gong et al. 2024). Third, fitness costs associated with *Wolbachia* infection, such as effects on mating competitiveness, fecundity, or longevity, must be thoroughly evaluated (Kyritsis et al. 2019, Suárez et al. 2019). Studies have shown that proper management of *Wolbachia* infections results in acceptable fitness outcomes (Ross et al. 2017, Maciel-de-Freitas et al. 2024). For example, research on *B. oleae* genetic sexing strains has shown that *Wolbachia* do not affect mating compatibility or performance (Zabalou et al. 2009), but in *C. capitata*, the infection has been shown to reduce male sexual signaling, especially in protein-fed and younger males (Kyritsis et al. 2022).

Moreover, environmental factors are also important in determining the effectiveness of IIT. Temperature has been shown to influence *Wolbachia* replication and CI expression (Bordenstein and Bordenstein 2011, Ulrich et al. 2016, Hague et al. 2020) that could impact suppression efforts in field conditions. For example, in *D. suzukii* optimal mating and survival were observed when both fertile and sterile flies were maintained at around 25 °C or at high relative humidity (81% to 100%). In contrast, temperatures of 10 or 35 °C and humidity levels below 60% significantly weaken mating (Krüger et al. 2021). Furthermore, exposing pupae to chilling temperature with hypoxia during transportation can preserve flight ability and overall performance in adult *D. suzukii* (Enriquez et al. 2021).

Establishing stable laboratory colonies of tephritid flies is essential to observe the effects of *Wolbachia*, including CI crosses and life-history traits, in these species. Therefore, future research involving experimental CI crosses and detailed analysis of life-history traits is necessary to confirm the functional roles of the *cif* genes and evaluate their practical use in biocontrol strategies. Despite these limitations,

*w*Zap represents a promising candidate for further exploration, provided its ability to induce strong CI is established.

In conclusion, we report a low incidence of *Wolbachia* detection in *Bactrocera* and *Zeugodacus* species. Of the 8 species collected, only *Z. apicalis* tested positive for *Wolbachia*. The 1.3 Mb-long *w*Zap genome contains 1,248 genes belonging to supergroup B. The presence of *cifA* and *cifB* in the genome suggests that this strain could induce CI. Further experiments focusing on the role of *cif* genes in inducing incompatibility in tephritid flies could aid in understanding the potential for exploiting these genes in biocontrol strategies.

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Supplementary material

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Author contributions

Matsapume Detcharoen (Conceptualization [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Areeruk Nilsai (Investigation [equal], Resources [equal], Writing—review & editing [equal]), Narit Thaochan (Conceptualization [equal], Methodology [Equal], Resources [equal], Writing—review & editing [equal]), and Cholakan Nuansuwon (Resources [equal], Writing—review & editing [equal])

Ethics statement

Fly collection and rearing were approved by the Institutional Animal Care and Use Committee of Prince of Songkla University (Protocol Code 2564-01-029).

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Data availability

The sequences were deposited on NCBI under project number PRJNA1053509.

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