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Low Endosymbiont Incidence in *Drosophila* Species Across Peninsula Thailand

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Abstract

Arthropods are known to harbor several endosymbionts, such as *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*. *Wolbachia*, for example, are the most widespread known endosymbionts in the world, which are found in about half of all arthropod species. To increase their transmission, these endosymbionts must manipulate their hosts in several ways such as cytoplasmic incompatibility and male killing. In tropical regions, endosymbiont diversity has not been studied exhaustively. Here, we checked four endosymbionts, including *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*, in eleven *Drosophila* species found in Thai Peninsula. The *Wolbachia* strain *w*Ri-like was found in all populations of *Drosophila ananassae* and *Drosophila simulans*. Furthermore, we found two new strains, *w*MalA and *w*MalB, in two populations of *Drosophila malerkotliana*. Besides *Wolbachia*, we did not find any of the above endosymbionts in all fly species. This work reveals the hidden diversity of endosymbionts in *Drosophila* and is the first exhaustive study on *Drosophila* in the region.

Keywords Arthropod · *Cardinium* · *Rickettsia* · *Spiroplasma* · *Wolbachia*

Introduction

Insects harbor several symbiotic bacterial species inside their cells. These endosymbionts manipulate their hosts to obtain maximal transmission to the next generation via various mechanisms, such as cytoplasmic incompatibility (i.e., death of embryos in crosses between infected males with uninfected females), male killing (i.e., death of infected male embryos), and thelytokous parthenogenesis (i.e., infected females can produce offspring without mating with males) [1, 2]. Many endosymbionts are parasites or mutualists, while some of them are obligate mutualists [3]. Bacterial endosymbionts are transmitted vertically, but horizontal transmission between different insect species can also be observed [4–6]. Common bacterial endosymbionts in insects are *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia* [3].

Bacterial endosymbionts are diverse, and their diversity is poorly understood [7, 8]. *Wolbachia*, for example, are the most widespread known endosymbionts in the world but just less than 1% of their diversity is currently known [7]. It is expected that around 40–60% of all arthropod species are infected by *Wolbachia*, 24% by *Rickettsia*, and 13% by *Cardinium* [9, 10].There are more than 2000 *Drosophila* species worldwide [11], and hundreds of species are found in tropical parts of Thai-Malay Peninsula (https://www.taxodros.uzh.ch, retrieved on September 15, 2020). Yet, although the fruit fly is found in every part of Thailand, research on *Drosophila* is lacking, both traditional and molecular systematics.

Despite their extreme diversity in terms of both described species and symbiotic associations, the evolution and diversity of endosymbionts in *Drosophila* are still poorly understood. Knowing endosymbiont diversity can help us understand evolution of both hosts and endosymbionts because their interactions are one of the factors influencing host biology and speciation [12, 13]. Here, we aimed to fill this knowledge gap by investigating and characterizing endosymbionts, *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*, in *Drosophila* species found in Peninsula Thailand. We used *COI* barcoding to identify fly species. Furthermore, we used endosymbiont specific primers to understand the relationship between *Drosophila* and their endosymbionts. We found eleven fly species. *Wolbachia* infections were found in every fly checked in all populations of *Drosophila ananassae*. In addition, two new *Wolbachia* strains of supergroups A and B were found in *Drosophila malerkotliana*.

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Methods

Drosophila species were caught using fruit bait in various habitats in the Thai Peninsula, ranging from dry evergreen forests (TK, SL, and SK), orchards (PT, SR, and SN), peat swamp (NK), tropical evergreen rainforests (NS, NN, and PA), and urban areas (SC, SH, and PM) (Fig. 1, Table 1). The samples were collected during the rainy season in 2020. Flies were stored immediately in 96% ethanol and kept in a cool place until arriving at the laboratory. We identified the flies based on morphology according to Mather [14], Bock [15], and Hihara & Lin [16]. Spermatheca, male and female genitalia were dissected using 10% potassium hydroxide, stored in glycerol, and photographed (Leica Microsystems, Switzerland). Additionally, molecular barcoding was used to confirm the fly species. Briefly, the DNA of an individual female fly was extracted using NucleoSpin tissue mini kit (Macherey–Nagel, Germany) and we amplified cytochrome c oxidase subunit I (*COI*) gene using LCO and HCO primers [17].

Different gene fragments of endosymbionts were amplified using PCR. We checked every sample for *Cardinium* 16S gene using primers CLO-f1 and CLO-r1 [18] and Ch-F and Ch-R [19]; *Spiroplasma dnaA* and *p18* genes using ApDnaAF1 and ApDnaAR1 [20] and p18-f and p18-r [21], respectively; *Rickettsia* 17-kDa antigen gene using R1 and R2 [22]; and *Wolbachia wsp* gene using wsp81F and wsp691R [23]. For samples with *Wolbachia* infection, five additional genes (*coxA*, *fbpA*, *ftsZ*, *gatB*, and *hcpA*) of the multilocus sequence typing (MLST) were used for *Wolbachia* strain identification [24]. The PCR products were sequenced using dideoxy method. The MLST sequences were checked against the *Wolbachia* MLST database on PubMLST [25]. The DNA sequences were deposited on GenBank under accession numbers MZ520835-MZ520856 for *COI* gene and MZ566522-MZ566563 for *Wolbachia* MLST and *wsp* genes.

For phylogenetic analysis, we added *COI* sequences from GenBank (JQ679118, KP863293, EU493590, EU493585, KX052956, KX052951, KX052947, EU493593, MN448089, KX052975, and EU493584) and MLST and *wsp* sequences from PubMLST [25]. The alignments of both *COI* gene and concatenated MLST and *wsp* genes were conducted using Clustal Omega version 1.1.0 [26] implemented in Seaview version 5.0.4 [27]. Evolutionary models were calculated using jModelTest version 2.1.10 [28]. For phylogenetic tree construction of *Drosophila COI* gene, GTR + F + I + G4 model was used, and *Hirtodrosophila duncani* was selected as an outgroup. For concatenated *Wolbachia* MLST and *wsp* genes, we used TPM2u + F + G4, TPM3 + F + G4, TIM + F + I, TIM + F + G4, TN + F + G4, and TIM2 + F + G4 models for *coxA*, *fbpA*, *ftsZ*, *gatB*, *hcpA*, and *wsp* genes, respectively. *Wolbachia* strain *w*Bm was chosen as an outgroup. The maximum likelihood phylogenetic trees were constructed using IQ-TREE version 2.1.3 [29] with 10,000 ultrafast bootstraps.

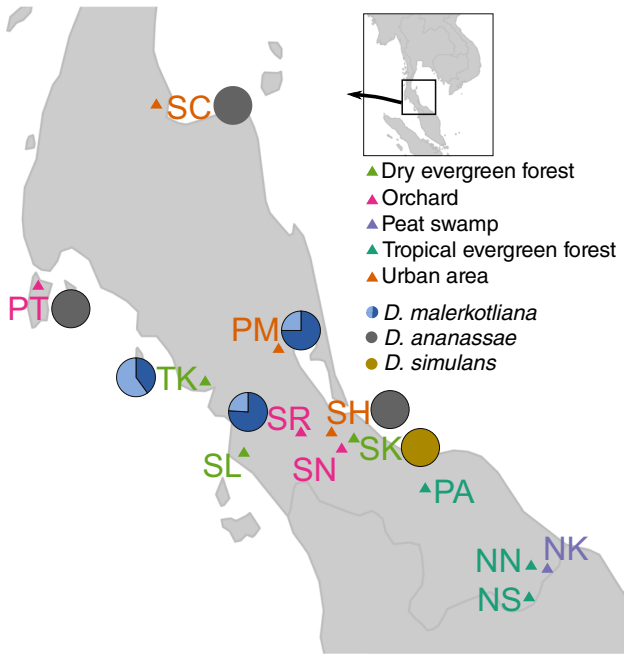

Fig. 1 Thirteen sampling sites in various habitats across Peninsula Thailand. Pie charts shows percent of positive samples in three species infected with *Wolbachia*. *Drosophila ananassae* (gray pies) and *Drosophila simulans* (yellow) were 100% infected. *Wolbachia* infections in *Drosophila malerkotliana* (dark blue) were between 40 and 76%

Results

We decided to use molecular barcoding of *Drosophila* collected in this work because many of them had similar morphology especially genitalia of those within the same group, for example, between *Drosophila neohypocausta* and *Drosophila nasuta* (Fig. S1d, f). With species identification using a fragment of the *COI* gene, we identified eleven *Drosophila* species from thirteen locations across Peninsula Thailand. Among all the habitats, the highest fly diversity was in the dry evergreen forests (Table 1).

We found *Drosophila ananassae* most frequently, followed by *D. malerkotliana* (262 and 180 flies, respectively). Apart from the two most collected species, there were 97 *Drosophila neohypocausta*, 45 *Drosophila rubida*, 23 *Drosophila nasuta*, 17 *Drosophila eugracilis*, 14 *Drosophila pseudoananassae*, 14 *Drosophila hypocausta*, 12 *Drosophila simulans*, 10 *Drosophila mimetica*, and 6 *Drosophila albomicans* (Table 1).

Table 1 Prevalence of *Wolbachia* in female *Drosophila* species collected from thirteen locations, number of flies collected, number of positive/number of samples checked, percent of infection, and *Wolbachia* strains found. Asterisks indicate the new *Wolbachia* strains

Habitat	Site	Code	Species	Flies collected	Positive/checked (%)	*Wolbachia* strain
Dry evergreen forest	TK, Trang, Kantang (7°24′14.1″N 99°31′14.0″E, 72 m.a.s.l.)	TK01	*D. malerkotliana*	12	2/5 (40)	*w*MalA*
	SL, Satun, La-ngu (6°56′19.1″N 99°48′57.0″E, 21 m.a.s.l.)	SL01	*D. albomicans*	6	0/3 (0)	-
	SK, Songkhla, Kho Hong (7°00′23.1″N 100°30′38.1″E, 97 m.a.s.l.)	SK01	*D. malerkotliana*	77	0/44 (0)	-
		SK02	*D. rubida*	22	0/13 (0)	-
		SK03	*D. eugracilis*	17	0/7 (0)	-
		SK04	*D. neohypocausta*	66	0/48 (0)	-
		SK05	*D. hypocausta*	9	0/4 (0)	-
		SK06	*D. simulans*	12	5/5 (100)	*w*Ri-like
Orchards	PT, Phuket, Thalang (8°04′28.9″N 98°20′22.7″E, 37 m.a.s.l.)	PT01	*D. ananassae*	25	16/16 (100)	*w*Ri-like
	SR, Songkhla, Rattaphum (7°02′25.2″N 100°12′59.2″E, 82 m.a.s.l.)	SR01	*D. malerkotliana*	35	19/25 (76)	*w*Ri-like
	SN, Songkhla, Namom (6°58′06.3″N 100°35′10.2″E, 46 m.a.s.l.)	SN01	*D. rubida*	8	0/6 (0)	-
		SN02	*D. hypocausta*	5	0/3 (0)	-
Peat swamp	NK, Narathiwat, Su-ngai Kolok (6°04′05.5″N 101°58′06.9″E, 12 m.a.s.l.)	NK01	*D. neohypocausta*	8	0/5 (0)	-
Tropical evergreen forest	NS, Narathiwat, Sukhirin (5°49′51.2″N 101°50′21.5″E, 103 m.a.s.l.)	NS01	*D. malerkotliana*	40	0/27 (0)	-
		NS02	*D. pseudoananassae*	14	0/3 (0)	-
		NS03	*D. neohypocausta*	9	0/5 (0)	-
	NN, Narathiwat, Su-ngai Kolok (6°06′08.3″N 101°50′53.8″E, 40 m.a.s.l.)	NN01	*D. nasuta*	10	0/6 (0)	-
		NN02	*D. mimetica*	10	0/7 (0)	-
	PA, Pattani, Khok Pho (6°39′26.1″N 101°05′57.6″E, 275 m.a.s.l.)	PA01	*D. albomicans*	15	0/8 (0)	-
		PA02	*D. rubida*	13	0/7 (0)	-
Urban areas	SC, Surat-thani, Chaiya (9°22′03.7″N 99°11′22.2″E, 9 m.a.s.l.)	SC01	*D. ananassae*	214	25/25 (100)	*w*Ri-like
	SH, Songkhla, Hat Yai (7°00′18.8″N 100°28′16.9″E, 28 m.a.s.l.)	SH01	*D. ananassae*	23	8/8 (100)	*w*Ri-like
	PM, Patthalung, Mueang (7°37′01.1″N 100°05′04.0″E, 11 m.a.s.l.)	PM01	*D. neohypocausta*	14	0/6 (0)	-
		PM02	*D. malerkotliana*	16	6/8 (75)	*w*MalB*

The flies were classified into several groups, namely, *D. malerkotliana* and *D. pseudoananassae* of *Drosophila bipectinata* species complex; *D. hypocausta* and *D. rubida* within *D. hypocausta* subgroup; and *D. albomicans*, *D. nasuta*, and *D. neohypocausta* within *D. nasuta* subgroup (Fig. 2a).

The tested *Drosophila* samples were not infected by *Cardinium*, *Rickettsia*, and *Spiroplasma* (Fig. S2). *Wolbachia* were found in three fly species, *D. simulans*, *D. ananassae*, and *D. malerkotliana*, collected from seven locations (TK, SK, PT, SR, SC, SH, and PM) (Table 1). We found 100% *Wolbachia* infection in *D. ananassae* collected from orchards (PT) and urban areas (SC and SH), and *D. simulans* collected from dry evergreen forest (SK). For *D. malerkotliana*, the *Wolbachia* infection ranged from 0–76%. No *Wolbachia* were detected in the samples collected from

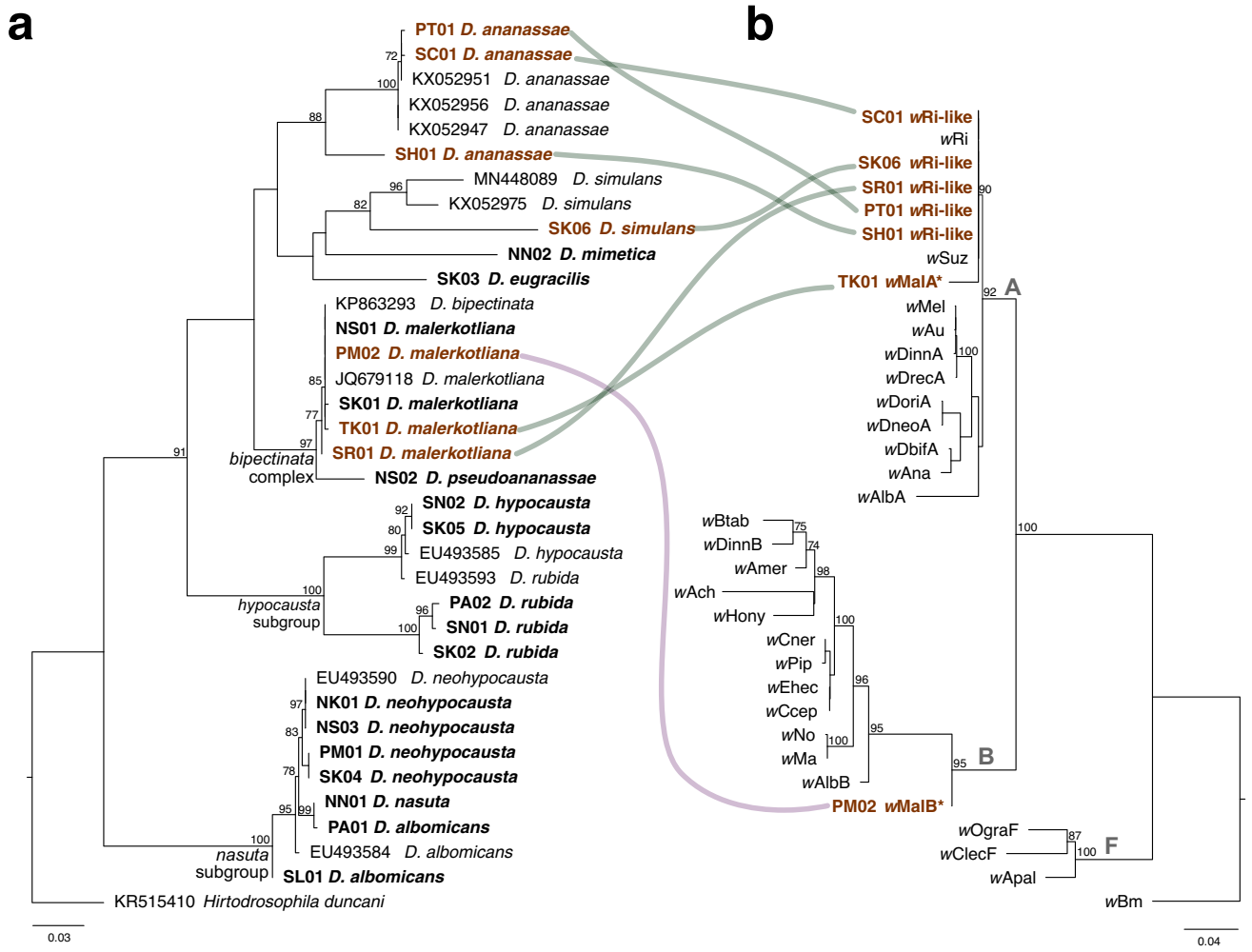

Fig. 2 Rooted maximum likelihood phylogenetic trees of *Drosophila* species collected across Peninsula Thailand based on cytochrome c oxidase subunit I gene (**a**) and *Wolbachia* strains based on five concatenated multilocus sequence typing (MLST) and *wsp* genes (**b**). Bootstrap values more than 70% are shown. *Wolbachia* supergroups are indicated with capital letters. Samples from this study are in bold. Brown color indicates samples with *Wolbachia* infection. *Drosophila* samples and their *Wolbachia* are linked with lines

peat swamp (NK01) and tropical evergreen forests (SL01, NS01-03, NN01-02, and PA01-02).

For *Wolbachia* strain characterization using concatenated MLST and *wsp* genes, it was found that *D. ananassae* and *D. simulans* samples from every location were infected with *Wolbachia* strain *w*Ri-like (supergroup A). *Drosophila malerkotliana* collected from site SR01 were infected by *w*Ri-like, but the same species collected from the other two locations, TK01 and PM02, were infected with two new different *Wolbachia* strains, *w*MalA and *w*MalB, belonging to supergroups A and B, respectively (Fig. 2b). In detail, there were 48 position differences at the *coxA* locus between *w*MalA and *w*Ri-like strains. The *w*MalB, however, is highly divergent from other supergroup B strains (Fig. 2b). Based on the *wsp* sequences, *w*MalA and *w*MalB were identical to those found in *Psyttalia incise* and *Fopius vandenboschi* (Hemiptera: Braconidae), parasitoids of the tephritid fruit flies.

Discussion

This study is the most exhaustive survey on endosymbionts of wild-caught *Drosophila* species in the tropical Peninsula of Thailand. Eleven *Drosophila* species were found. This diversity is higher than ever reported in the TaxoDros database, which records the distributions of *Drosophila* in Thailand from 1958 (https://www.taxodros.uzh.ch/, retrieved on September 15, 2020). Of the four tested endosymbionts, we only found *Wolbachia* infections in three species. The low *Wolbachia* prevalence we found conformed to other *Drosophila* studies in other regions [30, 31].

We depended on molecular barcoding to identify the flies because of morphological similarities among species (e.g., between *D. neohypocausta* and *D. nasuta*) and because there were taxonomic conflicts regarding the description species using just morphology, such as between *D. rubida* and *D. hypocausta* [16, 15]. A recent study also found that the shape of the male genitalia can change with respect to temperature [32], and this may provide an explanation for taxonomy incongruence among drosophilids. The phylogenetic tree based on *COI* gene confirmed several species complex groups (Fig. 2a). Yet, we might expect better classification of our samples using whole-genome data, as shown in the *D. nasuta* species complex [33].

Drosophila malerkotliana was found in almost every site, from urban areas to tropical evergreen forests. It is a cosmopolitan species that originated and is widely spread in Southeast Asia [34]. Another cosmopolitan species, *D. ananassae*, was frequently found in urban areas and orchards because its distribution is mainly associated with human activities [35]. This study also reported the first information about the habitats (i.e., forest types and elevation) of *Drosophila eugracilis* and *D. mimetica* in the Thai Peninsula.

We expected to observe higher *Drosophila* diversity in the region due to great habitat diversity. For example, we found only one species in the peat swamp, despite the fact that it consisted of diverse topography and tree species, which, in turn, gives rise to several microhabitats [36]. The sampling method we used, such as using only fruit bait and seasonal factors, could affect the number of flies we caught. Having a different kind of bait other than the fruit bait may help to catch different fly species. For example, using fungus bait might help catching mycophagous species [37, 38]. Some *Drosophila* species are specialists [39, 40], and even generalist species can be seasonal specialists [41]. Yet, knowledge on the food source of drosophilids in this region is poorly known. Precipitation is another factor that determines the abundance of *Drosophila*. *Drosophila malerkotliana*, for example, was less abundant, while some species like *Drosophila willistoni* and *Drosophila paulistorum* were more abundant during the rainy season [42, 43].

All *D. ananassae* samples were infected with *Wolbachia* *w*Ri-like. This is in line with other studies of *D. ananassae* populations [44, 45]. However, we could not specify the exact strain because several *w*Ri-like strains, such as *w*Ana and *w*Ri, are closely related, having the same MLST and *wsp* profiles, and only minor differences were found between genomes [46–48].The low sensitivity of *Wolbachia* strain characterization using MLST method has been demonstrated in some *Wolbachia* strains and, thus, whole genome data are preferred over MLST for characterization [49, 50]. Bleidorn and Gerth [50] compared the MLST loci with over two hundred single copy loci and showed that many of these loci are better at strains characterization than the five loci used in MLST. However, the use of whole genome data for strain characterization can be costly and time consuming in some studies.

Here, we found two new *Wolbachia* strains, *w*MalA and *w*MalB, in *D. malerkotliana* from two populations, TK01 and PM02, with infection frequencies of 40% and 75%, respectively (Table 1). These two *Wolbachia* strains were not fixed in the populations. Other *D. malerkotliana* populations in this study were found not to be infected by any of these strains. By blasting the *wsp* sequences of the new strains, we found that the closest matches were from parasitoids of tephritid fruit flies. Thus, it is likely that the two new strains were horizontally transmitted from the parasitoids. However, there is no information that these parasitoids attack *Drosophila* species.

This study found 100% *Wolbachia* infection in all populations of *D. ananassae* and *D. simulans*. Cosmopolitan species like *D. ananassae*, *D. simulans*, and *D. melanogaster* are commonly infected by *Wolbachia* and the infection frequency can rise up to 100% in many populations [45, 51–54]. Apart from the cosmopolitan species, we found that the incidence of *Wolbachia* infections in many *Drosophila* species were at 0%, which were low like in other regions [30, 31].

Geographical barriers, such as mountain range, distance between populations, host genetic background, and *Wolbachia* themselves play essential roles in *Wolbachia* distribution. For instance, less gene flow between geographically distant populations prevents *Wolbachia* transmission in the parasitoid *Leptopilina clavipes* [55]. As *Wolbachia* are only maternally transmitted, *Wolbachia* with strong cytoplasmic incompatibility can lead to high infection frequency of natural populations in a short period [54, 56]. Moreover, beneficial effects on their hosts, such as protection against pathogens and providing nutrients, can help *Wolbachia* spread within a host population [13]. Apart from *Wolbachia*, *Cardinium*, *Rickettsia*, and *Spiroplasma* were not found in our samples. Some populations of *D. simulans*, *D. melanogaster*, and *D. ananassae* were infected by *Spiroplasma* but at low frequencies [57, 58]. For *Cardinium*, our results were in line with other studies on *Drosophila* [19, 59].

To our knowledge, this is the first analysis of *Wolbachia* infection in wild *Drosophila* in Thailand and the whole Malay Peninsula. We found two new *Wolbachia* strains in supergroups A and B in *D. malerkotliana*. There were no *Cardinium*, *Rickettsia*, and *Spiroplasma* infections in the wild *Drosophila* populations throughout Peninsula Thailand. With more than 200 *Drosophila* species reported in the whole Thai-Malay Peninsula, we expect high endosymbiont diversity to be discovered in the future.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00248-022-01982-1.

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Author Contribution MD designed, collected the samples, analyzed the data, and wrote the manuscript. AN collected the samples and edited the manuscript.

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Data Availability The DNA sequences were deposited on GenBank under accession numbers MZ520835-MZ520856 for COI gene and MZ566522-MZ566563 for *Wolbachia* MLST and *wsp* genes.

Code Availability Not applicable.

Declarations

Ethics Approval This study was approved by the Institutional Animal Care and Use Committee of the Prince of Songkla University (Ref. 10/2021).

Consent to Participate The authors agreed to participate in this study.

Consent for Publication The authors have seen and approved the submitted manuscript and given consent to publish it in Microbial Ecology.

Conflict of Interest The authors declare no competing interests.

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