Wolbachia infection in butterflies and moths from Khao Yai and Kaeng Krachan National Parks, Thailand

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หัวข้อวิทยานิพนธ์	การศึกษาการติดเชื้อแบคทีเรีย *Wolbachia* ในผีเสื้อกลางวันและผีเสื้อกลางคืนจากอุทยานแห่งชาติเขาใหญ่และแก่งกระจาน ประเทศไทย
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บทคัดย่อ

Wolbachia เป็นแบคทีเรียอาศัยร่วมที่จัดเป็นปรสิตทางการสืบพันธุ์ต่อแมลงอาศัยและมีการศึกษาเพื่อนำมาใช้ประโยชน์ในการควบคุมแมลงศัตรูที่สำคัญ การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาการติดเชื้อ *Wolbachia* ในผีเสื้อกลางวันและผีเสื้อกลางคืน สายพันธุ์ต่างๆ จาก 3 ภูมิภาคที่แตกต่างกันของประเทศไทย โดยเก็บตัวอย่างผีเสื้อกลางคืน จำนวน 1,235 ตัวอย่าง รวม 58 ชนิด ใน 13 วงศ์ และผีเสื้อกลางวัน จำนวน 623 ตัวอย่าง รวม 46 ชนิด ใน 5 วงศ์ ซึ่งเก็บตัวอย่างผีเสื้อจากอุทยานแห่งชาติเขาใหญ่ (ภาคกลางและภาคตะวันออกเฉียงเหนือ) และอุทยานแห่งชาติแก่งกระจาน (ภาคตะวันตก). การติดเชื้อแบคทีเรีย *Wolbachia* ได้รับการตรวจคัดกรองโดยใช้ปฏิกิริยาลูกโซ่โพลีเมอเรสกับไพรเมอร์ยีน *16S rRNA*, *ftsZ* และ *wsp* ผลการวิจัย พบว่า มีอัตราการติดเชื้อ *Wolbachia* ในผีเสื้อกลางคืนในปริมาณที่สูง ในขณะที่ ผีเสื้อกลางวันมีอัตราการติดเชื้อในปริมาณที่ต่ำ *Wolbachia* ถูกตรวจพบในประชากรผีเสื้อกลางคืนที่มีภูมิศาสตร์ที่แตกต่างกันรวมทั้งหมด 625 ตัวอย่างจาก 28 ชนิด ใน 9 วงศ์ ซึ่งมีจำนวน 144, 156 และ 325 ตัวอย่างจากภาคกลาง ภาคตะวันออกเฉียงเหนือ และภาคตะวันตก ตามลำดับ และในผีเสื้อกลางวันจำนวนทั้งหมด 291 ตัวอย่างจาก 17 ชนิดใน 2 วงศ์ ซึ่งมีจำนวน 86, 72 และ 133 ตัวอย่างจากภาคกลาง ภาคตะวันออกเฉียงเหนือ และภาคตะวันตก ตามลำดับ ในผีเสื้อกลางคืนอัตราการติดเชื้อสูงสุด คือ 90.47% ในตัวอย่างจากภาคตะวันตกและมีอัตราการติดเชื้อเฉลี่ย 61.90% ในขณะที่ อัตราการติดเชื้อในผีเสื้อกลางวันอยู่ระหว่าง 40.00% - 87.50% โดยเฉลี่ย 63.75%. ผลจากการศึกษาเรื่องความหนาแน่นสัมพัทธ์ของ *Wolbachia* พบว่า มีความหนาแน่นของการติดเชื้อ *Wolbachia* ในปริมาณที่ต่ำทั้งในประชากรผีเสื้อกลางคืนและผีเสื้อกลางวันที่พบในประเทศไทย

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ABSTRACT

Wolbachia is a co-living bacteria classified as a reproductive parasite against living insects and has been studied for its use in controlling major insect pests. The objective of this research was to study *Wolbachia* infection in butterflies and moths. Different breeds from 3 different regions of Thailand, A total of 1,235 moth samples were screened for 58 species from 13 families, and 623 specimens from 46 species of butterflies from 5 families. Samples were collected from Khao Yai National Park (Central and Northeastern) and Kaeng Krachan National Park (Western Region). *Wolbachia* infections were screened using PCR with *16S rRNA*, *ftsZ,* and *wsp* gene primers. The results indicate that *Wolbachia* infection rates in moths are high, while in butterfly are low. It is found in a total of 625 different geographical populations from 28 moth species from 9 families: 144, 156, and 325 individuals from the Central, the Northeast, and the West, respectively, and in total 291 individuals of 17 butterfly species from 2 families, including 86, 72, and 133 individuals from the Central, the Northeast, and the West, respectively. In moths, the highest infection rate is 90.47% in the Western population and the average infection rate is 61.90%. Whereas the infection rate in butterflies was ranging from 40.00% - 87.50% with an average of 63.75%. As a result of *Wolbachia's* relative density, low *Wolbachia* infection density was found in both moth and butterfly populations from Thailand.

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CHAPTER 1
INTRODUCTION

1.1 Statement and significance

Destruction of forest ecosystems results in biodiversity degradation. Cutting and destroying forest cover degrades the environment and reduces biodiversity. Forests support biodiversity by providing habitat for wildlife. Apart from that, forests are conducive to the conservation of medicinal plants. For this reason, Deforestation can irretrievably destroy genetic diversity.

Insects are a group of organisms that have a higher diversity in terms of species than other living things. There are about 1.7 million known species in the world, or about 64 percent, of which are insects. It plays a role in the ecosystem, such as helping to pollinate plants and being a decomposer. It helps to break down and circulate nutrients in the food fiber in the ecosystem. Some groups of insects such as ants, beetles, butterflies, and termites It can be used as an index to assess the diversity of forest conditions, and moths can be used to monitor climate change.

Butterfly (Lepidoptera). It is one of the most diverse insecticide orders with more than 157,000 species described. (Bipinchandra *et al.* 2012). Lepidoptera plays an important role primarily in the functioning of ecosystems as pollinators and herbivores. (Banziger, 1992; Pierce, 1995; Plotkin and Goddard, 2013; Zaspel, 2011). However, it was found that in the caterpillar stage will eat the young leaves of various plants, including many important agricultural cash crops such as the caterpillar of tomato leaf. (Tomato leafminer). It is a serious pest on a global scale because it can destroy many crops and damage tomato-growing sites in South America, Europe, as well as Thailand, etc.

Wolbachia is a that must live inside cells, found in many arthropods. It plays a role in the host as a reproductive parasite in many forms. (Cordaux *et al.* 2001; Werren *et al.* 2008). It can induce cytoplasmic incompatibility or CI disorder that occurs during mating between males with *Wolbachia* and females without *Wolbachia* or between males and females with different species of *Wolbachia* and can spread in the nature of insect populations on its own. Through the reproductive system because it can be

transmitted from mother to child. (Stouthamer *et al.* 1999). It can also be transmitted between one host and another (horizontal transmission) (Brownstein *et al.* 2003).

Wolbachia is currently being studied for pest control, a new method that has been studied recently to control pests without using chemicals. It is the use of inducing reproductive disorders, especially inducing CI, such as controlling fruit flies (*Ceratitis capitata*) by pairing females without *Wolbachia* with males with *Wolbachia*, as well as a hybrid pairing between females and males with different species of *Wolbachia*. It was found to produce 100% embryo mortality. (Yamada *et al.* 2011). Control of leafworm flies (*Liriomyza trifolii*). By pairing females without *Wolbachia* with males with *Wolbachia*, they found that the number of eggs and hatchability rates were lower than their normal counterparts. (Wiwatanaratanabutr and Kittayapong, 2006) and mosquito control. (*Aedes aegypti*). By mixing males with *Wolbachia* and females in nature without *Wolbachia*, it was found that the percentage of mosquito hatchability decreased. (Wiwatanaratanabutr and Kittayapong, 2009).

In addition, *Wolbachia* has also been found to have a reduced effect on increasing the number of viral cells in mosquitoes. Mosquitoes are less likely to transmit the virus that causes dengue fever. (Walker *et al.* 2011) and reports of the use of *Wolbachia* to control the increase in mosquito populations in real-world environments such as Brazil, Australia, and Indonesia. *Wolbachia* has been found to reduce mosquito populations and reduce dengue outbreaks.

Currently, *16S rRNA* (a ribosomal gene), *wsp* (a gene for a cell membrane protein), *ftsZ* (a regulatory gene of the bacterial cell cycle), *groEL* (heat shock protein), *coxA* (cytochrome c oxidase subunit I), and *gltA* (citrate synthase) It has been used to detect *Wolbachia* and study its evolutionary relationships. In addition, the technique of labeling probes with phosphors is used (FISH). The technique involves matching the probe with the target nucleic acid in the cell or directly in the tissue. (Werren *et al.* 2008). The target nucleic is RNA decoded from the gene to be examined. (Heddi *et al.* 1999). Study the habitats of *Wolbachia* in *Sitophilus oryzae*. *Wolbachia* was found to be mainly located around the ovaries and testes. (Zhao *et al.* 2013). Study the habitats of *Wolbachia* from *Tetranychus urticae*. *Wolbachia* was found to be located on the testes of males. While females find *Wolbachia* in both the ovaries and the egg sac and

(Saurav *et al.* 2016). Study the habitats of *Wolbachia* in *Thrips palmi*. *Wolbachia* is found mainly in the abdominal area.

This project will document the dominance, geographic distribution, and biodiversity of butterflies and moths, their *Wolbachia* endosymbionts, and relative plants in the tropical forest ecosystem in Thailand. Our study will lay the foundation for further biologically investigation into *Wolbachia's* impact on Lepidoptera hosts. To the best of our knowledge, *Wolbachia* infection in butterflies and moths together with their biodiversity and geographic distribution in the tropical forest ecosystem in Thailand has never been reported so far. Therefore, this project will be the first investigation of *Wolbachia* infection in butterflies and moths in Thailand as well as the first systematic survey of the biodiversity and geographic distribution of butterflies and moths in the tropical forest ecosystem in Thailand.

1.2 Objectives

1.2.1 To study *Wolbachia* infection in butterflies and moths from Khao Yai and Kaeng Krachan National Parks, Thailand.

1.2.2 To examine the biodiversity of butterflies and moths in the tropical forest ecosystem in Thailand.

1.3 Expected benefits from Research

The detection of *Wolbachia* bacteria in butterflies and moths has a positive effect on farmers who want to control pests without chemicals since *Wolbachia* bacteria can inhibit the incubation of butterflies and moths, which are pests that often destroy agricultural products.

CHAPTER 2

REVIEW LITERATURE

Rainforest is one of the world's most important ecosystems because of its abundance and diversity in terms of species, species of diversity, the genetic diversity that exists in each living unit that is grouped together in populations, and habitat-based ecology. It is also the world's most important source of oxygen production. In addition. Rainforests in different regions of the world are similar in appearance but differ in structure, composition, quantity, and species of life. Studies have shown that: Rainforests occur in highly humid areas, with rainfall all year round. Rainfall is not less than 2,000 milliliters per year. Found in areas with an altitude of up to 400 meters. However. Currently, tropical rainforests occupy only 6% of the earth's surface and are rapidly being invaded. Most of the rainforests are located near the equator, including South America, Africa, and Southeast Asia, and Thailand is mainly in the southern zone.

Climate change is a long-term change in weather patterns in an area that persists for decades or longer. There can be several reasons for climate change, one of which is due to: global warming from increasing the concentration of greenhouse gas (GHG) in the atmosphere related to human activities. In addition. Global temperature increases are changing several other weather patterns, such as rising temperatures causing global warming. As a result, the ice mass melts and evaporates increases. These have several physical consequences. consist of sea level rise Increased variability of weather patterns and extreme weather events These physical changes are a consequence of global warming appearing and affecting the livelihoods of animals, including humans. (Santer *et al.* 1996). The causes of climate change consist of

1) Energy Production: The production of electrical and thermal energy by burning fossil fuels contributes to large global greenhouse gas emissions. Most of the electricity is still produced by burning coal. This releases carbon dioxide and nitrous oxide. These two gases are greenhouse gases that have the power to cover the earth and trap heat from the sun only about a quarter of the electricity used worldwide is produced by wind, solar and other renewable energy sources, with little or no greenhouse gas emissions or pollutants emitting into the air, in contrast to fossil fuels.

2) Product of production: Activities in the manufacturing and industrial sectors generate greenhouse gases, mainly from burning fossil fuels to generate energy for the production of goods such as cement, steel, metals, electronics, plastics, clothing, and others. manufacturing machinery is often powered by coal. Oil or Gas Some materials, such as plastics, are made from chemicals derived from fossil fuel sources. Manufacturing is one of the sectors with the highest greenhouse gas emissions worldwide.

3) Deforestation: Each year about 75 million hectares of forest have been destroyed, deforestation for farming or pasture, or for other purposes. It produces greenhouse gas emissions because cut trees emit carbon dioxide that they absorb. Deforestation thus reduces nature's ability to store greenhouse gases from entering the atmosphere. Deforestation of agriculture and changes in land use patterns contribute to one-quarter of global greenhouse gas emissions.

4) Transportation: Most cars, trucks, boats, and planes are powered by fossil fuels. Transportation thus becomes the main causes of greenhouse gas emissions, especially carbon dioxide. Vehicles on the road emit the highest greenhouse gases. because internal combustion engines rely on the combustion of petroleum-derived products such as gasoline, followed by ships and planes. The transport sector's emissions account for nearly a quarter of global energy-related carbon dioxide emissions, and the transport sector's fuel consumption is likely to increase significantly in the coming years.

5) Food production: Food production produces carbon dioxide, methane, and other greenhouse gases. Other activities related to food production are no exception, such as deforestation and clearing of land for agriculture and pasture. The process of digestion of cows and sheep, the production and use of fertilizers and manure, the use of energy for agricultural tools, and most fishing vessels often use fossil fuels. Food packaging and distribution also generate greenhouse gases.

6) Energy consumption in residential buildings: Commercial and residential buildings consume more than half of the electricity consumed globally. Oil and natural gas for heating and cooling Buildings will continue to be a source of massive greenhouse gas emissions. The demand for energy for heating and cooling has increased markedly as more people have air conditioners. Greater electricity

consumption for lighting Appliances and connected devices has all contributed to the higher carbon associated with energy consumption in homes in recent years.

7) The temperature rises: As greenhouse gas concentrations increase, the Earth's surface temperature will also increase. Almost all regions of the planet are increasingly exposed to heat waves. During the daytime, the number of hot weather increases. 2020 was the hottest year on record. Higher temperatures can cause heat-related diseases and illnesses and make work or travel more difficult. Wildfires will be easier and spread faster. Temperatures in the Arctic region are rising at least twice the global average.

8) Extinction of species: Climate change threatens the survival of all living organisms on land and water. The higher the temperature, the higher the temperature. Natural disasters are becoming more severe. Climate extremes, or the spread of pests and epidemics, some species may migrate to survive. While some cannot. Now the world is losing species at a rate 1,000 times faster than any period recorded in human history.

9) Food is scarce: Extreme and extreme weather exacerbates famine and malnutrition and damages fisheries, farming, and livestock because heat causes water supplies to dry up and pasture areas for grazing. More acidic oceans damage marine resources that feed billions of people. Changes in snow and ice in the Arctic region have a heavy impact on the amount of feed that comes from livestock farming. Hunting and fishing Extreme heat also shrink the amount of water and pastures for grazing. Impact on crop and livestock supply.

10) Sea water is hotter and higher: The oceans must absorb most of the heat caused by global warming. As a result, ice melting and sea levels are rising, threatening coastal communities and islands. In addition to heat. The oceans also need to absorb carbon dioxide from reaching the atmosphere, acidifying seawater and harming marine life.

A butterfly is a group of small insects of medium and large size which has several species and biodiversity. The destruction will occur in the worm phase. (caterpillar) by eating a worm of plants causing quantity and quality of production to decrease If there is a severe outbreak, it may cause the plant to die There are approximately 157,000 butterflies in the world more than half of them are prevalent in tropical countries in the Asian region. In Thailand, there are approximately 15,000 species of butterflies the species can be classified. Many other butterflies cannot be classified which can now be divided into two groups: butterflies and moths.

1) Butterflies The tip of the tentacles swelled out thick like a club. Some had tentacles at the end, curving into the shape of the body of a fairly long butterfly. In comparison the width of the wings and rarely covered. Most of them will fly during the 6 days time, only some of them will feed in the morning. And at dusk, welding both wings of the butterfly to wave together day is different from moths. Which will have wide wings spread over each other island resting in the butterfly. Usually lift the wings straight up on the body, see the underside of the wings.

2) Moths There are many different shapes of tentacles, such as slender, yarn-shaped, comb-shaped teeth. Flew in the evening short fat body. Bonding both wings to waving the butterfly together Moths have a hard coat from the base of the pair of hind wings. Inserting about a small request the base of the lower wing of the front wing. The island resting on butterflies, moths will place their wings flat on the island with the front wing edge falling below the side of the back, looking like a gable roof and covering the rear pair of wings etc. (Fig 2.1)

Fig. 2.1 Comparison (A= front, B= back) butterflies and (C =front, D=back) moths (Source: Panukorn boonsit, 2021)

Lepidoptera has an early life cycle from the egg stage, worm stage, and larval stage until the amorphous stage enters the adult stage with colorful wings to the human eye. In entomology, the classification of this group of insects uses wing lines for classification. (Banziger, 1992; Pierce, 1995; Plotkin and Goddard, 2013; Zaspel, 2011). In addition, scientists estimate that butterflies may have originated as early as the Cretaceous Period, 66 million years ago, with the oldest fossil being Skipper, *Thymelicus lineola*, around the Paleocence Epoch, about 57 million years ago. Found in Fur, Kingdom of Denmark, and Dominican amber fossils of the 25-million-year-old metalmark butterfly (*Voltinia dramba*).

Currently, butterflies are commonly distributed in the cold and arid terrain. It is estimated that there are now more than 17,500 species of butterflies in the superfamily, Papilionoidea, and 180,000 species of Lepidoptera. In addition, butterflies have been found to be an important component of the ecosystem by helping to reproduce and propagate various plant species. They are both prey and predators in the food chain in the ecosystem.

Butterflies are insects that have 4 life cycles: eggs, worms, pupa, and adults in which each stage of the butterfly is important to the ecosystem (Dionysopoulou *et al*. 2020). Whether it is a phase in which the larvae feed on leaves for use in growing to molt and preparing for pupa if in the area there is a leaf that is the food plant of that type of caterpillar Eating the leaves of caterpillars may also be beneficial to the ecosystem in terms of reducing the density of the leaves resulting in sunlight shining down to the ground the diversity of butterflies reflects the diversity of plants which is a food plant of butterflies also in the worm phase and the adult phase of butterflies it is also food for other organisms such as birds, lizards, etc., female butterflies that consume nectar from flowers as the main food and will fly between a flower to another flower Cause pollination between male stamens and pistil the distribution of that plant species in which all these results in balanced ecosystem butterflies are considered an organism that is important to the ecosystem.

2.1 General Characteristics of Butterflies

Butterflies are one of the most biologically diverse groups of insects the main characteristics are: Vivid, eye-catching, beautiful, and unusual Order: Lepidoptera Class: Insecta in the Phylum Arthropoda. (Fig.2.2). It has the appearance of an invertebrate body structure However, there is a hard shell, which is a chitin substance, covering the outside of the body. It is divided into 3 parts: the head, the thorax, and the abdomen consist of multiple ring sides in a tile welded with a thin membrane it has a hard shell appearance the inside has a hard rod fastened between muscles for movement the rings connected to the butterfly's body total 14 internodes divided into 1-part head, 3-part chest, and 10-part belly. In addition, other organs including the mustache look like a pair of batons between the eyes and serve to smell. Eye Section consists of 2 types of eyes ocelli and compound eyes. Single eyes serve to perceive bright darkness. The combined eye consists of thousands of small lenses. It serves to get animated images and is very effective within vision. In addition, the mouth is a surfing band that acts as a sucker for liquid food such as nectar & minerals while not sucking food The butterfly's mouth is curled up in a circle, resembling a clock curl. Swings have 2 pairs the front and rear wings. The front wings are attached between the middle stripping. The rear pair of wings is attached between the third stripping. (The sides attached to the abdominal side) Thorax consists of 3 interspecies that are jointed between the joints. There's a pair of legs attached to the chest. The abdomen, which is the organ at the end of the butterfly consists of the genitals. It has different shape characteristics according to the type of butterfly. And the legs have a joint shape. divided into 5 parts namely the coxa, the trochanter, the femur, the tibia, and the tarsus. It can be found in the tropical and subtropical forests regions of southeast Asia, as well as in Thailand.

Butterflies of life cycle

Butterflies have Holometabolous growth is a growth that changes shape completely metamorphosis growth at each stage butterflies are not the same shape. The advantage of this type of growth is that each part of the life cycle requires different foods and may live in different environments. There are different kinds of enemies. This causes growth at each stage. Less risk of leaving it is divided into 4 phases as follows: Egg stage namely the phase at which butterflies have been bred will fly to

the plants to lay eggs they have different style shapes such as a round shape or in some species a conical appearance. In addition, the spawning of butterflies is placed under the leaves of plants in clusters or singly that egg of butterflies the average duration is about 3-5 days. Therefore, it hatched into a caterpillar. The portion larva stage is the period after the caterpillar hatches from the eggs there are different characteristics at first, the caterpillars eat their eggshells then the caterpillars begin to eat leaves and molt to enlarge their size about 4-5 times In addition, the caterpillar has other organs, including 3 pairs of real legs on the chest and 4-5 pairs of prosthetics on the abdomen and when the time is right the caterpillar stops feeding to get into the pupa this period takes about 15 days of the growth range. While the Pupa stage is when the caterpillars are fully grown they are ready to be Pupas and will not be able to move. But inside the Pupa shell, there will be various aspects of growth and development. The Pupa takes about 7-1 day and the adult stage is the stage at which the pupa develops like an adult. So butterflies the legs push the bark of the Pupa out through the back of the thorax and move out of pupa then it hangs its head and wings down the bottom and excretes the waste generated while it is a pupa and it takes time to rest to dry the wings completely and breathe in so that blood flows in the wings about 1-2 hours also it can continue to before eating and breeding and it serves to find food, which is minerals including nectar from pollen, ground or sand, rotten fruits, creeks, water streams, manure.

Fig. 2.2 General Characteristics of Butterflies.

(Source: Panukorn boonsit, 2021)

2.2 General Characteristics of Moths

Moths are one of the largest number of insects of their kind Phylum: Arthropoda, Class: Insecta Order: Lepidoptera. (Fig.2.3). It has an invertebrate structure the main characteristics are: The hard outer shell envelops the chitin inside the hard shell is the anchor of the muscles used to move. The fuselage consists of: The ring-like internode is welded with thin membranes consisting of 3 parts namely his head which is the organ at the front of the moth has a sense organ that is ocelli serves to perceive bright darkness and compound eyes officiate get animations high visibility performance between the eyes there are 2 antennae used to smell the antennae of moths have in many forms such as style, pectinate, filiform and plumose under the head, there is a reciting proboscis used for sucking food. Thorax has 3 Pericles that is prothorax, mesothorax, and metathorax in each area, there are legs attached to 1 pair of legs. In addition, the thorax consists of wings 2 pairs of splices wherewith fore wings attached to the mesothorax and hind wings attached to the metathorax and abdomen has characteristics amount 6-7 segments on the sides of each side there is a 1 dual of spiracles eat at dusk it is important for ecosystems and balancing nature adults help pollinate, which is the origin of the life of a variety of species moths can be found in all areas of the high mountains. mixed forest meadows, rainforests, aerations, mangroves, and pong. In addition, moths it is also the most diverse group of biological species of insects around 200,000 species, and found in Thailand 20,000 species

Moths of life cycle

Moths have Holometabolous growth is a complete metamorphosis growth throughout the life span that can be divided into 4 stages: egg, larva, pupa, and adult stage, each of which takes a different development time there are details as follows: Eggs have a characteristic of size. Shape, Color, and pattern vary. The size of the egg is very small. Therefore, it is necessary to rely on a microscope to study the eggs of a moth. Egg shells contain chitin. It is the same substance as the body shell of butterflies and other insects. And when looking through the microscope, a small open hole is found. It's called a micropyle It is a hole that causes male semen to mix with the eggs of females. It takes about 3-5 days to hatch into a caterpillar at this stage. The caterpillar has a different characteristic. The first food that caterpillars eat is the shells

of their Own eggs After that, the caterpillar. Start the soft leaves first. The eating characteristics of the caterpillar begin from the edge of the leaves to the middle of the leaves. And the molting will be enlarged 4-5 times. Throughout this worm period, There's been a change. In addition to the larger size, some species of color and shape vary. Like an inspired caterpillar, lemon worm. In the early stages, It's like bird droppings. But as the caterpillar grows, the color changes. It is green, with an eye-like pattern on the chest, etc. But all of them have one thing, which makes it possible to identify caterpillars. The caterpillar has 3 pairs of real legs on the chest. And 4-5 pairs of prosthetics on the stomach. Common caterpillars are often single. But there is some kind of early stages that are tricky in groups. It takes a total of 15 days. When the worm matures, you need to look for something to molt on to get into the pupa. It is not possible to move, but inside the pupa, shell developments are constantly evolving as a period of fully accumulated food, which attracts the beans each caterpillar chooses a different pupa. The pupa distance takes about 7-10 days. Adult stage is a beautiful colorful butterfly. Starting from the pupa. By butterfly using a pressure pin. The pupa peel shatters the prism and the butterflies come out in a way that dangles the head down and shoots out pink waste in the early stages, the wings of butterflies are still not able to radiate a liquid pump is required called hemolysins. Into the wing line. And it takes an hour to make the wings hard enough to fly 2-3 some species can last for 1-2 weeks depending on the type and each life expectancy range. (Biodiversity research center her majesty the queen's 72nd birthday anniversary, 2553).

Fig. 2.3 General Characteristics of Moth.

(Source: Panukorn boonsit, 2021)

2.3 The role of co-living bacteria in Arthropod insects

symbiosis namely the coexistence between two organisms which is a large creature called the host and small creatures inhabiting large creatures called symbiont consists of 3 types namely cohabitation with one another benefits and the other is wasted (parasitism) coexistence with dependency on both sides benefits (mutualism) and living with one another benefits, but the other party does not or does not lose benefits (commensalism). Many insects live together with bacteria (bacterial endosymbionts). It is a bacterium that lives inside insects it can be divided into 2 species namely primary co-dependent bacteria (primary symbiont) namely bacteria live in a group of cells that gather into tissues or are special structures called bacteriomes each cell comes together called bacteriocyte position and structural characteristics of bacteriome different depending on the type of insect living such as *Diaphorina citri* (Family Psyllidae) have Characteristics of bacteriome the curved structure resembles the letter U yellow living in the abdomen (Fig. 2.4) (WangKeeree and Hanboonsong, 2016) and bacteria co-live secondary (secondary symbiont) namely bacteria found in the tissues of different parts of insects example digestive tract, salivary glands, and ovary genetic traits can be transmitted from parents to their child's generation or transfer between different insects. (Tewaruxsa, 2017).

In addition, this type of co-living bacteria is not necessary for the growth or reproduction of insects, living the same as the first type. But relationship characteristics come in many forms it can be beneficial to both sides either party benefits or benefit only bacteria, but insects are a waste of benefits. Some species can be isolated, and cultured outside the insect such as aphids that have the bacteria *Hamiltonella defensa.* It can help increase resistance to bien insects. (Montllor *et al.* 2002) etc. And this type of co-living bacteria may or may not be specific to insects. This depends on the species of bacteria and insects. which S-symbiont Some species can be found in many groups of insects. While S-symbiont some species can’t be found in all insects of the same species. But it is found only in certain groups of insects in the population. However, nowadays it has been studied and discovered a type of co-habitat bacteria that are reproductive parasites, as well as have properties that can induce reproductive disorders in living insects such as *Wolbachia* bacteria.

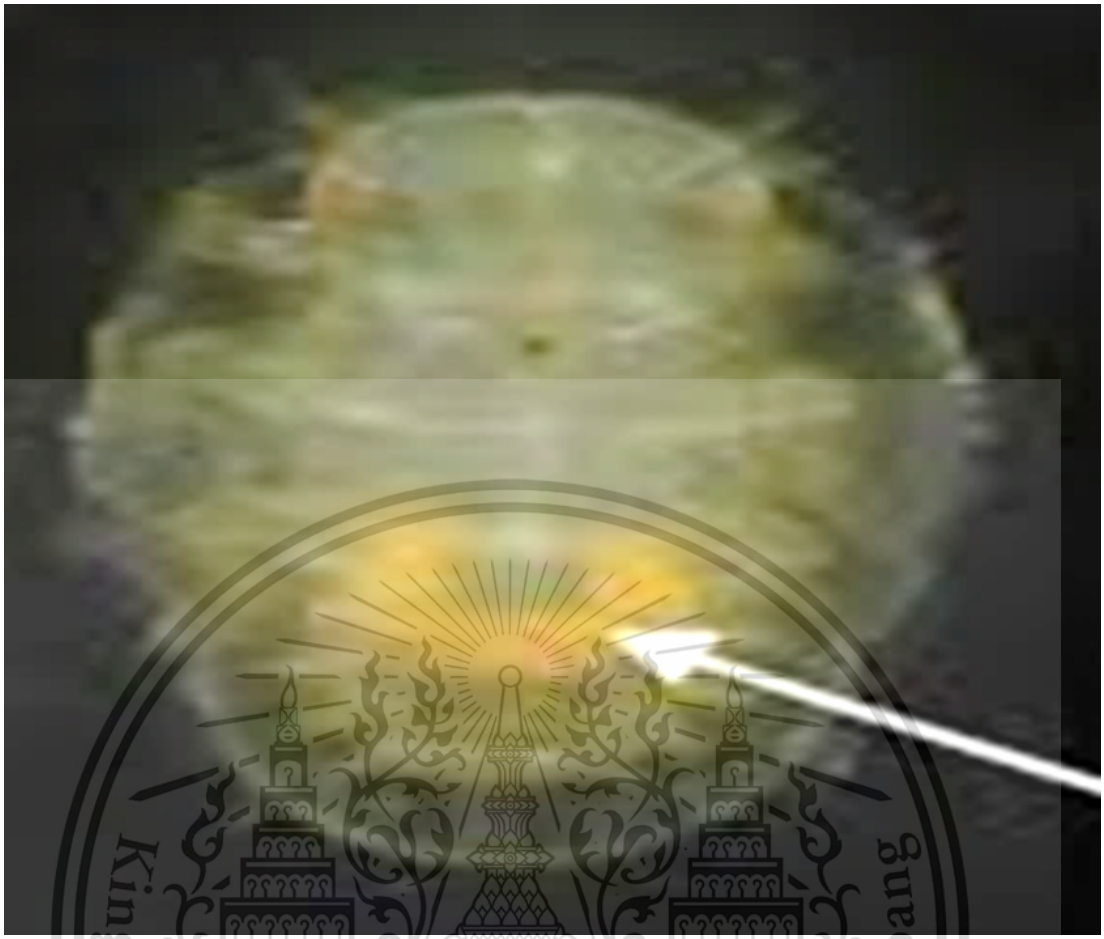

Fig. 2.4 Position and Characteristics structure of bacteriome in the *D. citri.* (Source: WangKeeree and Hanboonsong, 2016)

2.4 General Characteristics of the *Wolbachia* bacteria

Wolbachia It is a gram-negative bacterium that must live only in living cells. (Obligate intracellular bacteria) *Wolbachia* is a species of bacteria classified as alpha-Proteobacteria order Rickettsiales There are 2 types of characteristics namely shape rodlike small irregular (lengthily 0.5-3 μm) and shape small coccoid (diameter 0.25-0.5 μm) or large (diameter 1-1 .8 μm) (Hertig, 1936). As the hostages. The amount of *Wolbachia* will increase as well. (Wright, 1979). *Wolbachia* It can be found in a vacuole that is encapsulated in 3 layers of tissue. *Wolbachia* has been found mainly in the cytoplasm of cells, reproductive tissues, either around the ovaries or testes of mosquitoes, as well as other types of living hosts, but sometimes it can be found around the excretory organ called the malpician tube (Werren *et al*. 1995; Saurav *et al*. 2016).

Wolbachia It can be found everywhere in insect organisms including mites, isopod, and filarial nematode based on insect species surveys 20-76% of *Wolbachia* infections (Cordaux *et al.* 2012; Werren *et al.* 2008; Wiwatanaratanabutr, 2013ab, 2015, 2016ab). Such as tropical insects of the americas at least 16% are infected with *Wolbachia*. subtropical insects at least 23% of *Wolbachia* infections are infected (Stouthamer *et al.* 1999). and tropical insects at least 34% of *Wolbachia* infections (Werren *et al.* 2008). In addition, ants in indonesia about 50% of all species including spider mite 6 in 16 species, predatory mite 4 in 7 species, terrestrial isopod 34% of all kinds, and filarial worms 9 in 10 species *Wolbachia* infection (Clancy and Hoffmann, 1998; Wiwatanaratanabutr and Kittayapong, 2009). However, *Wolbachia* in arthropod organisms has been attracting the attention of scientists for almost 80 years. In 1924 Hertig and Wolbach. Reports of the discovery of bacteria of the genus Rickettsia inside the ovarian area cells of *Culex pipiens* when viewed under a microscope (Hertig and Wolbach, 1942) and in 1936. Hertig. The bacterium found in *Wolbachia pipientis* was named in honor of Wolbach. This is the first reported occurrence of *Wolbachia* in insecticides.

According to the study of phylogenetic relationships, *Wolbachia* has a close relationship with bacteriogram. Ria belongs to the order Rickettsiales family anaplasmataceae. The study of *Wolbachia* in *16S rRNA*, *ftsZ* gene, *wsp* gene, and other genes found that *Wolbachia spp*. can be divided into 8 supergroups (A-H), i.e.

supergroups A and B, mainly found in arthropod hosts, supergroups C and D are normally found in *Philarian helminth* worm hosts. E supergroups are found in hosts, tailed insects. Supergroup F is found in arthropods and *Philarian helminths*, and supergroup H is found in termite hosts. (Fig 2.5). In addition, the phylogenetic tree around *Wolbachia's 16S rRNA* found in different insect species showed little difference in nucleotide sequence and percentage of guanine (G) and cytosine (C), so *Wolbachia* can be divided into two groups of arthropods, supergroup A and B (Tewaruxsa, 2017).

The phylogenetic tree around *Wolbachia's 16S rRNA* gene found in different insect species found that there was little difference between nucleotides and the percentage of guanine (G) and cytosine (C), so *Wolbachia* could be divided into two groups of insects: supergroup A and B (Breeuwer *et al*. 1992; Stouthamer *et al*. 1993).

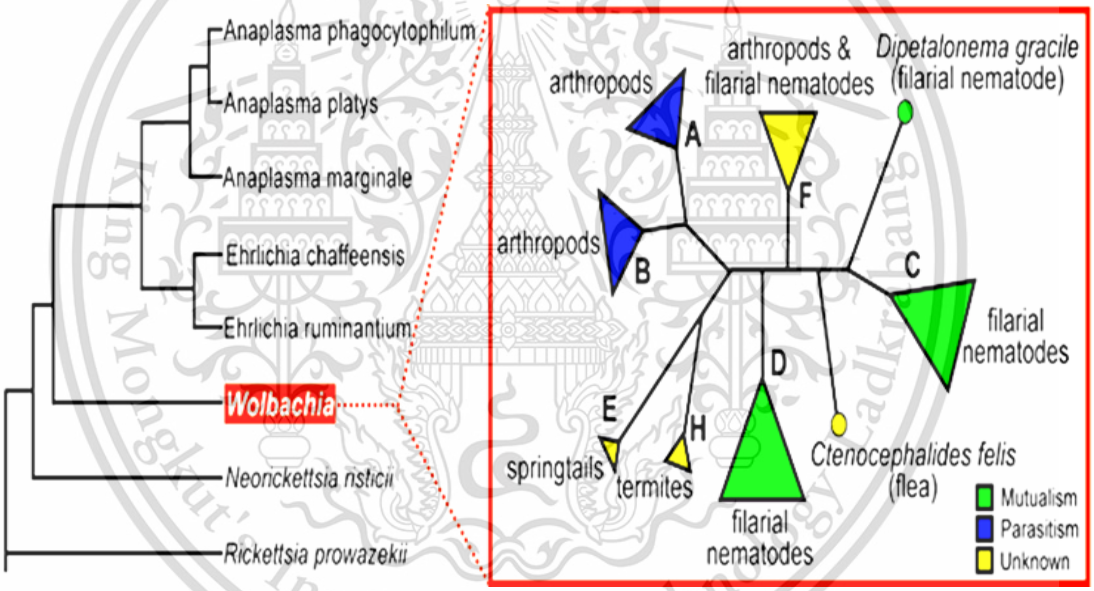

Fig. 2.5 Phylogenetic *Wolbachia* by size of the triangle represents the diversity of the *Wolbachia* species, the circular part represents. Single species of *Wolbachia* [Green: dependency coexistence silver: one side benefits and the other loses. Yellow: The relationship pattern is still unknown. (Source: Werren *et.al.* 2011).

Wolbachia is found in hosts belonging to different orders of insects, but there are few genetic differences between them. *Wolbachia* is transmitted by contact with insects or carriers to help transmit *Wolbachia*, as well as possibly by transmitting *Wolbachia* in the same host (intra-species) or different types of hosts (inter-species). This kind of transmission is called horizontal transmission (Ross *et al*. 2017). such as *Wolbachia* is detected in hornets (*Nasonia giraulti*). With green-headed flies (Protocalliphora sp.) Become a live host, Hornet Bien (*Leptopilina boulardi*). With flies (*Drosophila simulans*). Become live host.

Wolbachia is a parasitic bacterial that live in the cells of arthropod insects. Especially the insect group and one of the most common parasitic microorganisms in the world, over 70% of all insect species. It is a bacterium that must live in the cells of living organisms *Wolbachia* bacteria can be found in cells of the reproductive organs of the host in Arthropod insects. (PruksaKorn and Nuchprayoon, 2001) (Fig 2.6). In addition, *Wolbachia* is a bacterium found in many insects and a role in insects by being a reproductive parasite this can induce Cytoplasmic Incompatibility or CI. When mating is matched between infected *Wolbachia* bacteria male insects with female insects with uninfected *Wolbachia* bacteria or between male and female insects with different species of *Wolbachia* namely caused embryo death. *Wolbachia* can spread like the insect population itself by passing from mother to child.

Therefore, *Wolbachia* bacteria have attracted a lot of attention in many countries around the world including Thailand. To study the application guidelines for controlling the population of important pests. include insect population carriers of many diseases by adopting such features of *Wolbachia* bacteria causes reproductive disorders or phenomenal occurrence Cytoplasmic Incompatibility or CI.

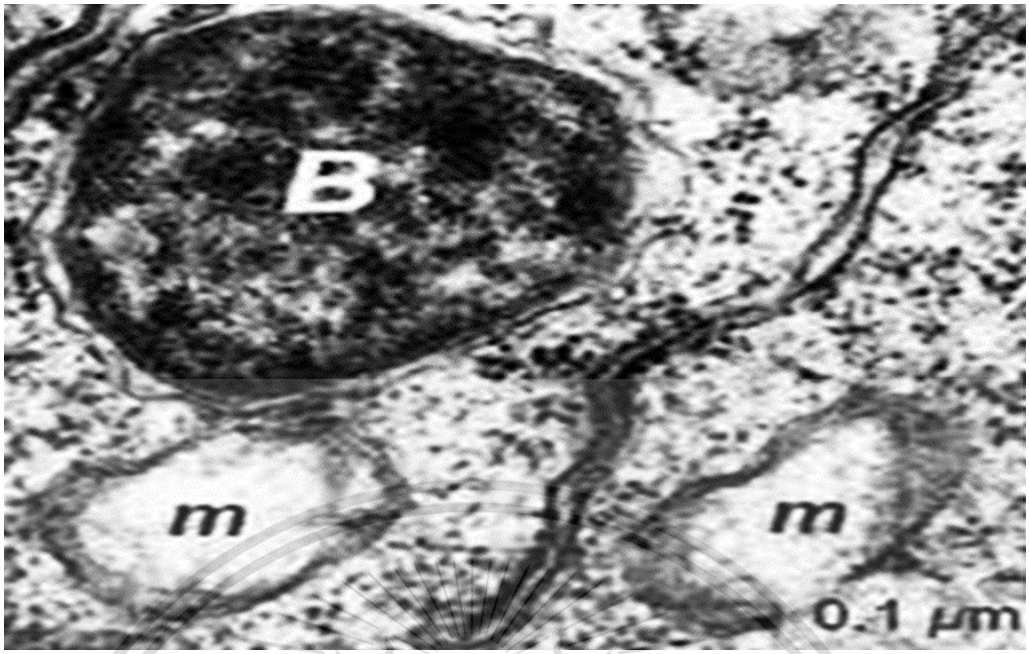

Fig. 2.6 General characteristics of *Wolbachia.*
(Source: Werren *et al.* 2011)

2.5 The role of *Wolbachia* bacteria in Arthropod insects

Wolbachia is a bacterium that coexisted with a variety of host organisms. The role of *Wolbachia* bacteria on the host was first reported in 1971 by Yen and Barr. *Wolbachia* was found to be the cause of cytoplasmic incompatibility phenomena (CI). By matching the combination of male mosquitoes with *Wolbachia* and females without *Wolbachia* in the mosquito population, *Culex pipiens.* as result, the embryo (Zygote) is unable to grow and eventually death and according to the test match breeding between *mosquitoes Cx. pipiens,* males with *Wolbachia* and females without *Wolbachia.* It found that the number of generations of children had a smaller birth rate or not, noticeably. But when bringing the population of male mosquitoes with *Wolbachia* infection to females with *Wolbachia.* It was found that the female mosquito population was able to leave their babies or lay eggs in a normal amount (compatibility). Therefore, the phenomenon of CI. It arises from matching mating with the population of male arthropod insects infected with *Wolbachia* with females that are not infected with *Wolbachia* bacteria. At the same time. The occurrence of the phenomenon of CI. It was found that it does not occur during mating pairings between female insects infected with *Wolbachia* and infected male insects. In addition, CI was also found to be formed by matching mating between male insects and female insects with different species of *Wolbachia* infection. With such features, many researchers around the world are interested in and studying the role of *Wolbachia* in the population of hosted arthropod insects live. (Noda, 1984). *Wolbachia* bacteria prosper and live in cells closely with a wide range of hosts and are associated with the host's reproductive organs in the group of arthropod insects. *Wolbachia* bacteria are Classified as reproductive parasites that can induce reproductive disorders 4 Formats namely (Dale and Welburn, 2001). (Fig 2.7)

1) Feminization is the introduction of having a female child male (Rousset ***et al***. 1992) is found in insects of the order Hymenoptera (ants, bees, and wasps) and Thysanoptera (thrips).

2) Parthenogenesis is the induction of a host to have female offspring. It is found in insects of the order Hemiptera (aphid), Isoptera (termites), and Lepidoptera (butterflies).

3) Male killing is the induction of the death of a male embryo after fertilization (Jiggins *et al*. 1998). It is found in insects of the order Coleoptera (beetle), Diptera (flies, whiteflies, glimpses, and mosquitoes), and Lepidoptera (butterflies).

4) Cytoplasmic incompatibility is the incompatibility induction of the cytoplasm of a living host with and without *Wolbachia* after fertilization. The result is the death of the embryo (Shoemaker *et al*. 1999; Hurst and Werren, 2001; Bordenstein and Wernegreen, 2004). Found in insects Order Coleoptera (beetle, beetle), Diptera (flies, whiteflies, glimpses, mosquitoes), Hemiptera (nets), Hymenoptera (ants, beetles, etc.) Isoptera (termites), Lepidoptera (butterflies), and Orthoptera (grasshoppers, crickets, cockroaches).

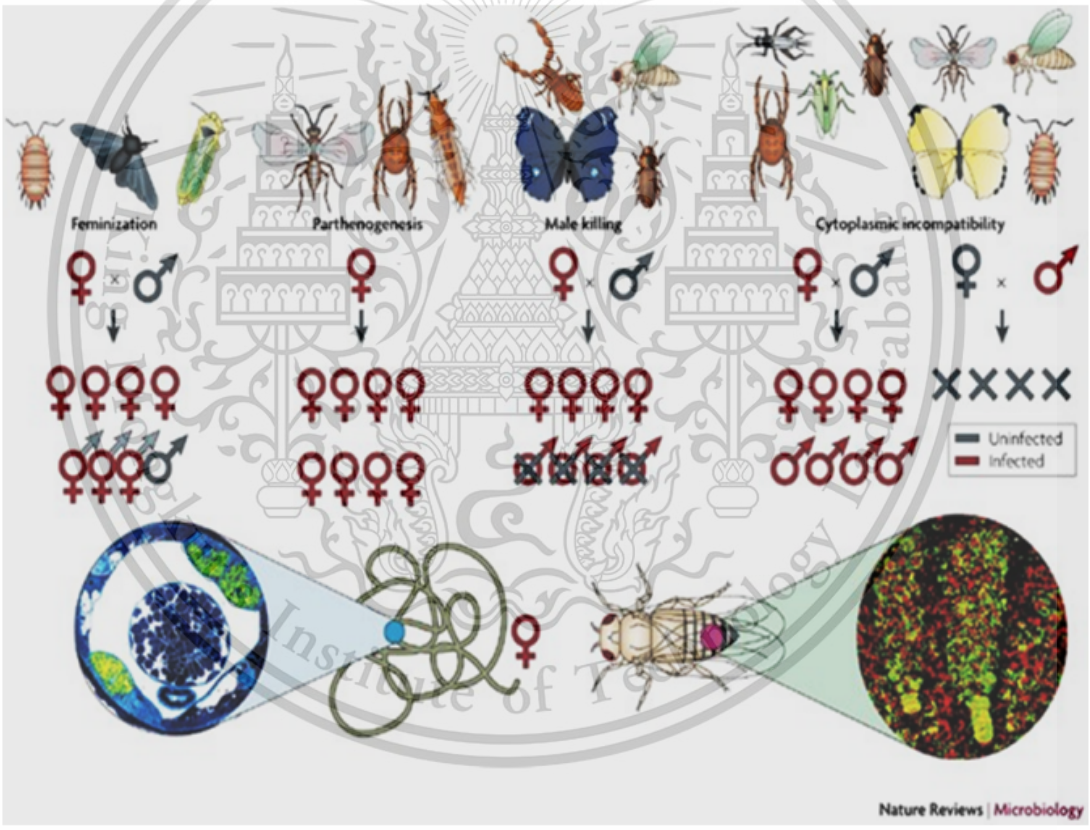

Fig. 2.7 *Wolbachia* - induced phenotypes.
(Source: Werren *et al.* 2008)

2.6 Guidelines for the application of *Wolbachia* bacteria to control pest outbreaks

Currently, *Wolbachia* bacteria. It has attracted widespread interest from scientists in many countries around the world, including Thailand. In the study, the role in controlling outbreaks of insects, pests, and carriers of many diseases by a butterfly in Order Lepidoptera It is one of the ranks of insects with the most biologically diverse and plays an important role in the ecosystem. However, a Study of the role of *Wolbachia* bacteria in butterflies and moths found in Kaeng Krachan National Park, Phetchaburi Province, and Khao Yai National Park, Nakhon Ratchasima Province hasn't been studied before. Which has relevant research details as follows: (Zabalou *et al.* 2004). The use of *Wolbachia* has been studied to control *Ceratitis capitata*. The study authors created a population infected with *Wolbachia* by transferring two *Wolbachia* strains, *w*Cer2 and *w*Cer4, from *Rhagoletis cerasi.* Collected from Austria and Italy. *C. capitata* were introduced by microinjection and mating between 100 females without *Wolbachia* aged 2-3 days and 100 males aged 1 day. Then, count the hatchability rate. It was found that mating pairs between female insects without *Wolbachia* and male insects with *Wolbachia*, as well as hybrids between females and male insects with different species of *Wolbachia*, increased the mortality rate of embryos. The hybrid between male and female insects with the same species of *Wolbachia* had an embryo mortality rate of 65%, and the hybrid pair between female insects with *Wolbachia* and male insects without *Wolbachia* had a normal embryo mortality rate of about 32%. (Tagami *et al*. 2006). Study the use of *Wolbachia* to control *Liriomyza trifolii*, a major insect pest of vegetable and ornamental plants grown around the world. The study collected samples of leafworm flies from Hamamatsu City. Hamamatsu, Shizuoka Prefecture, Japan. then raised in the nut plant at 25 °C, providing 16 hours of light and 8 hours of darkness. To collect adult insects born within 24 hours, eat bee juice mixed with 50 mg/g tetracycline hydrochloride for 1 day for *Wolbachia*. afterward match insects in plastic cups for 2 days (diameter 12 cm, height 9.5 cm). Remove the adult offspring and provide water containing 20 mg/ml tetracycline hydrochloride foliar plants feed until insects develop into adults by giving antibiotics at least 2 generations. Female insects extract DNA and use PCR in *Wolbachia* detection to confirm *Wolbachia* results. The

study authors divided insects into three groups: insects with *Wolbachia*, insects without *Wolbachia*, and insects classified with *Wolbachia* using tetracycline hydrochloride. Each group of insects is then bred for CI testing. The pupae are collected from each group and mated in plastic cups. After 3 days of Moving male and female pastes out of the cup, the PCR test found that the hybrid between female insects without *Wolbachia* and male insects with *Wolbachia* had about 20 eggs and an incubation rate of 0.83%. Including a hybrid between a female *Wolbachia* and a male *Wolbachia* with about 23 eggs. And it has an incubation rate of 1.45% at a relatively low percentage of hatchability due to females without *Wolbachia* and females who carry *Wolbachia* when mixed with males with *Wolbachia* do not hatch. The hybrid pair between female insects without *Wolbachia* and male insects without *Wolbachia* had approximately 27 eggs and a hatchability rate of 96.37%, as well as a hybrid pair between female insects with *Wolbachia* and males without *Wolbachia* with approximately 50 eggs and a hatchability rate of up to 98%. (Walker *et al*. 2011). Study on the effect of *Wolbachia* on increasing the amount of dengue virus in Aedes mosquitoes by transferring 2 strains of *Wolbachia*, namely *w*Mel and *w*MelPop-CLA, from *D. melanogaster* into Aedes mosquitoes by microinjection and then feeding the population. It was found that Aedes mosquitoes without *Wolbachia* had a dengue virus load. As much as 80.2%, Aedes mosquitoes with *Wolbachia* both *w*Mel and *w*MelPop-CLA strains have a 0% Denmark virus load. Increasing the amount of dengue virus inside the mosquito gives the virus There is not enough quantity to transmit to humans. (Dao – Hong zhu and Shao – gao, 2021). This is a study exploring the prevalence of *Wolbachia* in butterflies from southern China, studying the prevalence of *Wolbachia* in 52 species of butterfly from 5 families collected in southern China. *Wolbachia* infections were found in 18 of the 52 strains 35%: members of the family Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae and Pieridae. *Wolbachia* STs indicate that they belong mainly to supergroup B, which is found in 2 Ypthima spp. belonging to supergroup A. The most widespread species in our specimens, ST-41, was detected in seven butterfly species from four families. (Ilinsky *et al.* 2017). *Wolbachia* infection was studied in 120 species of the 13 Lepidoptera family, main butterflies from Western Siberia, according to sequential printing. Multilocus (MLST) and *wsp* locus and conduct a comprehensive survey on the distribution of *Wolbachia*

and genetic diversity in Lepidoptera around the world. It was found that genetic analysis of the *Wolbachia* strain revealed MLST allele cores in lepidopteran hosts worldwide, including ST-41 allele content.

2.7 Polymerase chain reaction or PCR

PCR is the basic molecular biology technique (Biomolecules) used to increase the number of chemicals or DNA in vitro from the amount of DNA used as a template (DNA template) just a little until the productions are billions of molecules which can be widely used such as medicine, research, industry and agriculture, and history Component of PCR consist of

1) DNA Template or specific DNA prototype that needs to increase the volume start in the range of 10-50 ng/reaction

2) Primers are oligonucleotides. A short string with a length of about 20-24 bp and an element of G and C between 40-60% will be the starting point for the creation of a new DNA line in the DNA template or gene range we want. The final concentration in the PCR reaction of each primer ranged from 0.1-2.0 μM

3) DNA polymerase. It is an enzyme used to create new DNA strands or PCR products in the desired gene position by applying deoxynucleotide triphosphate (dNTP), i.e. base A, T, C G, to the next position from the primers on each side with phosphodiester bonds, specific to the underlying DNA in direction 5' 3' (polymerase activity). In addition, there is another characteristic polymerase enzyme, which is to detect errors in the formation of new DNA strands (5' 3' exonuclease) or proofreading activity, the enzyme used must be effective at working at high temperatures, the most common enzyme in PCR applications is Taq DNA polymerase.

4) Deoxynucleotide triphosphate (dNTPs) It consists of dATP, dTTP, dCTP and dGTP, which are nucleo tides A, T, C, G that will be used to create a new line of PCR products.

5) PCR buffer and other elements in PCR reactions by PCR buffer will allow the PCR reaction to occur effectively, with the buffer corresponding to the type of DNA polymerase used, which is normally prepared as a concentration of 10 times the actual use (10X), in which the user must put 1 in 10 of the total volume in the PCR reaction so that the final concentration is 1X.

Another important element is $MgCl_2$, which acts as a co-factor of polymerase enzymes and contains Effect on reaction performance in general, the final concentration of MgCl2 used in PCR reactions is 1.5 mM, but in actual work, operators can adjust the concentration to be in the range of 1-5 mM, taking into account the

characteristics of the template using the primers used, as well as the optimal conditions when a new PCR is performed. The reaction of PCR consists of 3 main steps:

1) Denaturation uses a high temperature around 94-95 °C for 30-60 seconds to make DNA unscrew the pair from each other into a single wire and act as a model for DNA synthesis.

2) Annealing uses a temperature of 50-65 °C for about 30-60 seconds so that the primer binds to the DNA template in the area where the sequence matches.

3 Extension uses a temperature of 70-75 °C for approximately 30-120 seconds depending on the length of the DNA wanting to increase at this stage, the DNA polymerase acts as a base (A, T, C, G) for matching DNA the template connects to the ends of both primers to get a new DNA line (Fig. 2.8)

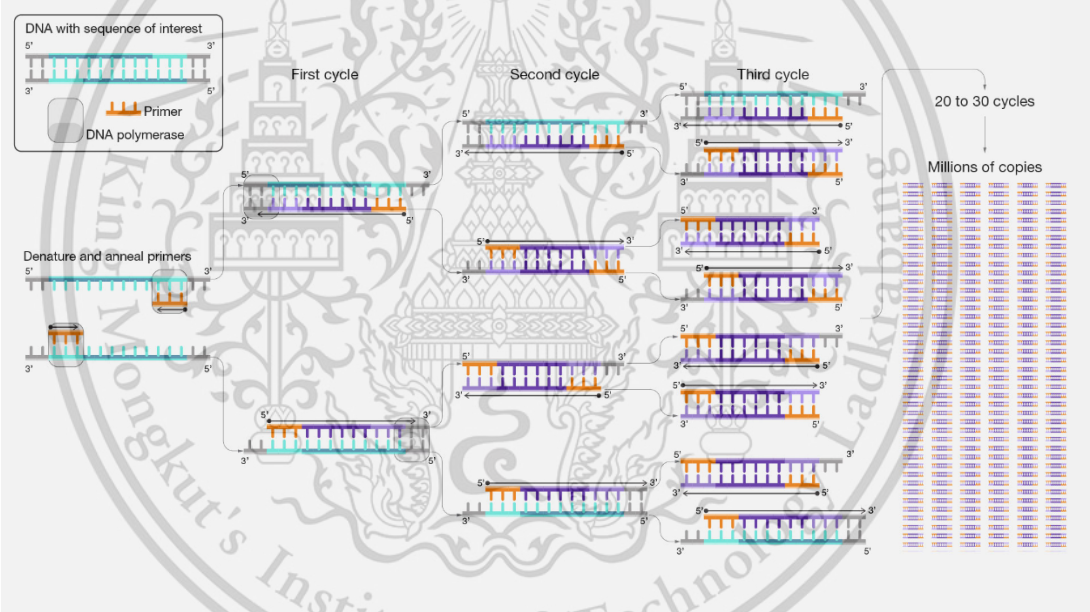

Fig. 2.8 Polymerase chain reaction

(Source: https: www.genome.gov)

At the beginning of the PCR reaction (before entering the denaturation step), the temperature is increased to the temperature of the denaturation procedure or slightly higher (up to 95 degrees Celsius) for a period of at least 3-5 minutes (called pre denaturation step or initial denaturation step) or perhaps more but not more than 15 minutes. (If activation Taq DNA polymerase is required) by following the manual of

each company used. For example, to help the DNA cord loosen better. This is especially true in DNA templates with a lot of GC base. In addition, it is also the activation period of some DNA polymerase enzymes, such as hot start Taq DNA polymerase, before entering the PCR reaction. Another temperature is called final extension or final elongation step, which uses the temperature during extension or elongation step for 5-15 minutes to ensure the reaction is generated. The DNA strands in each of the forward and reverse primers are complete, as well as to increase the stability of the newly synthesized PCR product. In general, the PCR condition is determined at the multiplier. Automatic genetic material (PCR machine).

2.8 Qualitative Polymerase Chain Reaction (qPCR)

It is a technique to increase the amount of nucleic acids from highly sensitive and convenient analytical samples, making it widely used Currently, PCR techniques are applied in laboratory diagnostics such as viral and bacterial infections. Genetic diseases and cancers, including applications in forensic science, molecular biology, etc. Since conventional PCR cannot perform quantitative analysis, although various forms of quantitative PCR have been developed, most of the methods are quite cumbersome in practice. Large sample analysis because there is a chance of mistakes and readings with reduced accuracy and low reproducibility. The demand for quantitative analysis in various tasks is increasing, such as treatment monitoring, diagnosis, etc. This has led to the development of real-time PCR techniques, which are the development of technology to detect PCR products in solutions using fluorescence reporters, and the development of thermal cyclers as real time thermal cyclers. Therefore, real-time PCR is an increase in DNA volume by measuring the actual amount of PCR products at that time, as opposed to conventional PCR that detects PCR products after the reaction is expanded.

Real-time PCR or Quantitative PCR (qPCR) is a technique developed from traditional PCR using fluorochrome fluorochrome labeling as a probe to label DNA in the primer stage. This makes it possible to quantify the initial target DNA from what needs to be measured, and to measure the amount of DNA that increases immediately, without having to wait for the process to finish. The real-time PCR method is a method of determining the amount of DNA that increases in each PCR reaction, resulting in a real increase in the amount of DNA from the value of the exponential phase obtained from the beginning of the target DNA. There are 5 types of phosphor substances used in Real-Time PCR consist of

1. Use colors that can be inserted with DNA The color samples used are SYBR-Green I Dye (SG), which is a fluorescence color that can bind to the minor groove of the double-stranded DNA. When this substance is stimulated by ultra-violet light, the energy is released into fluorescence light during denature to loosen the DNA strands from double strands into single strands. But when new DNA synthesis begins, SG begins to insert itself into DNA. When the PCR cycle reaches the denature phase again, the SG will fall out of the DNA, causing the glow to decrease again. In the case of multiple

DNA fragments in the sample, fluorescence can be separated by comparing Tm values, which is the optimum temperature for matching DNA strands with a matching bass group, because Tm is a unique feature of each pair of DNA, which corresponds to the percentage of base G and C in the DNA strand (%GC content) Fig 2.9

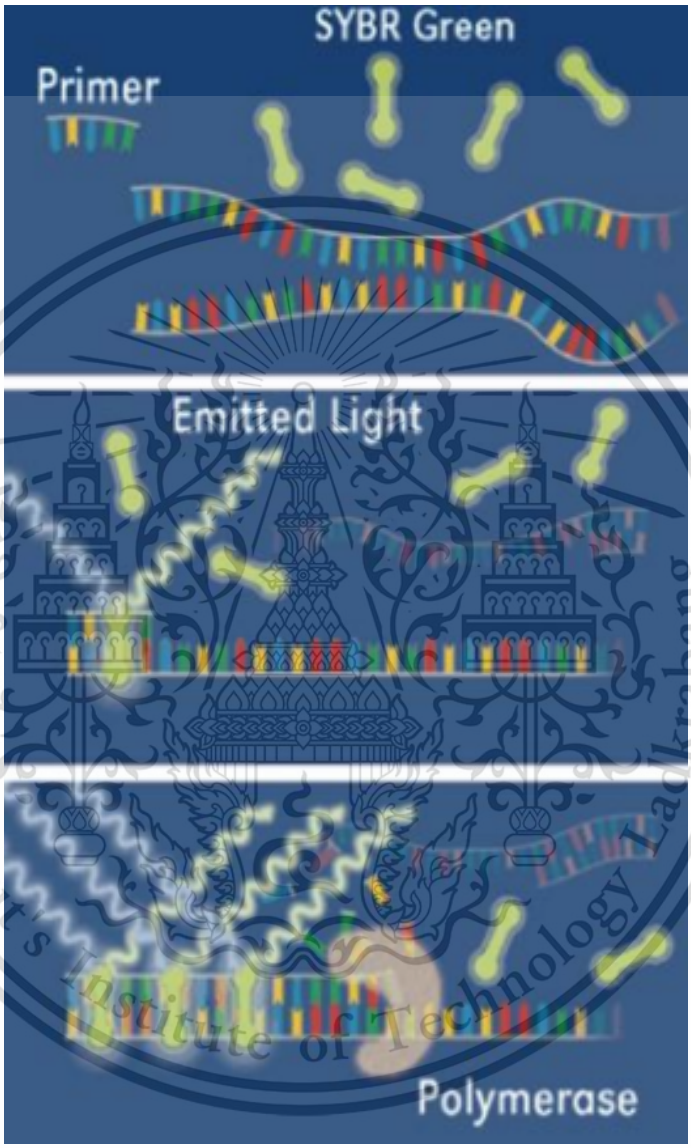

Fig. 2.9 SYBR Green I

(Source: Wechprasit, 2015)

2. Hydrolysis probes: probe. It is characterized by dual-labelled probe using a probe labeled with fluorescence color, which is a fluorescent substance this method is used when high specificity is required, which using SG is not possible. When the

probe is stimulated by high-energy light, the first fluorochrome (Quencher) absorbs energy and transfers energy to the second fluorochrome (Reporter Dye) without losing energy to the external system. When Reporter receives energy from Quencher, it releases energy into the external system in the form of light. When an amplify target sequence occurs the enzyme DNA polymerase, which has the properties of 5' exonuclease activity, hydrolyse probe, causes the separation of Reporter and Quencher fluorophore, So the fluorescence of the reporter fluorophore is measured during each cycle of the PCR cycle, fluorescence light increases as more and more independent fluorophore reporters accumulate and used in hybridization, there are 3 types as follows:

2.1 TaqMan hybridization probes. It is a single line probe consisting of reporter and quencher, where reporter dye is fluorescence, captured at the end of 5′ of the probe, about 5′ away from the end of 5′ fluorescence including of FAM : 6-carboxy fluorescence; TET : tetrachloro-6-carboxyfluorescein; HEX : hexachloro-6-carboxy fluorescein of quencher dye including of TAMRA (6-carboxytetramethylrhodamine) captured at the end of 3′ of the probe when hybridization, the fluorescein color of the reporter is stimulated (excite) and emits light (emit) in real-time PCR. When an extension reaction occurs, Taq DNA polymerase with 5′ nuclease activity cuts the reporter dye from the probe, causing the reporter dye to fall away from the quencher dye and can release energy in the form of fluorescence when stimulated by high energy. (Fig 2.10)

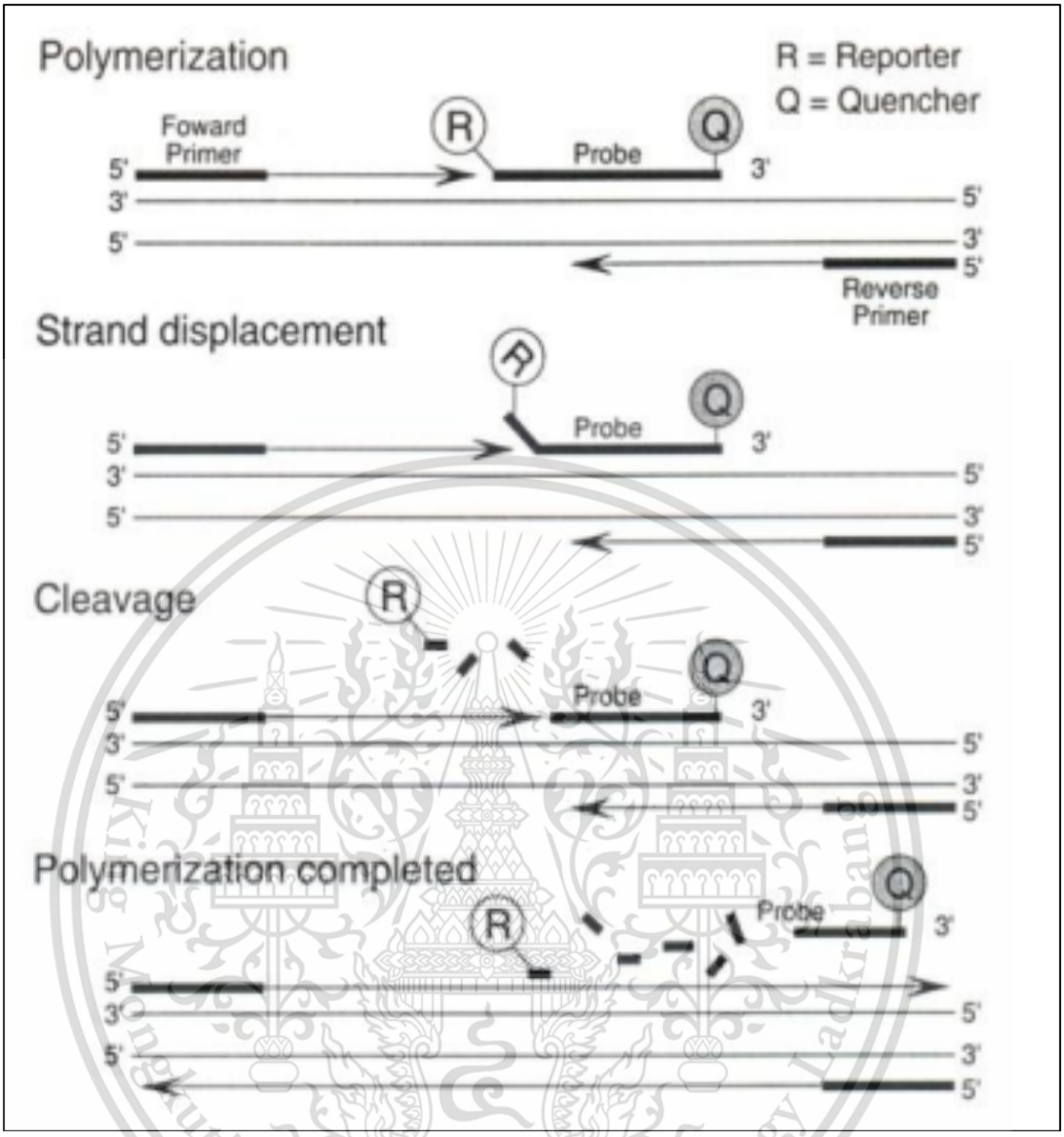

Fig. 2.10 TaqMan hybridization probes
(Source: Wechprasit, 2015)

2.2 Molecular beacon probes. It is a probe that is structured as a hairpin loop when it is not hybridization with the target DNA (selfcomplementary) this is stem sequence it is attached to a hydrogen bond of about 5-7 nucleotides and has a very GC bass pair, so that the end 5′ labeled with reporter fluor and the end 3′ labeled with quencher dye come close together until the quencher dye can absorb the energy from the reporter fluor. The hairpin area is created to have a bass sequence that matches the target DNA. If the probe is not hybridization with DNA, the target is in the

form of a hairpin loop, where there is no fluorescence this keeps Reporter Fluor away from Quencher Dye and releases fluorescence when stimulated by high energy. (Fig 2.11)

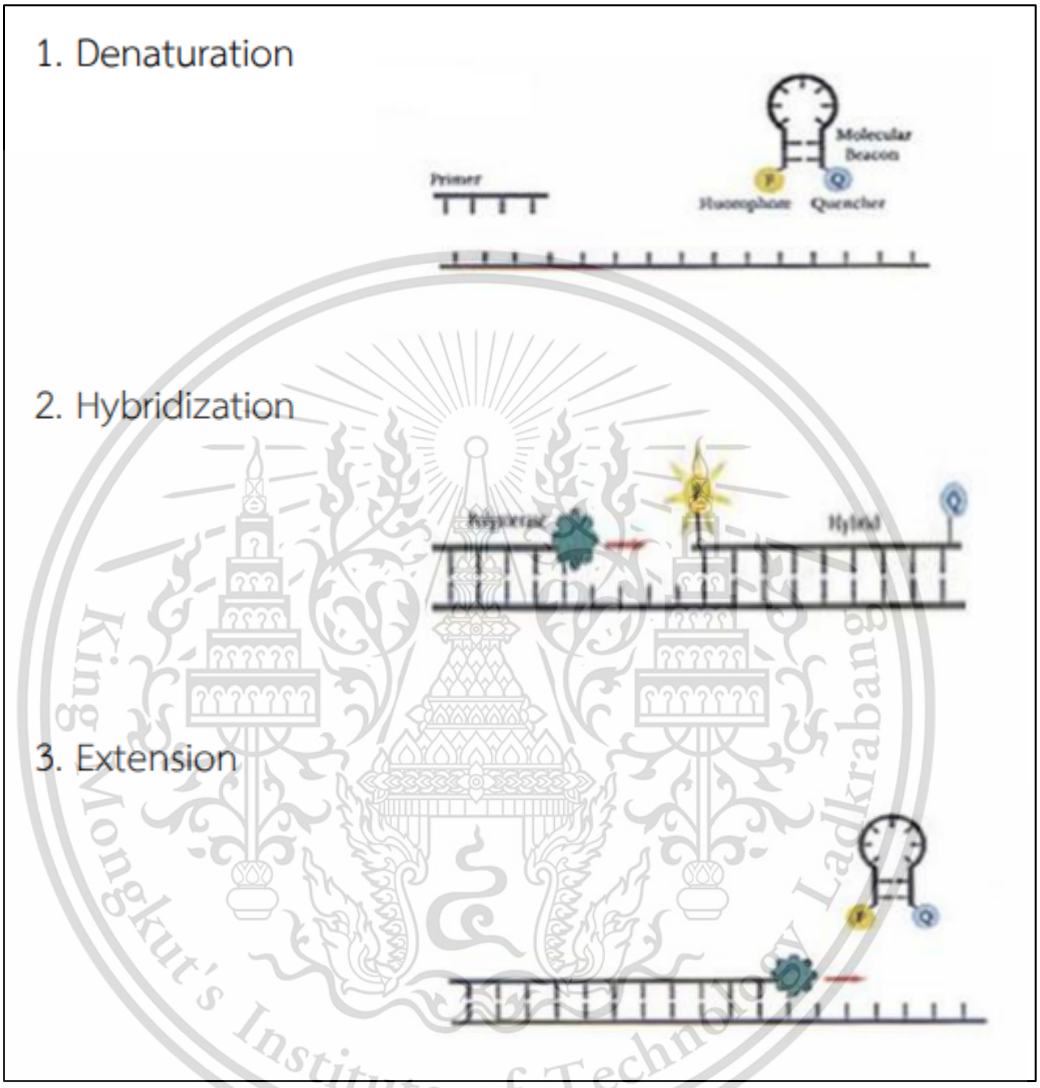

Fig. 2.11 Molecular beacon probes

(Source: Wechprasit, 2015)

2.3 Scorpion probes. It is a hairpin loop that is connected to the 5′ end of the primer. After the extension reaction, the probe can bind to a matching base within the target DNA molecule, causing the probe's hairpin loop molecule to open, no fluorescent quench, and an additional measurement signal is added Fig 2.12

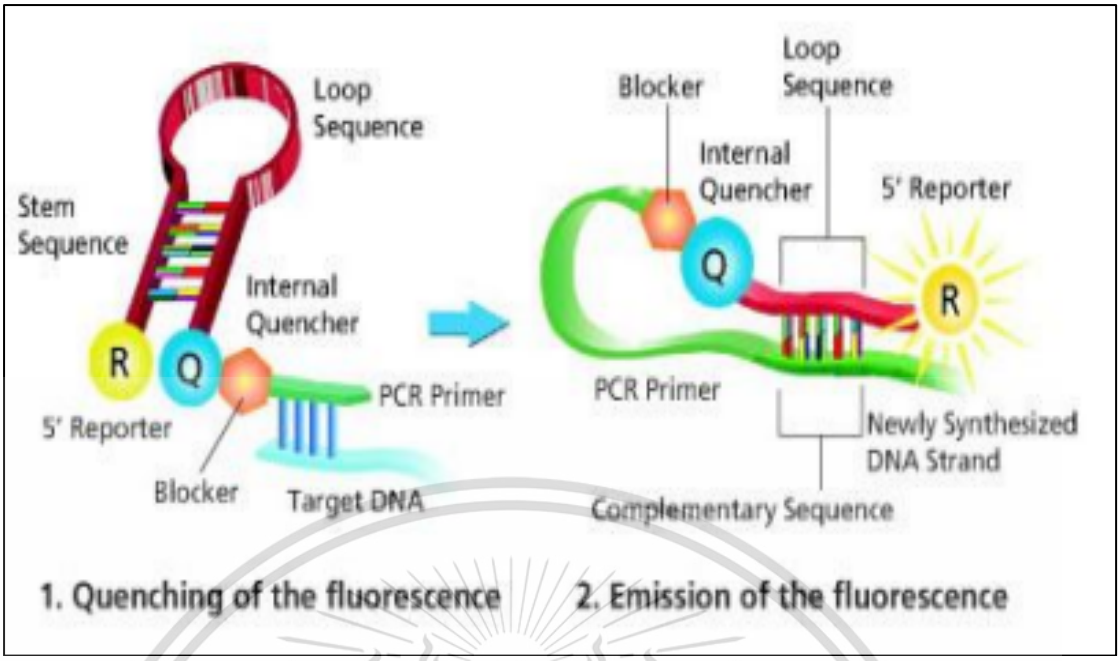

Fig. 2.12 Scorpion beacon probes

(Source: Wechprasit, 2015)

3. Hybridization probe or Fluorescence Resonance Energy Transfer (FRET) It is a probe that relies on the transfer of energy from fluorescence paint to another, with oligonucleotide with two specific base sequences, labeled with fluorescence color, the first line is upstream probe, which is a donor molecule at the end of 3′the other is a downstream probe, which is an acceptor molecule at the end of 5′ probe, designed to hybridize close to each other on the target DNA molecule, which causes fluorescence paint attached to the two probe molecules to come close together and transfer energy from the giver molecule to the receiver molecule. Emits signals at different wavelengths fluorescence light decreases in the giver molecule and increases in the receptor molecule Fig 2.13

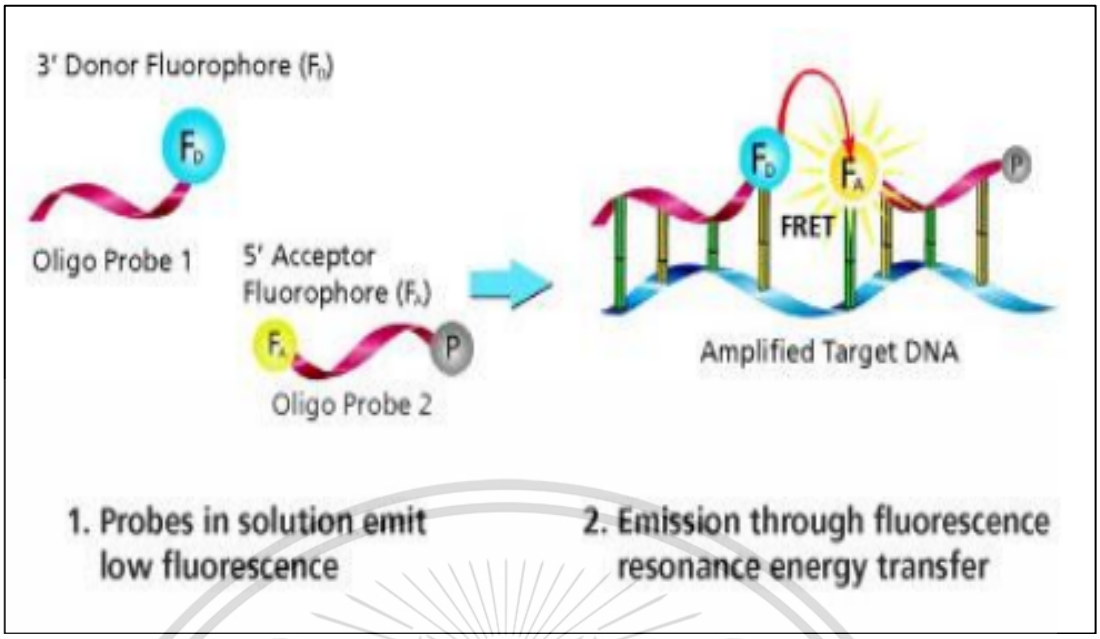

Fig. 2.13 Hybridization probe

(Source: Wechprasit, 2015)

The calculation of the initial DNA quantity can be performed by using standard DNA or target DNA with a known quantity to increase the quantity alongside the unknown DNA. The data obtained from standard DNA is used to create a calibration graph to compare with the target DNA and calculate the initial DNA content. Y-axis of calibration graph displays the amount of fluorescent light measured. The X-axis is a cycle number of PCR, the graph line is automatically generated based on data obtained by increasing the initial standard DNA volume, such as 10^6, 10^5, 10^4 copies. The real-time thermal cycler can detect the amplification signal that enters the linear log phase of each DNA sample at the crossing line after that a calibration graph is generated again during the number of cycles of the PCR process (Y-axis, cycle number). At the signal of increase in the number of target DNA that begins to enter the linear log phase (at the intersection of the crossing line). And standrad DNA concentration (X,log concentration) For samples that require the initial DNA quantity, the intersection of amplification signals displayed as the number of cycles of the PCR process on the calibration graph can be used to calculate the initial DNA content of the sample. Fig 2.14

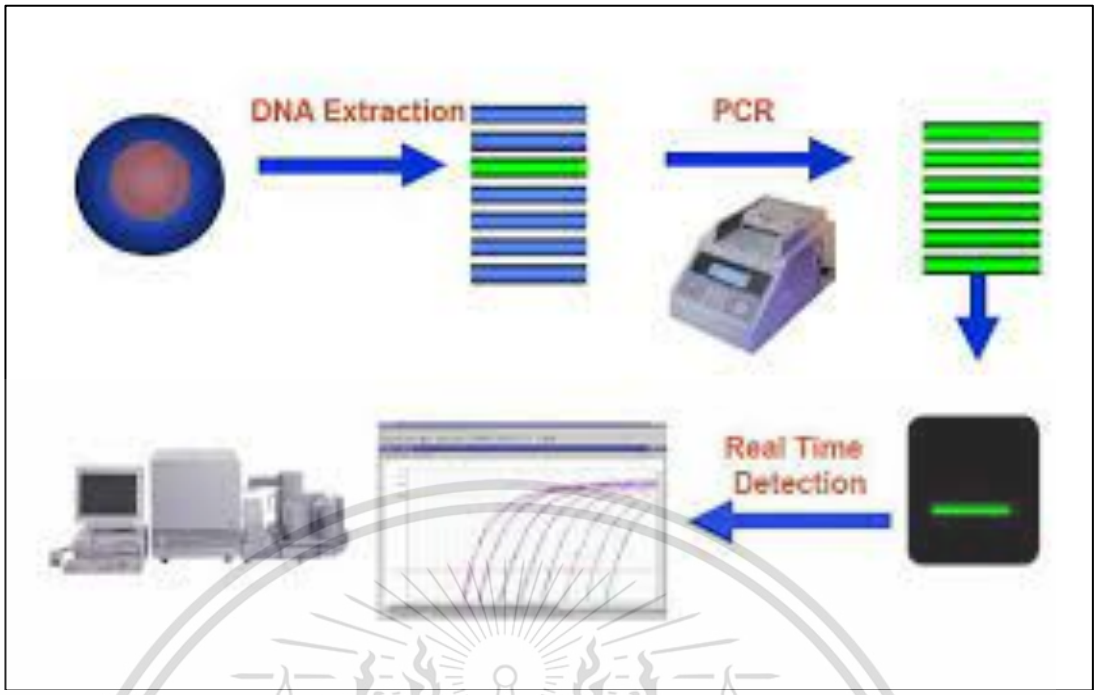

Fig. 2.14 The real-time thermal cycler

(Source: Wechprasit, 2015)

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Study area

Geographic distribution of the 5 field sites surveyed collected from 2 tropical forests in 3 different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand with different latitudes and longitudes temperate region of Thailand (Table 3.1).

Table 3.1 Geographical distribution of the 5 areas in two tropical forests of different latitudes and longitudes of Thailand.

Location	National Park	Region	Latitudes and longitudes
Pha Kuai Mai	Khao Yai	Northeast	14°26'04.02"N,101°23'45.41"E
Pha Diao Dai	Khao Yai	Northeast	14°21'58.56"N,101°24'10.72"E
Krok I Dok Waterfall	Khao Yai	Central	14°27'10.98"N,101°12'54.68"E
Ta Khor Waterfall	Khao Yai	Central	14°10'54.75"N,101°35'33.58"E
Phanoen Thung	Kaeng Krachan	West	12°49'31.55"N,99°21'47.51"E

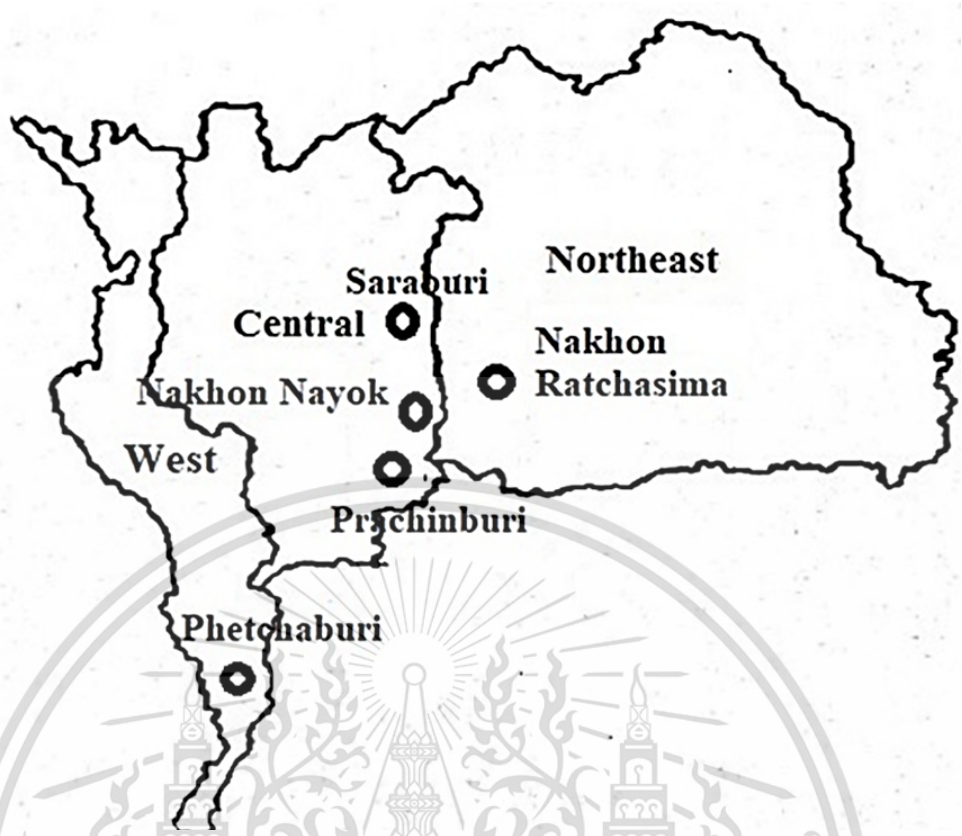

Fig. 3.1 Geographic distribution of the 5 field sites surveyed in 2 tropical forests from 3 different geographic regions including Khao Yai National Park (the Central and the Northeast regions including 4 provinces - Saraburi, Nakhon Nayok, Nakhon Ratchasima and Prachinburi) and Kaeng Krachan National Park (the West region including 1 province - Phetchaburi) of Thailand.

3.2 Insect collection

The butterflies and moths used in this study were collected from 2 tropical forest ecosystems in 3 regions (Central, Middle, and Female). These include Khao Yai National Park (Central and Northeastern) and Kaeng Krachan National Park (West). Using a light trap. It has been morphologically identified at the species (or genus) level by researcher Mr. Paradorn dokchan using a guide to photographing butterflies and moths in Thailand. (Suwannaphak, 2012) at the Department of Entomology, Faculty of Agriculture, Kasetsart University. The abdomen of butterflies and moths is removed and preserved in absolute ethanol at -20 °C.

3.3 Extraction of DNA

Remove all ethanol from the soaked butterfly sample. Then Take a sample on the belly of the butterfly, grind it thoroughly, and place it in a 1.5 ml centrifuge tube. add Extraction Solution 200 µl to allow the decomposition of the cell wall, bacteria, and DNA will drift in the solution together with various cell fragments add Proteinase K 20 µl to decompose proteins included in the solution. It is then used to homogenize the substances in vitro to maximize the reaction using a Vortex machine and heat to 56 °C for 30 minutes. Then take it to the centrifuge at 14,000 rpm for 5 minutes. The suction of the clear solution was put in a new 1.5 ml microcentrifuge tube. Add RNase A 4 µl to digest proteins and RNA mixed in the solution. Then place it at room temperature for 5 minutes. Add Binding Solution 200 µl. Shake to vortex machine and heat to 70 °C for 10 minutes. After that, add 95% Ethanol 200 µl blend to a homogeneous consistency and suck the clear solution into Spin Colum and bring it to the centrifuge at a speed of 14,000 rpm for 1 minute. Add a Wash solution of 700 µl for removing protein and RNA residues on the filter of the column. And take it to Centrifuge 14,000 rpm 1 for 1 minute and move the Spin Colum into a 1.5 ml centrifuge tube. Add pre-heat Elution solution Which is a highly concentrated salt solution. For making DNA escape from the filter in the column down to a 1.5 ml micro-centrifuge tube. Centrifuge at 14,000 g rpm for 1 minute. (Fig. 3.4). Measure the concentration of DNA exaction by nanodrop spectrophotometer for DNA

concentration, DNA purity and DNA quality take DNA to measure OD and find the ratio of OD 260/280 DNA and the extracted DNA was kept at -20 °C for later use. One microliter of the supernatant was used to PCR-screen for *Wolbachia*.

Fig. 3.2 DNA exaction and preparation.

(Source: Panukorn boonsit, 2021)

3.4 Polymerase Chain Reaction (PCR) Amplification

DNA extracted from all collected samples is quality checked using PCR (Werren *et al.* 1995). All samples were screened for *Wolbachia* detection using *wsp* (Zhou *et al.* 1998), *16S rRNA* (O'Neill *et al.* 1992), and *ftsZ* genes (Baldo *et al*. 2006). Contamination was checked using double distilled H_2O as a negative control. PCR expansion is done on the heating circuit using a reaction mixing volume of 20 containing: 2 µl 25 mM $MgCl_2$, 10x 2 µl PCR buffer, 20 µM forward and reverse primers, 1.5 µl (10 mM) dNTPs each) and 1 unit of Taq DNA polymerase. The PCR protocols of *wsp* and *16S rRNA* primers include denaturation starting at 94 °C for 3 minutes, followed by 35 cycles with denaturation procedure at 94 °C for 1 minute, annealing at 55 °C for 1 minute, expansion at 72 °C for 2 minutes, and final expansion at 72 °C for 10 minutes. The *ftsZ* protocol for PCR combines denaturation at 94 °C for 3 minutes, followed by denaturation 10 cycles at 94 °C for 10 seconds, annealing at 65 °C for 30 seconds, and amplification at 68 °C for 1 minute, followed by 25 cycles with denaturation steps at 94 °C for 10 seconds, annealing at 65 °C. for 30 seconds and an extension at 68 °C for 1 minute. Each 10 µl of PCR product runs on 1% agarose gel with 1 kb ladder to check for the presence and size of the expanded DNA.

3.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The qPCR method was performed to determine the density of *Wolbachia* in individual butterflies and moths using SYBR green for fluorescence detection in Light Cycler 480 systems. 1 pair of *Wolbachia ftsZ* primers, consisting of *ftsZ* (*77 Bf1* and *ftsZ 77 Br1*) that amplifize 111 bp of the *ftsZ* gene in PCR products, are used for quantity. (Noda *et al*. 2001). The insect host single copy gene, which increases the number of cell nuclear antigens (*PCNA*), is quantified so that it can be standardized for density comparisons between species. One pair of *PCNA* primers (*PCNAF* and *PCNAR*) are used for testing. Each run consists of a set of standard DNA samples for unknown DNA sample volumes (double replication of each sample) and ddH_2O is a negative control to check for contamination. The reaction mixture in each well is a total volume of 25 µl, consisting of DNA samples, 200 mM dNTP, 50 nM SYBR green, 300 nM primer, 0.2 U AmpErase UNG, 5 mM $MgCl_2$, 0.6 U Ampli Taq Gold and 8% glycerol. The qPCR temperature profile is 50 °C for 2 minutes to run AmpErase UNG and 95 °C for 10 minutes to activate Ampli Taq Gold, followed by 40 cycles of 95 °C. for 15 seconds and 60°C for 1 minute. For each standard reaction, DNA as well as dilute kits of PCR products (10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies/µl) were included to estimate the absolute number of copies of the target sequence in the DNA. In the preparation of standard samples, the gel DNA of PCR products is cut off and purified by Wizard SV. The copy number of the DNA standard sample is evaluated by concentration measured with a spectrophotometer and calculated based on the molecular weight data of the nucleotides presented. An absolute quantitative analysis using the second derivative maximum method used in Light Cycler Tool operator software version 1.5 (Roche) was used to analyze the qPCR data obtained in this study.

CHAPTER 4

RESULTS

4.1 *Wolbachia* infection in moths and distribution in tropical forest ecosystems.

Wolbachia infection in moths and their distribution in tropical forest ecosystems. The data was collected from field surveys in 5 provinces (Saraburi, Nakhon Nayok, Nakhon Ratchasima, Prachinburi and Phetchaburi) from 2 tropical forests in 3 areas, namely Khao Yai National Park (Central and Northeastern) and Kaeng Krachan National Park (Western Region) from Thailand. The results showed that 625 people (male = 209 and female = 416) from 1,235 (sample) (50.61%) or 28 of 58 species (48.28%) from Thailand's three populations tested positive for *Wolbachia* infection. *wsp* and *ftsZ* PCR products are around 600 bp while *Wolbachia's 16S rRNA* PCR products are around 900 bp. Similar PCR results were obtained from all three pairs of primers, although there were slight, but insignificant differences among them. In a total of 625 positive samples of *Wolbachia*, they included 28 species from 9 families, such as: Crambidae (1, 18), Endromidae (2, 52), Erebidae (2, 63), Geometridae (10, 233), Lasiocampidae (1, 11), Lymantriidae (1, 19), Noctuidae (4, 69), Sphingidae (6, 140), Uraniidae (1, 20) (The number in parentheses indicates the number of positive species in each family and the number of positive specimens in each family, respectively). Distribution of *Wolbachia* using PCR in 58 moth species from 13 families in Lepidoptera included 1,235 specimens collected from two tropical forests in three different geographical regions. (C = Central, NE = Northeast and W = West) from Thailand: species name, family, location (province). Geographical regions the number of samples tested, the number of positive samples (% infection) and the number of samples (by gender) are shown in Table 4.1.

The 28 *Wolbachia*-infected moth species included Abraxas sp., *Acosmeryx shervillii,* Ambulyx sp., Artena sp., *Buxura inoui, Celenna festivaria, Cleora determinate,* Comibaena sp., *Creatonotos transiens, Daphnis hypothous, Herochroma flavibasalis,* Hippotion sp., *Ischyja marapok, Lyssa zampa, Mustilizans dierli, Mustilia sphingiformis, Olene mendosa,* Olepa sp., Ourapteryx sp., *Oxyodes scrobiculata, Pergesa acteus, Parotis marginate, Plutodes flavescens, Ruttellerona pallicostaria, Thyas honesta ,* Theretra sp., *Testa montana* and Trabala sp. was listed in Table 4.2 For *Wolbachia*-uninfected species, *A. plana* had the highest number of specimens collected in this study (25 individuals) whereas *P. bicolor*, the green rice moth in the family Limacodidae, had the lowest number of collected specimens (6 individuals). The number, family, and species name of *Wolbachia*-infected specimens of 9 families in the order Lepidoptera across geographic regions (C = the Central, NE = the Northeast, and W = the West) in Thailand in Table 4.3.

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown.

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Abraxas sp.*	Geometridae	Saraburi	C	11	9 (81.81)	3	6	0	2
		Nakhon Nayok	C	10	7 (70.00)	4	3	1	2
		Phetchaburi	W	18	16 (88.89)	7	9	1	1
Acherontiia styx	Sphingidae	Nakhon Ratchasima	NE	8	0	0	0	3	5
		Phetchaburi	W	4	0	0	0	1	3
		Prachinburi	C	5	0	0	0	3	2
Acosmeryx	Sphingidae	Prachinburi	C	3	0	0	0	1	2
sericeus		Phetchaburi	W	7	0	0	0	3	4
Acosmeryx	Sphingidae	Nakhon Ratchasima	NE	8	6 (75.00)	2	4	1	1
shervillii		Phetchaburi	W	15	13 (86.67)	6	7	2	1
		Nakhon Nayok	C	9	7 (77.78)	3	4	0	2

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Amata sp.	Erebidae	Prachinburi	C	5	0	0	0	2	3
		Nakhon Ratchasima	NE	7	0	0	0	1	6
		Saraburi	C	4	0	0	0	2	2
Ambulyx sericeipennis	Sphingidae	Nakhon Nayok	C	8	0	0	0	3	5
		Phetchaburi	W	11	0	0	0	5	6
Ambulyx sp.	Sphingidae	Saraburi	C	7	3 (42.85)	1	2	1	3
		Nakhon Ratchasima	NE	12	8 (66.67)	2	6	2	2
		Phetchaburi	W	14	11 (78.57)	4	7	2	1
Antheraea assamensis	Saturniidae	Prachinburi	C	6	0	0	0	3	3
		Nakhon Nayok	C	9	0	0	0	4	5
		Phetchaburi	W	8	0	0	0	4	4
Antheraea frithi	Saturniidae	Nakhon Nayok	C	7	0	0	0	2	5
		Phetchaburi	W	3	0	0	0	0	3

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
		Nakhon Ratchasima	NE	5	0	0	0	1	4
Artena sp.	Noctuidae	Phetchaburi	W	8	6 (75.00)	2	4	0	2
		Nakhon Nayok	C	3	2 (66.67)	0	2	1	0
		Nakhon Ratchasima	NE	5	4 (80.00)	1	3	1	0
Asota plana	Erebidae	Saraburi	C	7	0	0	0	2	5
		Phetchaburi	W	12	0	0	0	4	8
		Prachinburi	C	6	0	0	0	2	4
Attacus atlas	Saturniidae	Nakhon Ratchasima	NE	8	0	0	0	2	6
		Phetchaburi	W	7	0	0	0	3	4
		Nakhon Nayok	C	3	0	0	0	2	1
Brahmaea	Brahmaeidae	Phetchaburi	W	5	0	0	0	1	4
hearseyi		Nakhon Ratchasima	NE	7	0	0	0	2	5
		Prachinburi	C	4	0	0	0	1	3

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Buxura sp.	Geometridae	Nakhon Nayok	C	6	0	0	0	2	4
		Phetchaburi	W	4	0	0	0	2	2
		Nakhon Ratchasima	NE	8	0	0	0	3	5
Buxura inoui	Geometridae	Phetchaburi	W	13	11 (84.61)	5	6	0	2
		Nakhon Nayok	C	6	3 (42.85)	1	2	1	2
		Prachinburi	C	5	4 (80.00)	1	3	1	0
Celenna	Geometridae	Phetchaburi	W	15	13 (86.67)	5	8	0	2
festivaria		Nakhon Ratchasima	NE	10	6 (60.00)	2	4	3	1
		Nakhon Nayok	C	6	4 (66.67)	1	3	1	1
Cleora	Geometridae	Nakhon Ratchasima	NE	7	3 (42.85)	1	2	1	3
determinata		Phetchaburi	W	11	7 (63.63)	2	5	2	2
		Saraburi	C	5	2 (40.00)	0	2	1	2

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Cleora	Geometridae	Saraburi	C	5	0	0	0	2	3
tenebrata		Phetchaburi	W	8	0	0	0	3	5
		Nakhon Ratchasima	NE	4	0	0	0	2	2
Comibaena sp.	Geometridae	Nakhon Nayok	C	7	4 (57.14)	1	3	1	2
		Nakhon Ratchasima	NE	9	6 (66.67)	2	4	0	3
		Phetchaburi	W	13	11 (84.61)	4	7	1	1
Creatonotos	Erebidae	Saraburi	C	8	6 (75.00)	1	5	0	2
transiens		Phetchaburi	W	18	16 (88.89)	6	10	1	1
		Nakhon Ratchasima	NE	11	9 (81.81)	3	6	2	0
Daphis	Sphingidae	Phetchaburi	W	21	19 (90.47)	7	12	1	1
hypothous		Nakhon Nayok	C	9	6 (66.67)	2	4	1	2
		Nakhon Ratchasima	NE	13	11 (84.61)	4	7	2	0

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Dudusa vethi	Notodontidae	Phetchaburi	W	6	0	0	0	2	4
		Nakhon Nayok	C	2	0	0	0	2	0
		Prachinburi	C	4	0	0	0	1	3
Dysphania	Geometridae	Nakhon Ratchasima	NE	2	0	0	0	0	2
militaris		Prachinburi	C	5	0	0	0	3	2
		Phetchaburi	W	7	0	0	0	2	5
Euhampsonia	Notodontidae	Saraburi	C	3	0	0	0	1	2
roepkei		Nakhon Ratchasima	NE	6	0	0	0	3	3
		Nakhon Nayok	C	4	0	0	0	3	1
Euplocia	Erebidae	Phetchaburi	W	5	0	0	0	2	3
membliaria		Prachinburi	C	3	0	0	0	1	2
		Nakhon Nayok	C	5	0	0	0	2	3

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Eudocima phalonia	Noctuidae	Nakhon Ratchasima	NE	4	0	0	0	2	2
		Saraburi	C	3	0	0	0	2	1
		Prachinburi	C	2	0	0	0	2	0
Fodina contigua	Erebidae	Nakhon Nayok	C	4	0	0	0	0	4
		Phetchaburi	W	7	0	0	0	4	3
		Nakhon Ratchasima	NE	5	0	0	0	2	3
Herochroma flavibasalis	Geometridae	Phetchaburi	W	19	17 (89.47)	6	11	0	2
		Prachinburi	C	6	4 (66.67)	2	2	1	1
		Nakhon Ratchasima	NE	15	13 (86.67)	4	9	1	1
Hippotion sp.	Sphingidae	Prachinburi	C	8	5 (62.50)	2	3	2	1
		Nakhon Ratchasima	NE	11	7 (63.63)	3	4	1	2
		Phetchaburi	W	7	4 (57.14)	1	3	1	2

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Ischyja marapok	Noctuidae	Nakhon Nayok	C	5	4 (80.00)	2	2	0	1
		Saraburi	C	9	6 (66.67)	1	5	2	1
		Phetchaburi	W	20	17 (85.00)	4	13	0	3
Lyssa zampa	Uraniidae	Nakhon Ratchasima	NE	8	5 (62.50)	2	3	1	2
		Phetchaburi	W	14	11 (78.57)	5	6	2	1
		Nakhon Nayok	C	7	4 (57.14)	1	3	1	2
Marumba	Sphingidae	Prachinburi	C	7	0	0	0	3	4
cristata		Nakhon Nayok	C	4	0	0	0	1	3
		Nakhon Ratchasima	NE	6	0	0	0	2	4
Mustilizans dierli	Endromidae	Phetchaburi	W	20	18 (90.00)	6	12	0	2
		Nakhon Ratchasima	NE	9	7 (77.78)	3	4	1	1
		Prachinburi	C	8	6 (75.00)	2	4	0	2

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Mustilia sphingiformis	Endromidae	Nakhon Ratchasima	NE	10	7 (70.00)	1	6	2	1
		Phetchaburi	W	12	9 (75.00)	2	7	1	2
		Nakhon Nayok	C	7	5 (71.42)	1	4	1	1
Neochera inop	Erebidae	Saraburi	C	4	0	0	0	3	1
		Prachinburi	C	2	0	0	0	0	2
		Phetchaburi	W	5	0	0	0	3	2
Neochera dominia	Erebidae	Nakhon Ratchasima	NE	3	0	0	0	1	2
		Nakhon Nayok	C	1	0	0	0	0	1
Olene mendosa	Lymantriidae	Nakhon Nayok	C	6	3 (50.00)	1	2	1	2
		Phetchaburi	W	14	12 (85.71)	5	7	1	1
		Prachinburi	C	5	4 (80.00)	0	4	0	1

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Olepa sp.	Erebidae	Nakhon Ratchasima	NE	12	10 (83.33)	3	7	2	0
		Phetchaburi	W	18	16 (88.89)	6	10	1	1
		Nakhon Nayok	C	8	6 (75.00)	1	5	0	2
Ourapteryx sp.	Geometridae	Nakhon Nayok	C	9	6 (66.67)	2	4	1	2
		Prachinburi	C	5	3 (60.00)	1	2	0	2
		Phetchaburi	W	16	14 (87.50)	3	11	1	1
Oxyodes	Noctuidae	Nakhon Ratchasima	NE	8	6 (75.00)	1	5	1	1
scrobiculata		Phetchaburi	W	10	8 (80.00)	2	6	2	0
		Saraburi	C	5	2 (40.00)	0	2	1	2
Parasa bicolor	Limacodidae	Nakhon Nayok	C	2	0	0	0	2	0
		Prachinburi	C	4	0	0	0	3	1

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Pergesa acteus	Sphingidae	Phetchaburi	W	13	11 (84.61)	4	7	1	1
		Nakhon Ratchasima	NE	10	8 (80.00)	3	5	2	0
		Nakhon Nayok	C	7	4 (57.14)	1	3	1	2
Pareuchaetes	Erebidae	Saraburi	C	2	0	0	0	0	2
pseudoinsulata		Phetchaburi	W	4	0	0	0	3	1
Parotis	Crambidae	Phetchaburi	W	11	7 (63.63)	3	4	1	3
marginata		Nakhon Ratchasima	NE	9	8 (88.89)	2	6	0	1
		Prachinburi	C	4	3 (75.00)	1	2	0	1
Prooedema		Nakhon Nayok	C	2	0	0	0	0	2
inscisala		Prachinburi	C	1	0	0	0	1	0
		Phetchaburi	W	5	0	0	0	2	3

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Plutodes	Geometridae	Nakhon Nayok	C	7	4 (57.14)	2	2	1	2
flavescens		Nakhon Ratchasima	NE	15	12 (80.00)	4	8	2	1
		Phetchaburi	W	18	16 (88.89)	5	11	1	1
Ramadosa pavo	Noctuidae	Saraburi	C	6	0	0	0	2	4
		Phetchaburi	W	10	0	0	0	2	8
Ruttellerona	Geometridae	Nakhon Ratchasima	NE	11	9 (81.81)	3	6	0	2
pallicostaria		Phetchaburi	W	15	13 (86.67)	5	8	1	1
		Prachinburi	C	7	5 (71.42)	3	2	1	1
Semiothisa	Geometridae	Nakhon Nayok	C	4	0	0	0	1	3
eleonora		Nakhon Ratchasima	NE	8	0	0	0	3	5
		Prachinburi	C	5	0	0	0	2	3

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Spodoptera litura	Noctuidae	Nakhon Ratchasima	NE	7	0	0	0	2	5
		Saraburi	C	3	0	0	0	0	3
		Phetchaburi	W	8	0	0	0	2	6
Sympis rufibasis	Noctuidae	Nakhon Nayok	C	4	0	0	0	2	2
		Phetchaburi	W	5	0	0	0	1	4
Thyas honesta	Noctuidae	Nakhon Ratchasima	NE	6	3 (50.00)	1	2	2	1
		Phetchaburi	W	11	9 (81.81)	4	5	0	2
		Prachinburi	C	5	2 (40.00)	0	2	1	2
Theretra sp.	Sphingidae	Phetchaburi	W	12	9 (75.00)	3	6	1	2
		Nakhon Nayok	C	5	2 (40.00)	0	2	0	3
		Nakhon Ratchasima	NE	9	6 (66.67)	2	4	2	1

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Testa montana	Geometridae	Prachinburi	C	4	2 (50.00)	2	0	1	1
		Phetchaburi	W	8	6 (75.00)	1	5	1	1
		Saraburi	C	6	4 (66.67)	1	3	2	0
Thosea siamica	Limacodidae	Nakhon Nayok	C	1	0	0	0	1	0
		Phetchaburi	W	3	0	0	0	2	1
Trabala sp.	Lasiocampidae	Phetchaburi	W	7	5 (71.42)	1	4	1	1
		Nakhon Nayok	C	5	3 (60.00)	2	1	2	0
		Nakhon Ratchasima	NE	6	2 (33.33)	0	2	1	3
Tarsolepis elephantorum	Notodontidae	Prachinburi	C	3	0	0	0	1	2
		Phetchaburi	W	5	0	0	0	2	3
		Nakhon Ratchasima	NE	4	0	0	0	2	2

Table 4.1 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera, included 1,235 samples collected. from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia.* * For positive samples, the number of positive samples by sex was shown. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Xanthomelaena schematias	Crambidae	Saraburi	C	2	0	0	0	0	2
		Phetchaburi	W	4	0	0	0	1	3
		Nakhon Nayok	C	1	0	0	0	1	0

Table 4.2 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera throughout the region in Thailand. The 28 species (625 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name.

Species name	Family	No. of samples tested	No. of positive samples (% infection)	No. of positive males (% infection)	No. of positive females (% infection)
Abraxas sp.*	Geometridae	39	32 (82.05)	14 (43.75)	18 (56.25)
Acherontiia styx	Sphingidae	17	0	0	0
Acosmeryx sericeus	Sphingidae	10	0	0	0
*Acosmeryx shervillii**	Sphingidae	32	26 (81.25)	11 (42.30)	15 (57.69)
Amata sp.	Erebidae	16	0	0	0
Ambulyx sericeipennis	Sphingidae	19	0	0	0
Ambulyx sp.*	Sphingidae	33	22 (66.67)	7 (31.81)	15 (68.18)
Antheraea assamensis	Saturniidae	29	0	0	0
Antheraea frithi	Saturniidae	15	0	0	0
Artena sp. *	Noctuidae	16	12 (75.00)	3 (25.00)	9 (75.00)
Asota plana	Erebidae	25	0	0	0
Attacus atlas	Saturniidae	18	0	0	0
Brahmaea hearseyi	Brahmaeidae	16	0	0	0
Buxura sp.	Geometridae	18	0	0	0
*Buxura inoui**	Geometridae	24	18 (75.00)	7 (38.89)	11 (61.11)

Table 4.2 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera throughout the region in Thailand. The 28 species (625 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name. (continued).

Species name	Family	No. of samples tested	No. of positive samples (% infection)	No. of positive males (% infection)	No. of positive females (% infection)
*Celenna festivaria**	Geometridae	31	23 (74.19)	8 (34.78)	15 (65.21)
*Cleora determinata**	Geometridae	23	12 (52.17)	3 (25.00)	9 (75.00)
Cleora tenebrata	Geometridae	17	0	0	0
Comibaena sp.*	Geometridae	29	21 (72.41)	7 (33.33)	14 (66.67)
*Creatonotos transiens**	Erebidae	37	31 (83.78)	10 (32.25)	21 (67.74)
*Daphis hypothous**	Sphingidae	43	36 (83.72)	13 (36.11)	23 (63.89)
Dudusa vethi	Notodontidae	12	0	0	0
Dysphania militaris	Geometridae	14	0	0	0
Euhampsonia roepkei	Notodontidae	13	0	0	0
Euplocia membliaria	Erebidae	13	0	0	0
Eudocima phalonia	Noctuidae	9	0	0	0
Fodina contigua	Erebidae	16	0	0	0
*Herochroma flavibasalis**	Geometridae	40	34 (85.00)	12 (35.29)	22 (64.70)
Hippotion sp.*	Sphingidae	26	16 (61.53)	6 (37.50)	10 (62.50)
*Ischyja marapok**	Noctuidae	34	27 (79.41)	**7** (25.92)	20 (74.07)

Table 4.2 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera throughout the region in Thailand. The 28 species (625 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name. (continued).

Species name	Family	No. of samples tested	No. of positive samples (% infection)	No. of positive males (% infection)	No. of positive females (% infection)
*Lyssa zampa**	Uraniidae	29	20 (68.96)	8 (40.00)	12 (60.00)
Marumba cristata	Sphingidae	17	0	0	0
*Mustilizans dierli**	Endromidae	37	31 (83.78)	11 (35.48)	20 (64.51)
*Mustilia sphingiformis**	Endromidae	29	21 (72.41)	4 (19.04)	17 (80.95)
Neochera inops	Erebidae	11	0	0	0
Neochera dominia	Erebidae	4	0	0	0
*Olene mendosa**	Lymantriidae	25	19 (76.00)	6 (31.57)	13 (68.42)
Olepa sp.*	Erebidae	38	32 (84.21)	10 (31.25)	22 (68.75)
Ourapteryx sp.*	Geometridae	30	23 (76.66)	6 (26.08)	17 (73.91)
*Oxyodes scrobiculata**	Noctuidae	23	16 (69.56)	3 (18.75)	13 (81.25)
Parasa bicolor	Limacodidae	6	0	0	0
*Pergesa acteus**	Sphingidae	30	23 (76.66)	8 (34.78)	15 (65.21)
Pareuchaetes pseudoinsulata	Erebidae	6	0	0	0

Table 4.2 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera throughout the region in Thailand. The 28 species (625 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name. (continued).

Species name	Family	No. of samples tested	No. of positive samples (% infection)	No. of positive males (% infection)	No. of positive females (% infection)
*Parotis marginata**	Crambidae	24	18 (75.00)	6 (33.33)	12 (66.67)
Prooedema inscisala	Crambidae	8	0	0	0
*Plutodes flavescens**	Geometridae	40	32 (80.00)	11 (34.37)	21 (65.62)
Ramadasa pavo	Noctuidae	10	0	0	0
*Ruttellerona pallicostaria**	Geometridae	33	27 (81.81)	11 (40.74)	16 (59.25)
Semiothisa eleonora	Geometridae	17	0	0	0
Spodoptera litura	Noctuidae	18	0	0	0
Sympis rufibasis	Noctuidae	9	0	0	0
*Thyas honesta**	Noctuidae	22	14 (63.63)	5 (35.71)	9 (64.28)
Theretra sp.*	Sphingidae	26	17 (65.38)	5 (29.41)	12 (70.58)
*Testa montana**	Geometridae	18	12 (66.67)	4 (33.33)	8 (66.67)
Thosea siamica	Limacodidae	4	0	0	0
Trabala sp.*	Lasiocampidae	18	10 (55.56)	3 (30.00)	7 (70.00)

Table 4.2 Distribution of *Wolbachia* using PCR in 58 moth species from 13 families, respectively, Lepidoptera throughout the region in Thailand. The 28 species (625 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name. (continued).

Species name	Family	No. of samples tested	No. of positive samples (% infection)	No. of positive males (% infection)	No. of positive females (% infection)
Tarsolepis elephantorum	Notodontidae	12	0	0	0
Xanthomelaena schematias	Crambidae	7	0	0	0

Table 4.3 The number, family, and species name of *Wolbachia*-infected specimens of 9 families in the order Lepidoptera across geographic regions (C = the Central, NE = the Northeast, and W = the West) in Thailand.

Family	Species name	No. of individuals
Geometridae	Abraxas sp.	32
	Buxura inoui	18
	Celenna festivaria	23
	Cleora determinata	12
	Comibaena sp.	21
	Herochroma flavibasalis	34
	Ourapteryx sp.	23
	Plutodes flavescens	32
	Ruttellerona pallicostaria	27
	Testa montana	12
Sphingidae	*Acosmeryx shervillii*	26
	Ambulyx sp.	22
	Daphis hypothous	36
	Hippotion sp.	16
	Pergesa acteus	23
	Theretra sp.	17
Noctuidae	Artena sp.	12
	Ischyja marapok	27
	Oxyodes scrobiculata	16
	Thyas honesta	14
Erebidae	*Creatonotos transiens*	31
	Olepa sp.	32
Uraniidae	*Lyssa zampa*	20
Endromidae	*Mustilizans dierli*	31
Lymantriidae	*Mustilia sphingiformis*	21
Crambidae	*Olene mendosa*	19
Lasiocampidae	*Parotis marginata*	18
	Trabala sp.	10
	Total	**625**

Most of the *Wolbachia* infections come from Kaeng Krachan National Park, located in Phetchaburi province in western Thailand. Although Khao Yai National Park covers 4 provinces in 2 regions of Thailand, namely Saraburi, Nakhon Nayok, Prachinburi, Central and Nakhon Ratchasima, it is the largest city in Thailand in the Northeast. The frequency of infection of *Walbachia* in different moth populations, collected from three different geographical regions of Thailand (Central Thailand). Significant differences (F = 2.3650, $p < 0.01$, df = 18) ranged from 33.33 % to 90.47 %, with an average of 61.90% of infections based on the number of positive samples. (Fig 4.1). The highest frequency of infection was observed in Phetchaburi (West) (90.47%). While the lowest frequency was recorded in the Nakhon Ratchasima (Northeastern) population (33.33%), although each region (west and northeast) had only one province in this study, Phetchaburi and Nakhon Ratchasima respectively. 28 species of *Wolbachia*-infected moth are included Abraxas sp., *Acosmeryx shervillii,* Ambulyx sp., Artena sp., *Buxura inoui, Celenna festivaria, Cleora determinate,* Comibaena sp., *Creatonotos transiens, Daphnis hypothous, Herochroma flavibasalis,* Hippotion sp., *Ischyja marapok, Lyssa zampa, Mustilizans dierli, Mustilia sphingiformis, Olene mendosa,* Olepa sp., Ourapteryx sp., *Oxyodes scrobiculata, Pergesa acteus, Parotis marginate, Plutodes flavescens, Ruttellerona pallicostaria, Thyas honesta ,* Theretra sp., *Testa montana* and Trabala sp. was listed in Table 4.2.

the 28 species (625 *Wolbachia*-infected moths), 144 were infected. Male (male = 46, female = 98) 156 Male = 49, Female = 107) and 325 (Male = 114, Female = 211) They can be found in the Central, Northeastern, and Western regions, respectively. All these 28 species could be found at all three regions above. Interestingly, the number of positive females seems to be higher than males out of the entire population. In addition, the total number of moths collected in the western regions is significantly higher than that of moths collected in the other two regions. (F = 2.1750, $p < 0.01$, df = 13). Regarding *Wolbachia*, *D. hypothous*, hawkmoth infected specimens, jade of the family Sphingidae had the highest number of infected specimens collected in this study (36 people), while Trabala sp. in the Lasiocampidae family had the fewest number of infected specimens collected here (10 people). For uninfected strains, *Wolbachia A. plana* had the highest number of samples collected in this study (25 people), while *P. bicolor*, the green rice moth of the Limacodidae family, had the

fewest samples collected (6 people). The number, family, and species names of nine families of *Wolbachia*-infected specimens, respectively, cross-regional Lepidoptera (C = Central, NE = Central). Northeast and W = West) in Thailand in Table 4.3.

Wolbachia infection is not detected from all collected species of the four families: Brahmaeidae, Limacodidae, Notodontidae and Saturniidae although these families include some important species such as the family Saturniidae which contains some of the largest species of moths in the world. Comparisons between moth species indicate that the frequency of strains infected with *Wolbachia* differs significantly ($F = 2.4830$, $p < 0.01$, df = 14). The conclusion regarding the number of infected specimens was shown as follow (In parentheses is the family name): *D. hypothous* (Sphingidae) 36 individuals, *H. flavibasalis* (Geometridae) 34 individuals, Abraxas sp. (Geometridae) 32 individuals, *P. flavescens* (Geometridae) 32 individuals, Olepa sp. (Erebidae) 32 individuals, *M. dierli* (Endromidae) 31 individuals, *C. transiens* (Erebidae) 31 individuals, *I. marapok* (Noctuidae) 27 individuals, *R. pallicostaria* (Geometridae) 27 individuals, *A. shervillii* (Sphingidae) 26 individuals, *C. festivaria* (Geometridae) 23 individuals, *P. acteus* (Sphingidae) 23 individuals, Ourapteryx sp. (Geometridae) 23 individuals, Ambulyx sp. (Sphingidae) 22 individuals, Comibaena sp. (Geometridae) 21 individuals, *M. sphingiformis* (Endromidae) 21 individuals, *L. zampa* (Uraniidae) 20 individuals, *O. mendosa* (Lymantriidae) 19 individuals, *B. inoui* (Geometridae) 18 individuals, *P. marginata* (Crambidae) 18 individuals, Theretra sp. (Sphingidae) 17 individuals, O. scrobiculata (Noctuidae) 16 individuals, Hippotion sp. (Sphingidae) 16 individuals, *T. honesta* (Noctuidae) 14 individuals, *T. montana* (Geometridae) 12 individuals, Artena sp. (Noctuidae) 12 individuals, *C. determinata* (Geometridae) 12 individuals and Trabala sp. (Lasiocampidae) 12 individuals in total of 28 species in 625 individuals.

The relative density of *Wolbachia* from 28 infected moths was determined by a quantitative, real-time PCR test using the *ftsZ* gene as a target sequence (Fig 4.2). The relative density of *Wolbachia* within each species is different. *Olene mendosa*, a brown moth or shaggy moth of the Erebidae family, has the highest relative Wolbachia density compared to 27 other infectious species, while *M. dierli* has the lowest density among all infected species. The sequence of relative *Wolbachia* density in these 28 species from highest to lowest is as follows: *O. mendosa, T. honesta, Artena sp., D. hypothous,* Trabala sp.*,* Theretra sp.*, A. shervillii, P. marginata, B. inoui, H. flavibasalis,*

R. pallicostaria, P. acteus, O. scrobiculata, M. sphingiformis, C. transiens, P. flavescens, Comibaena sp.*,* Abraxas sp.*, C. festivaria, C. determinata, T. montana,* Ambulyx sp.*, L. zampa,* Olepa sp.*, I. marapok,* Hippotion sp.*,* Ourapteryx sp. *and M. dierli.* Interestingly, at least 10 species from these infected species are agricultural pests, including: *O. mendosa, O. scrobiculata, T. honesta, D. hypothous, A. shervillii, P. acteus, C. transiens, T. montana, I. marapok,* Hippotion sp. and *O. mendosa*, which has the highest relative density of *Wolbachia*, is the main agricultural pest of durian in Thailand. Jade Falcon *D. hypothous* with the highest number of infected specimens totaling 36 of the 43 collected had relative *Wolbachia* density in fourth place.

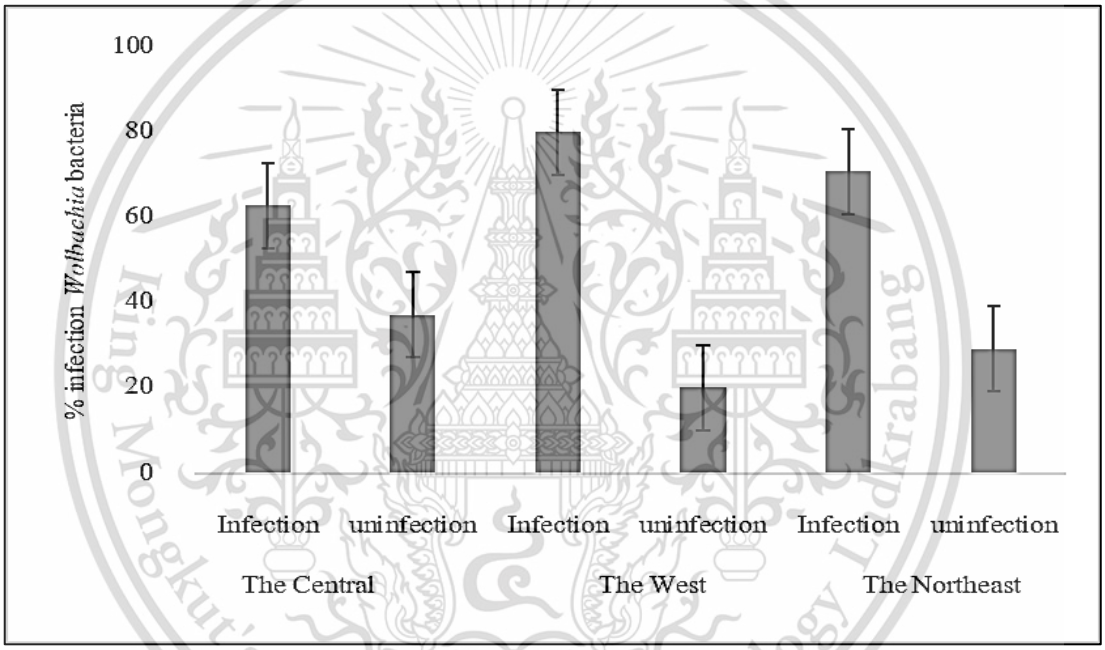

Fig. 4.1 Percentage of moth frequency *Wolbachia* infection (infection/uninfection) from each region (C = Central, NE = Central) Northeast and W = West) of Thailand.

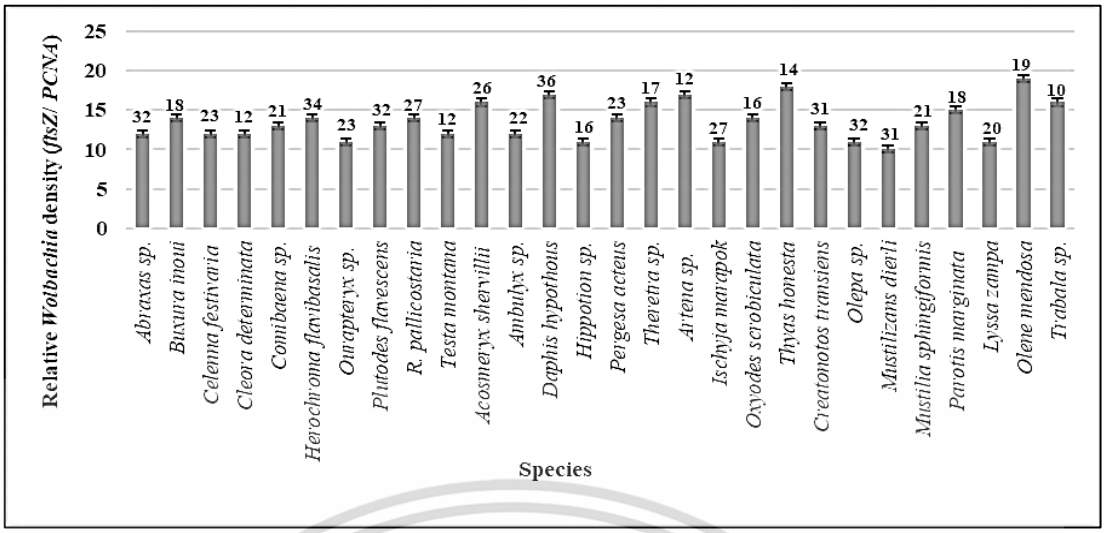

Fig. 4.2 Relative *Wolbachia* density in 28 *Wolbachia*-infected moths from Thailand. The error bars represent standard errors. The number of samples was added to the bar of each species. Two replications of each sample were done. The relative values (Y-axis) were obtained by comparing the ratio between the *Wolbachia ftsZ* gene copy number of each sample and the proliferating cell nuclear antigen gene (*PCNA*) of each host.

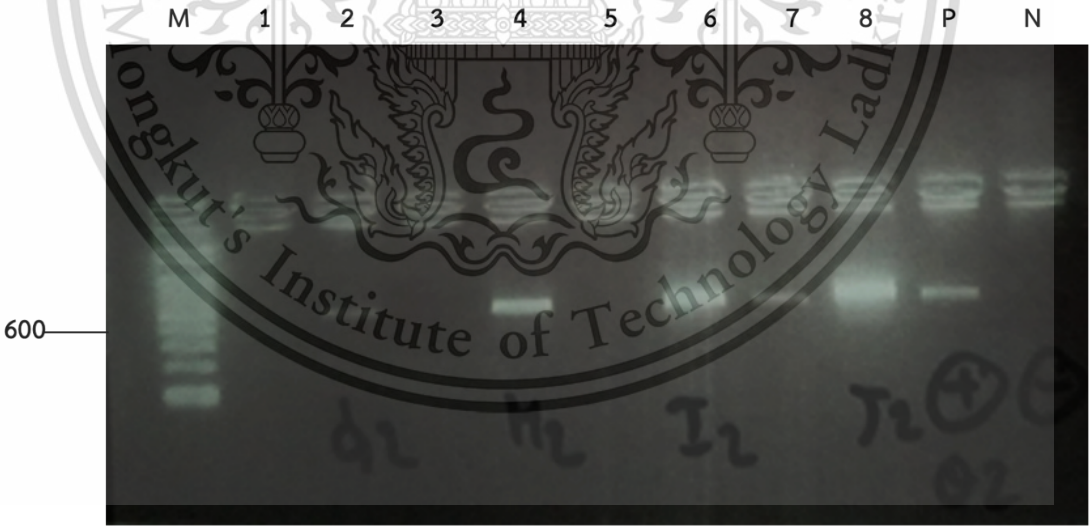

Fig. 4.3 An example of PCR results of *Wolbachia* infection. M: 100 bp DNA Ladder. P: Positive control, N: Negative control, 1 – 8: DNA of Moths Sample

4.2 *Wolbachia* infection in butterflies and distribution in tropical forest ecosystems.

Wolbachia infection in butterflies and distribution in tropical forest ecosystems. A total of 623 individuals representing 46 butterfly species belonging to 5 families in the order Lepidoptera collected during the field surveys in 5 provinces (Saraburi, Nakhon Nayok, Nakhon Ratchasima, Prachinburi and Phetchaburi) from two tropical forests in three different geographic regions including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West) from Thailand. were first screened for *Wolbachia* infection status by PCR assay using *Wolbachia*-specific *wsp*, *ftsZ* and *16S rRNA* gene primers. In total, the results showed that 291 (male = 103 individuals and female = 188 individuals) of 623 individuals (specimens) (46.71%) or 17 of 46 species (36.96%) from all three populations of Thailand were positive for *Wolbachia* infection.

The *ftsZ* and *wsp* PCR products were about 600 bp. while the *16S rRNA* PCR products of *Wolbachia* were around 900 bp. Similar PCR results were obtained from all three pairs of primers although there are a few differences but not significant among them. In total of 291 *Wolbachia* positive specimens, they included 17 species from 2 families including Nymphalidae (16, 273) and Papilionidae (1, 18) (The number in parentheses indicated the number of positive species in each family and the number of positive specimens in each family, respectively). PCR-based distribution of *Wolbachia* in 46 butterfly species from 5 families in the Lepidoptera in total of 623 specimens collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand including species name, family, location (province), geographic regions, no. of samples tested, no. of positive samples (% infection) and no. of positive samples (by sex) was listed in Table 4.4 and 4.5.

In a total of 17 *Wolbachia*-infected species of butterfly (291 individuals), 86 individuals (Males = 32, Females = 54), 72 individuals (Males = 25, Females = 47) and 133 individuals (Males = 46, Females = 87) could be found in the Central region, the Northeast region, and the West region, respectively. All these 17 species could be found at all three regions above. Interestingly, the number of positive females appeared to be higher than males from all populations (Table 4.6).

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name.

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
*Acraea violae**	Nymphalidae	Phetchaburi	W	10	4 (40.00)	2	2	1	5
		Nakhon Nayok	C	9	6 (66.67)	2	4	0	3
		Nakhon Ratchasima	NE	7	5 (71.42)	2	3	1	1
Appias libythea	Pieridae	Nakhon Nayok	C	2	0	0	0	0	2
		Phetchaburi	W	8	0	0	0	3	5
		Saraburi	C	1	0	0	0	0	1
Arhopala centaurus	Lymphalidae	Phetchaburi	W	2	0	0	0	0	2
		Nakhon Nayok	C	2	0	0	0	0	2
Catopsilia pomona	Pieridae	Nakhon Ratchasima	NE	8	0	0	0	3	5
		Prachinburi	C	6	0	0	0	2	4
		Phetchaburi	W	12	0	0	0	5	7
Cepora nerissa	Pieridae	Saraburi	C	2	0	0	0	1	1
		Phetchaburi	W	4	0	0	0	1	3

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
*Cethosia cyane**	Nymphalidae	Nakhon Nayok	C	5	3 (60.00)	1	2	1	1
		Phetchaburi	W	11	9 (81.81)	3	6	0	2
		Nakhon Ratchasima	NE	9	7 (77.78)	3	4	1	1
*Cynithia lepidea**	Nymphalidae	Saraburi	C	6	4 (66.67)	1	3	2	0
		Nakhon Nayok	C	3	2 (66.67)	0	2	0	1
		Phetchaburi	W	8	6 (75.00)	2	4	0	2
*Danaus chrysippus**	Nymphalidae	Nakhon Ratchasima	NE	10	8 (80.00)	3	5	1	1
		Nakhon Nayok	C	6	4 (66.67)	1	3	0	2
		Saraburi	C	5	3 (60.00)	2	1	1	1
*Danaus genutia**	Nymphalidae	Phetchaburi	W	9	7 (77.78)	2	5	0	2
		Prachinburi	C	3	2 (66.67)	0	2	1	0
		Nakhon Ratchasima	NE	6	4 (66.67)	3	1	1	1
		Nakhon Ratchasima	NE	2	0	0	0	0	2

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Danaus	Nymphalidae	Prachinburi	C	4	2 (50.00)	0	2	0	2
*melanippus**		Nakhon Nayok	C	5	3 (60.00)	1	2	1	1
		Phetchaburi	W	9	7 (77.78)	3	4	0	2
Delias hyparete	Pieridae	Saraburi	C	3	0	0	0	1	2
		Phetchaburi	W	8	0	0	0	2	6
		Prachinburi	C	2	0	0	0	1	1
Doleschallia	Nymphalidae	Nakhon Ratchasima	NE	8	6 (75.00)	2	4	0	2
*bisaltide**		Nakhon Nayok	C	5	4 (80.00)	3	1	1	0
		Phetchaburi	W	7	5 (71.42)	2	3	1	1
Euploea algea	Nymphalidae	Nakhon Ratchasima	NE	3	0	0	0	1	2
		Prachinburi	C	1	0	0	0	0	1
		Saraburi	C	2	0	0	0	0	2

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Euploea	Nymphalidae	Nakhon Nayok	C	2	0	0	0	0	2
camaralzeman		Phetchaburi	W	5	0	0	0	1	4
		Prachinburi	C	3	0	0	0	1	2
Euploea mulciber	Nymphalidae	Nakhon Ratchasima	NE	3	0	0	0	2	1
		Prachinburi	C	1	0	0	0	0	1
		Nakhon Nayok	C	2	0	0	0	0	2
Euploea sylvester	Nymphalidae	Nakhon Nayok	C	3	0	0	0	2	1
		Phetchaburi	W	5	0	0	0	1	4
		Saraburi	C	1	0	0	0	0	1
Eurema simulatrix	Pieridae	Nakhon Ratchasima	NE	3	0	0	0	2	1
		Prachinburi	C	1	0	0	0	1	0
		Nakhon Nayok	C	1	0	0	0	0	1

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Euthalia evelina	Nymphalidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	7	0	0	0	2	5
		Nakhon Ratchasima	NE	3	0	0	0	1	2
Graphium agamemnon	Papilionidae	Phetchaburi	W	6	0	0	0	2	4
		Nakhon Ratchasima	NE	4	0	0	0	1	3
		Nakhon Nayok	C	1	0	0	0	1	0
Graphium antiphates	Papilionidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	3	0	0	0	2	1
		Nakhon Ratchasima	NE	5	0	0	0	2	3
Graphium doson	Papilionidae	Saraburi	C	2	0	0	0	1	1
		Phetchaburi	W	3	0	0	0	1	2
		Nakhon Ratchasima	NE	1	0	0	0	0	1

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Hypolimnas bolina	Nymphalidae	Nakhon Nayok	C	1	0	0	0	0	1
		Phetchaburi	W	2	0	0	0	0	2
*Junonia atlites**	Nymphalidae	Nakhon Ratchasima	NE	4	3 (75.00)	0	3	1	0
		Phetchaburi	W	9	7 (77.78)	3	4	1	1
		Nakhon Nayok	C	7	5 (71.42)	1	4	0	2
*Junonia lemonias**	Nymphalidae	Saraburi	C	6	4 (66.67)	2	2	1	1
		Nakhon Ratchasima	NE	5	3 (60.00)	0	3	2	0
		Phetchaburi	W	10	6 (60.00)	2	4	1	3
*Junonia orithya**	Nymphalidae	Prachinburi	C	6	5 (83.33)	2	3	0	1
		Phetchaburi	W	10	7 (70.00)	2	5	2	1
		Nakhon Nayok	C	4	2 (50.00)	2	0	1	1
Lexias albopunctata	Nymphalidae	Saraburi	C	2	0	0	0	0	2
		Nakhon Ratchasima	NE	4	0	0	0	1	3

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
		Prachinburi	C	1	0	0	0	0	1
Loxura athymnus	Lymphalidae	Phetchaburi	W	2	0	0	0	0	2
		Nakhon Nayok	C	1	0	0	0	1	0
*Melanitis leda**	Nymphalidae	Nakhon Ratchasima	NE	7	5 (71.42)	2	3	1	1
		Phetchaburi	W	15	13 (86.67)	4	9	2	0
		Nakhon Nayok	C	8	7 (87.50)	2	5	0	1
*Mycalesis mineus**	Nymphalidae	Phetchaburi	W	14	12 (85.71)	5	7	1	1
		Prachinburi	C	9	6 (66.67)	2	4	1	2
		Saraburi	C	5	3 (60.00)	1	2	1	1
Mycalesis	Nymphalidae	Nakhon Nayok	C	7	5 (71.42)	1	4	0	2
*perseoides**		Nakhon Ratchasima	NE	10	8 (80.00)	2	6	1	1
		Phetchaburi	W	8	6 (75.00)	1	5	2	0

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
*Mycalesis perseus**	Nymphalidae	Nakhon Ratchasima	NE	9	7 (77.78)	4	3	1	1
		Saraburi	C	5	4 (80.00)	2	2	0	1
		Phetchaburi	W	15	13 (86.67)	5	8	2	0
Neomyrina nivea	Lymphalidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	3	0	0	0	1	2
		Nakhon Ratchasima	NE	1	0	0	0	0	1
Neptis hylas	Nymphalidae	Nakhon Nayok	C	2	0	0	0	0	2
		Prachinburi	C	1	0	0	0	1	0
Pachliopta	Papilionidae	Nakhon Ratchasima	NE	2	0	0	0	1	1
aristolochiae		Phetchaburi	W	5	0	0	0	1	4
		Nakhon Nayok	C	1	0	0	0	0	1
Papilio demoleus	Papilionidae	Saraburi	C	1	0	0	0	0	1
		Nakhon Ratchasima	NE	2	0	0	0	0	2

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
		Phetchaburi	W	4	0	0	0	1	3
Papilio helenus	Papilionidae	Phetchaburi	W	2	0	0	0	0	2
		Nakhon Ratchasima	NE	1	0	0	0	0	1
Papilio memnon	Papilionidae	Prachinburi	C	1	0	0	0	1	0
		Nakhon Ratchasima	NE	2	0	0	0	2	0
		Phetchaburi	W	1	0	0	0	1	0
Papilio nephelus	Papilionidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	2	0	0	0	0	2
*Papilio polytes**	Papilionidae	Nakhon Ratchasima	NE	8	5 (62.50)	2	3	2	1
		Nakhon Nayok	C	3	2 (66.67)	1	1	0	1
		Phetchaburi	W	13	11 (84.61)	4	7	1	1

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
*Parantica aglea**	Nymphalidae	Prachinburi	C	6	4 (66.67)	2	2	0	2
		Phetchaburi	W	15	12 (80.00)	3	9	2	1
		Nakhon Ratchasima	NE	9	7 (77.78)	1	6	1	1
*Parantica agleoides**	Nymphalidae	Phetchaburi	W	10	8 (80.00)	3	5	2	0
		Nakhon Ratchasima	NE	6	4 (66.67)	1	3	1	1
		Nakhon Nayok	C	8	6 (75.00)	3	3	1	1
Parthenos sylvia	Nymphalidae	Saraburi	C	2	0	0	0	0	2
		Phetchaburi	W	5	0	0	0	1	4
		Nakhon Ratchasima	NE	3	0	0	0	1	2
Pieris rapae	Pieridae	Prachinburi	C	1	0	0	0	0	1
		Nakhon Nayok	C	2	0	0	0	2	0
		Phetchaburi	W	3	0	0	0	2	1

Table 4.4 Distribution of *Wolbachia* using PCR in 46 butterfly species from five families, respectively, Lepidoptera from a total of 623 samples collected from two tropical forests in three different geographical regions. (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species that found positive for *Wolbachia* are indicated by an asterisk (*) at the end of each species name. (continued).

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex		No. of negative samples by sex	
						Male	Female	Male	Female
Suastus gremius	Hesperiidae	Nakhon Ratchasima	NE	1	0	0	0	0	1
		Phetchaburi	W	3	0	0	0	1	2
		Nakhon Nayok	C	1	0	0	0	0	1
Tanaecia julii	Nymphalidae	Nakhon Ratchasima	NE	4	0	0	0	2	2
		Saraburi	C	1	0	0	0	0	1
		Phetchaburi	W	5	0	0	0	2	3
Ypthima huebneri	Nymphalidae	Prachinburi	C	7	0	0	0	3	4
		Nakhon Nayok	C	3	0	0	0	1	2
		Nakhon Ratchasima	NE	5	0	0	0	2	3

Table 4.5 Distribution of *Wolbachia* using PCR in 46 butterfly species from 5 families, respectively, Lepidoptera throughout the region in Thailand. The 17 species (291 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name.

Species name	Family	No. of samples tested	No. of positive samples (% infection)		No. of positive males (% infection)		No. of positive females (% infection)	
*Acraea violae**	Nymphalidae	26	15	(57.69)	6	(40.00)	9	(60.00)
Appias libythea	Pieridae	11	0		0		0	
Arhopala centaurus	Lymphalidae	4	0		0		0	
Catopsilia pomona	Pieridae	26	0		0		0	
Cepora nerissa	Pieridae	8	0		0		0	
*Cethosia cyane**	Nymphalidae	25	19	(76.00)	7	(36.84)	12	(63.15)
*Cynithia lepidea**	Nymphalidae	17	12	(70.58)	3	(25.00)	9	(75.00)
*Danaus chrysippus**	Nymphalidae	21	15	(71.42)	6	(40.00)	9	(60.00)
*Danaus genutia**	Nymphalidae	18	13	(72.22)	5	(38.46)	8	(61.53)
*Danaus melanippus**	Nymphalidae	18	12	(66.67)	4	(33.33)	8	(66.67)
Delias hyparete	Pieridae	13	0		0		0	
*Doleschallia bisaltide**	Nymphalidae	20	15	(75.00)	7	(46.67)	8	(53.33)
Euploea algea	Nymphalidae	6	0		0		0	
Euploea camaralzeman	Nymphalidae	10	0		0		0	
Euploea mulciber	Nymphalidae	6	0		0		0	
Euploea sylvester	Nymphalidae	9	0		0		0	

Table 4.5 Distribution of *Wolbachia* using PCR in 46 butterfly species from 5 families, respectively, Lepidoptera throughout the region in Thailand. The 17 species (291 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name (Continued).

Species name	Family	No. of samples tested	No. of positive samples (% infection)		No. of positive males (% infection)		No. of positive females (% infection)	
Eurema simulatrix	Pieridae	5	0		0		0	
Euthalia evelina	Nymphalidae	11	0		0		0	
Graphium agamemnon	Papilionidae	11	0		0		0	
Graphium antiphates	Papilionidae	9	0		0		0	
Graphium doson	Papilionidae	6	0		0		0	
Hypolimnas bolina	Nymphalidae	3	0		0		0	
*Junonia atlites**	Nymphalidae	20	15	(75.00)	4	(26.67)	11	(73.33)
*Junonia lemonias**	Nymphalidae	21	13	(61.90)	4	(30.76)	9	(69.23)
*Junonia orithya**	Nymphalidae	20	14	(70.00)	6	(42.85)	8	(57.14)
Lexias albopunctata	Nymphalidae	7	0		0		0	
Loxura athymnus	Lymphalidae	3	0		0		0	
*Melanitis leda**	Nymphalidae	30	25	(83.33)	8	(32.00)	17	(68.00)
*Mycalesis mineus**	Nymphalidae	28	21	(75.00)	8	(38.09)	13	(61.90)
*Mycalesis perseoides**	Nymphalidae	25	19	(76.00)	4	(21.05)	15	(78.94)
*Mycalesis perseus**	Nymphalidae	29	24	(82.75)	11	(45.83)	13	(54.16)

Table 4.5 Distribution of *Wolbachia* using PCR in 46 butterfly species from 5 families, respectively, Lepidoptera throughout the region in Thailand. The 17 species (291 species) in bold are those found positive for *Wolbachia* (*) at the end of each species name (Continued).

Species name	Family	No. of samples tested	No. of positive samples (% infection)	No. of positive males (% infection)	No. of positive females (% infection)
Neomyrina nivea	Lymphalidae	5	0	0	0
Neptis hylas	Nymphalidae	3	0	0	0
Pachliopta aristolochiae	Papilionidae	8	0	0	0
Papilio demoleus	Papilionidae	7	0	0	0
Papilio helenus	Papilionidae	3	0	0	0
Papilio memnon	Papilionidae	4	0	0	0
Papilio nephelus	Papilionidae	3	0	0	0
*Papilio polytes**	Papilionidae	24	18 (75.00)	7 (38.89)	11 (61.11)
*Parantica aglea**	Nymphalidae	30	23 (76.67)	6 (26.08)	17 (73.91)
*Parantica agleoides**	Nymphalidae	24	18 (75.00)	7 (38.89)	11 (61.11)
Parthenos sylvia	Nymphalidae	10	0	0	0
Pieris rapae	Pieridae	6	0	0	0
Suastus gremius	Hesperiidae	5	0	0	0
Tanaecia julii	Nymphalidae	10	0	0	0
Ypthima huebneri	Nymphalidae	15	0	0	0

Table 4.6 The number, family, and species name of *Wolbachia*-infected specimens of 2 families in the order Lepidoptera across geographic regions (C = the Central, NE = the Northeast, and W = the West) in Thailand.

Family	Species name	No. of individuals
Nymphalidae	*A. violae*	15
	C. cyane	19
	C. lepidea	12
	D. bisaltide	15
	D. chrysippus	15
	D. genutia	13
	D. melanippus	12
	J. atlites	15
	J. lemonias	13
	J. orithya	14
	M. leda	25
	M. mineus	21
	M. perseoides	19
	M. perseus	24
	P. aglea	23
	P. agleoides	18
Papilionidae	*P. polytes*	18
	Total	**291**

Most of the *Wolbachia* infections come from Kaeng Krachan National Park, located in Phetchaburi province in western Thailand. Although Khao Yai National Park covers 4 provinces in 2 regions of Thailand, namely Saraburi, Nakhon Nayok, Prachinburi, Central and Nakhon Ratchasima, it is the largest city in Thailand in the Northeast. The frequency of infection of *Wolbachia* in different butterfly populations collected from three geographical regions of Thailand (Central, Northeast and West) differed significantly (F = 1.8340, $p < 0.01$, df = 14) ranging from 40.00% - 87.50%, with an average of 63.75% of infections based on the number of positive samples. (Fig. 4.4). The highest infection frequency was found in Nakhon Nayok (the Central) populations (87.50%), while the lowest frequency was recorded in Phetchaburi (the West) populations (40.00%). Each region (the West and the Northeast) includes only 1 province in this study, i.e., Phetchaburi and Nakhon Ratchasima, respectively. The 17 *Wolbachia*-infected butterfly species included *Acraea violae, Cethosia cyane, Cynthia lepidea, Danaus chrysippus, Danaus genutia, Danaus melanippus, Doleschallia bisaltide, Junonia atlites, Junonia lemonias, Junonia orithya, Melanitis leda, Mycalesis mineus, Mycalesis perseoides, Mycalesis perseus, Papilio polytes, Parantica aglea* and *Parantica agleoides.*

In addition, the number of all butterflies collected in the West region was significantly higher than those butterflies collected in the other two regions (F = 1.7460, $p < 0.01$, df = 18). Regarding the *Wolbachia*-infected specimens, *M. leda*, the common evening brown butterfly, in the family Nymphalidae, had the highest number of infected specimens collected in this study (25 from 30 individuals) whereas *C. lepidea* and *D. melanippus* in the family Nymphalidae had the lowest number of infected specimens collected here (12 from 17 and 18 individuals, respectively). For *Wolbachia*-uninfected species, *C. pomona* had the highest number of specimens collected in this study (26 individuals) whereas *H. bolina, L. athymmus, N. hylas, P. helenus, P. nephelus* in the family Nymphalidae, Lymphalidae, Nymphalidae, Papilionidae and Papilionidae, respectively had the lowest number of collected specimens (3 individuals).

The infections of *Wolbachia* were not detected from all collected species of these three families including Hesperiidae, Lymphalidae and Pieridae, although these families include some important species of the world. Comparisons among butterfly species indicated that the frequency of *Wolbachia*-infected species differed significantly ($F = 1.5960$, $p < 0.01$, $df = 12$). The conclusion regarding the number of infected specimens was shown as follow (In parentheses is the family name): *M. leda* (Nymphalidae) 25 individuals, *M. perseus* (Nymphalidae) 24 individuals, *P. aglea* (Nymphalidae) 23 individuals, *M. mineus* (Nymphalidae) 21 individuals, *C. cyane* (Nymphalidae) 19 individuals, *M. perseoides* (Nymphalidae) 19 individuals, *P. polytes* (Papilionidae) 18 individuals, *P. agleoides* (Nymphalidae) 18 individuals, *A. violae* (Nymphalidae) 15 individuals, *D. chrysippus* (Nymphalidae) 15 individuals, *D. bisaltide* (Nymphalidae) 15 individuals, *J. atlites* (Nymphalidae) 15 individuals, *J. orithya* (Nymphalidae) 14 individuals, *D. genutia* (Nymphalidae) 13 individuals, *J. lemonias* (Nymphalidae) 13 individuals, *C. lepidea* (Nymphalidae) 12 individuals and *D. melanippus* (Nymphalidae) 12 individuals in total of 17 species from 291 individuals.

The relative *Wolbachia* densities from 17 infected butterfly species were determined by a real-time quantitative PCR assay using the *ftsZ* gene as the target sequence (Fig.4.5) The relative *Wolbachia* densities within each species were different from each other. *D. genutia*, the common tiger butterfly in the family Nymphalidae, had the highest relative *Wolbachia* densities compared with the other 16 infected species while *J. orithya* and *M. perseoides* in the family Nymphalidae showed the lowest density among all infected species. The relative *Wolbachia* densities among these 17 species from the highest to the lowest are as follows: *D. genutia, D. hegesippus, J. atlites, A. violae, C. lepidea, M. perseus, C. cyane, P. polytes, M. leda, D. chrysippus, J. lemonias, P. agleoides, M. mineus, D. bisaltide, P. aglea, J. orithya* and *M. perseoides.*

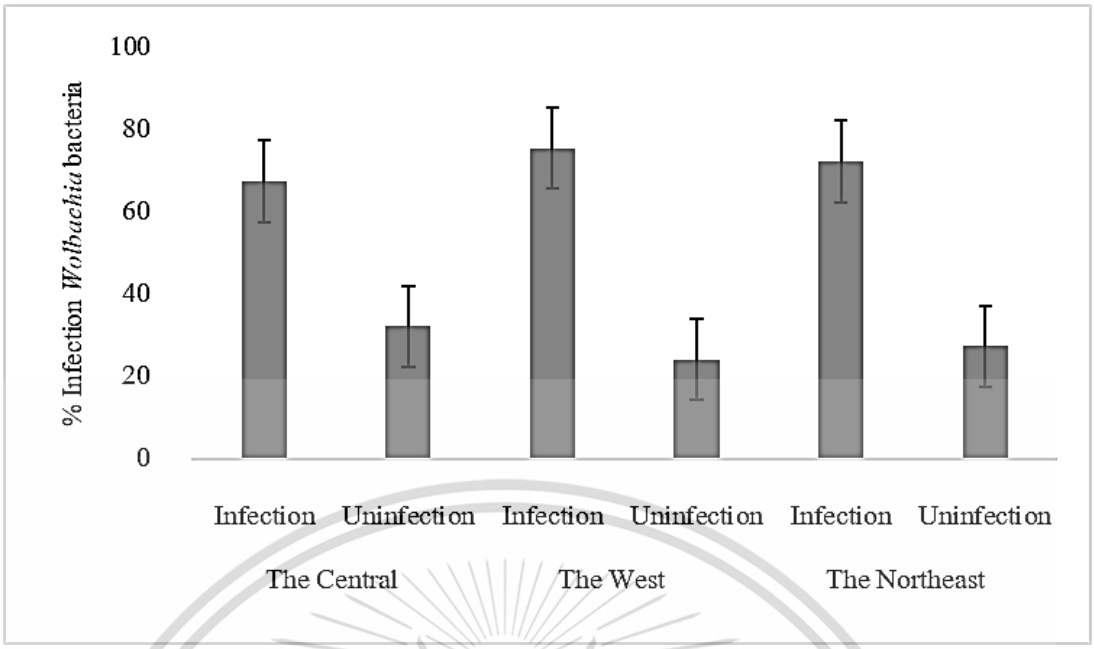

Fig. 4.4 Percent of *Wolbachia* infection butterfly frequency (Infection/ Unification) from each region. (C = the Central, NE = the Northeast and W = the West) from Thailand

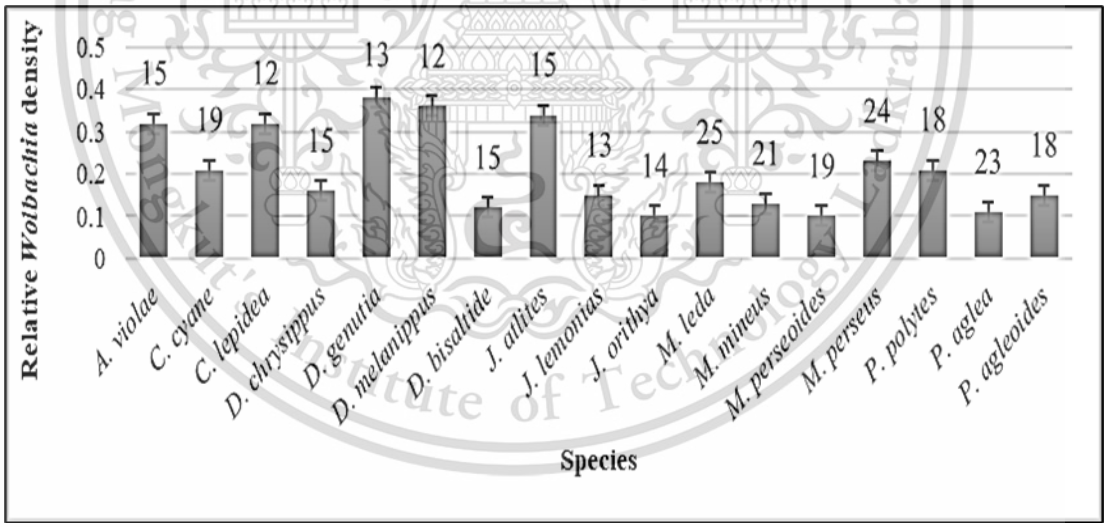

Fig. 4.5 Relative *Wolbachia* densities in 17 *Wolbachia*-infected butterflies from Thailand. The error bars represent standard error. The number of samples was added on the bar of each species. Two replications of each sample were done. The relative values were obtained by comparing the ratio between the *Wolbachia ftsZ* gene copy number of each sample and the proliferating cell nuclear antigen gene (*PCNA*) of each host.

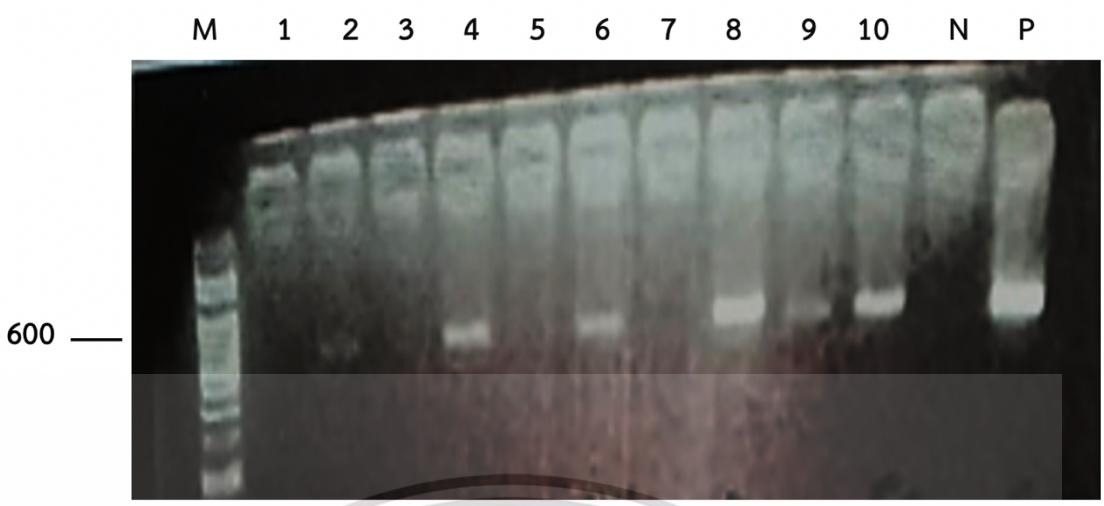

Fig. 4.6 An example of PCR screen by *wsp* gene primer results of *Wolbachia* infection. M: 100 bp DNA Ladder. P: Positive control, N: Negative control,1 – 10: DNA of Butterflies Sample.

CHAPTER 5
DISCUSSION

5.1 *Wolbachia* infection in moths and distribution in tropical forest ecosystems.

Wolbachia infection in moths and their distribution in tropical forest ecosystems. The percentage reported here may undoubtedly increase if additional samples are examined or different DNA or PCR extraction methods are used. This study implies that the widespread distribution of *Wolbachia* is unequal in the genus of moths and the geographical regions of Thailand. Species of moth that are positive for *Wolbachia* include many pests that are associated with Thai agriculture. Some moths from this study have been reported to be infected with *Wolbachia*, but they are not from Thailand. (Muhammad *et al*. 2015; Sumida *et al*. 2017). The distribution of all species in this study was mainly collected in the western regions, which are the main source of forest ecosystems in Thailand. However, all species can be found in all three regions of Thailand. Most *Wolbachia*-infected people were also collected from the western regions. In this region, habitat and temperature are somewhat different from other regions of Thailand. These results may indicate that climate differences may affect the survival of moths and infection with *Wolbachia*. (Muhammad *et al*. 2015). Infected individuals are found in many species, including a major pest in agriculture. So far, no previous studies have been reported on *Wolbachia* infection in moths from Thailand. Therefore, this is the first report on the discovery of *Wolbachia* infection in those species of moths from Thailand.

The field investigation has indicated that 50.61% (625 of 1,235 collected specimens) or 48.28% (28 of 58 collected species) of moths from Thailand were infected with *Wolbachia*. *Wolbachia* infection has been reported in many insects such as mosquitoes, planthoppers, leafhoppers, etc. But there have never been any reports of moths. (Wiwatanaratanabutr, 2013). Most of the moths infected with *Wolbachia* collected in this study belong to the family Geometridae. that has been known to be important pests in Thai agriculture. All individuals were collected from two tropical forests in five provinces of three regions from Thailand, which have been reported on

the abundance of these Lepidopteran throughout Thailand. Many species of collected moth are often found in many areas of Thailand. (Pierce, 1995; Suwannaphak, 2012). Although some are rare in the current situation under global warming and climate change. The difference in fertility of moth species collected from Thailand in current studies and previous studies in the period 2010-2012 (about 10 years ago) by Suwannaphak (2012). Reported on moths from Thailand (specimens collected before 2012) may be the result of global climate change, habitat, local temperatures, ecosystems. Time, as well as other related factors. (Schneider, 2013). Reported the Variable-Number-Tandem-Repeat molecular screening tool for the detection of *Wolbachia* infection in tsetse flies and showed that their infection in Glossina spp. could escape standard PCR screening methods by hiding it as a low-density infection below the detection threshold. Therefore, it is possible that not all infections were found in the current job due to *Wolbachia's* low-density infections.

In this study, another method using PCR was applied to this study to confirm *Wolbachia* infection. qPCR requires the quantification of the exact single-copy *ftsZ* gene per genome in *Wolbachia*, so a DNA standard of known concentration is needed and the host single-copy gene is quantified so that it can be standardized for comparison between species. Real-time PCR does not detect PCR products, but measures the fluorescence emitted by journalistic dyes. Thus, around the threshold, in which the glow begins to rise from the background level, is measured. Serial dilution of the standard produces a reliable standard curve that reduces the difference in primer adhesion performance to DNA samples. The relative density of *Wolbachia* within each host species of the three regions from Thailand is different. But they do not differ significantly as shown in Fig 4.2. ($P > 0.01$). According to Fig 4.2, there are more than 10 species of collected moths, which are agricultural pests of Thailand. Those moths have a similar level of *Wolbachia* density, do not differ significantly. The low density of infected moths may imply that *Wolbachia* may not spread through moth populations in Thailand due to climate change impacts or temperature differences. However, another possibility of this low-density detection, or the absence of *Wolbachia* in other moth species, is that *Wolbachia* loads may be below the threshold level of the real-time PCR detection system. Therefore, we need to continue

studying and increasing more about the diversity and distribution of *Wolbachia* infection, as well as the density of Lepidopteran infection from Thailand in the future.

Most of these species examined are agricultural pests. This may imply that *Wolbachia* have spread into almost all pest genera of moths. For these reasons, *Wolbachia* have attracted the interest of biologists as the basis for gene driving systems for the distribution of disease-blocking transgenes through populations of these moths, as well as for genetic control of other insect pests/vectors. As discussed above, *Wolbachia* infection often results in a CI phenotype, the consequences of which are non-viable insect eggs, especially in many insect species. (Sumida *et al*. 2017). Revealed that *Wolbachia* could induces costs to reproductive traits in the moth, *Ephestia kuehniella* whereas it might influence other species of moths in the other ways. This problem should be solved in our further studies. Thus, *Wolbachia* have been proposed as a potential gene-drive mechanism for modifying insects in order that they cannot transmit pathogens. By manipulating *Wolbachia*, these bacteria may ultimately prove useful as a control system for reducing pest populations and consequently decreasing transmission of many pests-borne diseases. Our findings involve ongoing efforts to manage pollinators such as moths and to understand the distribution of moth populations and infections *Wolbachia* from Thailand.

In addition, some of these results invite further thought and validate our understanding of the evolutionary dynamics of *Wolbachia* infections, their density in hosts and insects in the ecosystem. We found that geographic variation with close proximity was more likely to show similar levels of infection, and there was no evidence closely related to taxonomic groups indicating similar levels of infection. This observational study describes the intensive and important actions needed to address this discovery with well-designed research studies. We also found that latitudinal gradients can be an important factor in infection levels, such as lower frequencies to higher latitudes. This study demonstrates latitudinal gradients in *Wolbachia* infection at a broad taxonomic and geographical level in different regions, as well as implies that *Wolbachia* infection may be extrapolated from ecological variables. Therefore, the study of the density of *Wolbachia* infection will be the basis research for further biological investigation of the effect of Wolbachia on moths and very useful in the

application of natural enemies of insects for pest management, such as future caterpillar control.

5.2 *Wolbachia* infection in butterflies and distribution in tropical forest ecosystems.

Wolbachia infection in butterflies and distribution in tropical forest ecosystems. The percentage reported here may undoubtedly increase if different DNA extraction methods are used or more samples are examined. This report implies that *Wolbachia's* widespread distribution is unequal among the butterfly genus and geographical regions of Thailand. The species of butterflies that are positive for *Wolbachia* includes several pests that are important in relation to Thai agriculture. Some butterflies from this study were previously reported to be infected with *Wolbachia*, but did not come from Thailand. (Muhammad *et al*. 2015). The distribution of all species in this study was mainly collected in the western regions, which are the main source of forest ecosystems in Thailand. However, all species can be found in all three regions of Thailand. Most *Wolbachia* infected people were also collected from the western regions. In this region, habitat and temperature are somewhat different from other regions of Thailand. These results may imply that climate differences may affect the survival of butterflies and infection of *Wolbachia*. Sometimes multiple infections with *Wolbachia* that provoke different reproduction are found in some butterflies, such as *Eurema hecabe*. (Hiroki *et al*. 2004). Infected individuals are found in many species, including a major pest in agriculture. So far, no previous studies have been reported on *Wolbachia* infection in butterflies from Thailand. Therefore, this is the first report on the discovery of *Wolbachia* infection in those butterfly species from Thailand.

The field investigation has indicated that 46.71% (291 of 623 collected specimens) or 36.96% (17 of 46 collected species) of butterflies from Thailand were infected with *Wolbachia*. These findings were not much different from the previous report of Salunke *et al*. (2012) Regarding the determination of *Wolbachia* diversity in butterflies from India. *Wolbachia* infection has been reported in many insects such as mosquitoes, planthoppers, leafhoppers, etc. from Thailand. (Wiwatanaratanabutr, 2013; Wiwatanaratanabutr, 2015). But they have never been reported in butterfly. Most *Wolbachia*-infected butterfly species collected in this study were in the family Nymphalidae that has been known to be important pests in Thai agriculture. All individuals were collected from two tropical forests in five provinces from three regions

from Thailand, which have been reported on the abundance of these Lepidopteran throughout Thailand.

Many species of collected butterflies are commonly found in many areas of Thailand, although some species are rare in the current situation under global warming and climate change. The difference in the abundance of butterfly species collected from Thailand in the current study and previous studies during 2010-2012 (about 10 years ago) by Ek-Amnuay (2012) that reported on butterflies of Thailand (samples collected before 2012) may be the result of global climate change, habitat, local temperature, ecosystem, time and other relevant factors.

In this study, qPCR requires the quantification of the exact single-copy *ftsZ* gene per genome in *Wolbachia*, so a known concentration DNA standard is needed, and the single-copy *PCNA* host gene is quantified so that it can be standardized for comparison between species. Real-time PCR does not detect PCR products, but measures the fluorescence emitted by journalistic dyes. Thus, around the threshold, in which the glow begins to rise from the background level, is measured. Serial dilution of the standard produces a reliable standard curve that reduces the difference in primer adhesion performance to DNA samples. The relative density of *Wolbachia* within each host species of the three regions from Thailand is different. But there is no significant difference, as shown in Fig 4.5. ($P > 0.01$). According to Fig 4.5, there are more than 10 species of butterflies collected that are agricultural pests of Thailand. Those butterflies had a *Wolbachia* densities in the similar level, but significant difference among each other. The low density of infected butterflies may imply that *Wolbachia* may not spread through butterfly populations in Thailand due to climate change impacts or temperature differences. However, another possibility of this low-density detection, or the absence of *Wolbachia* in other species of butterflies, is that *Wolbachia* loads may be below the threshold level of the real-time PCR detection system. (Wiwatanaratanabutr, 2015). Therefore, we need to continue studying and increasing more about the diversity and distribution of *Wolbachia* infection, as well as the density of Lepidopteran infection from Thailand in the future.

Our findings involve ongoing efforts to manage pollinators such as butterflies and to understand the distribution of butterflies populations and infections *Wolbachia* from Thailand In addition, some of these results invite further thought and validate

our understanding of the evolutionary dynamics of *Wolbachia* infections, their density in hosts and insects in the ecosystem. We found that geographic variation with close proximity was more likely to show similar levels of infection, and there was no evidence closely related to taxonomic groups indicating similar levels of infection. This observational study describes the intensive and important actions needed to address this discovery with well-designed research studies. We also found that latitudinal gradients can be an important factor in infection levels, such as lower frequencies to higher latitudes. This study demonstrates latitudinal gradients in *Wolbachia* infection at a broad taxonomic and geographical level in different regions, as well as implies that *Wolbachia* infection may be extrapolated from ecological variables. Therefore, the study of *Wolbachia* infection density will be the basis research for further biological investigation of the effects of *Wolbachia* on butterflies and is very useful in the application of natural enemies of insects for pest management, such as future caterpillar control.

CHAPTER 6

CONCLUSION

6.1 *Wolbachia* infection in moths and distribution in tropical forest ecosystems.

Studies on *Wolbachia* infection in moths and their distribution in tropical forest ecosystems were conducted between January and December of 2019, with 1,235 samples screened in 58 moth species from 13 families. Specimens were collected from Khao Yai National Park (the Central and the Northeast regions), and Kaeng Krachan National Park (the West region). Infections of *Wolbachia* were screened by using PCR with *16S rRNA*, *ftsZ*, and *wsp* gene primers. The results showed that the rate of *Wolbachia* infection in the moth population from Thailand is high. It was found in a total of 625 different geographical populations in a total of 28 species of moths from 9 families, including 144 individuals (46 males and 98 females) from the central region, 156 individuals (49 males and 107 females) from the Northeast, and 325 individuals (114 males and 211 females) from the West. The highest infection rate is 90.47% in the Western population and the average infection rate is 61.90%. The relative density of *Wolbachia* within each person was determined using qPCR, and the results showed low *Wolbachia* infection density in these moth populations.

6.2 *Wolbachia* infection in butterflies and distribution in tropical forest ecosystems.

In this study, the first systematic survey of the *Wolbachia* infection status which was conducted between January to December 2019 in different species of butterflies (Order Lepidoptera) from tropical forests in 3 geographic regions of Thailand was reported. The 623 specimens from 46 butterfly species in 5 families were checked for *Wolbachia* infection. All samples were collected from two tropical forests consist of Khao Yai National Park (the Northeast and the Central regions) and Kaeng Krachan National Park (the West region) of Thailand. The *Wolbachia* infections were checked by using the PCR method with three gene primers including *16S rRNA*, *ftsZ*, and *wsp* which the results clearly showed evidence of *Wolbachia* infection in these butterflies. *Wolbachia* was detected in 291 individuals from 17 butterfly species of 2 families, comprising 86 individuals from the Central (32 males and 54 females), 72 individuals from the Northeast (25 males and 47 females), and 133 individuals from the West region (46 males and 87 females). The *Wolbachia* infection intensity from each infected species was examined and the results indicated that *D. genutia* had the highest infection intensity whereas the lowest intensity was found in *J. orithya* and *M. perseoides*.

6.3 Suggestion and Discussion.

The study of *Wolbachia* infection in butterflies and moths from Khao Yai and Kaeng Krachan National Parks, Thailand. *Wolbachia* bacteria were found to be highly genetically diverse. (high genetic diversity) Therefore, it can't be classified at the type level. Currently, the *Wolbachia* dance is divided into groups or Supergroup by analyzing the nucleotides of specific genes. Based on the research of Zhou *et al*. (1998). Grouped *Wolbachia* is two supergroups consisting of supergroup A and supergroup B and analyzing the nucleotides of the *wsp* gene. As technology evolves more thane the variety of *Wolbachia* can be detected increasingly. Currently, they are classified as sixteen supergroups including supergroups A-F and H-Q by analyzing the nucleotides of the *wsp*, *16S rDNA*, *ftsZ*, *groE,* และ *gltA*. Supergroups A and B it is more common in insects. (Augustinos *et al*. 2011; Bing *et al*. 2014; Glowska *et al*. 2015). So. In this case, the analysis of nucleotides as well as the evolutionary relationships of the sample populations should be studied for accuracy in the study. However. If more samples are selected for nucleotide analysis, other *Wolbachia* supergroups may be detected in the butterflies and moth sample populations in the future.

REFERENCES

Augustinos, A. A., Santos-Garcia, D., Dionyssopoulou, E., Moreira, M., Papapanagiotou, A., Scarvelakis, M., Doudoumis, V., Ramos, S., Aguiar, A. F., Borges, P. A. V., Khadem, M., Latorre, A., Tsiamis, G. and Bourtzis, K. 2011. "Detection and characterization of *Wolbachia* infections in natural populations of aphids: Is the hidden diversity fully unraveled". **PLoS ONE** 6: e28695.

Bagheri, Z., Talebi, A.A., Asgari, S., and Mehrabadi, M. 2019. "*Wolbachia* induce cytoplasmic incompatibility and affect mate preference in Habrobracon hebetor to increase the chance of its transmission to the next generation." **J. Invert Pathol**.163 : 1-7.

Baldo, L., Bordenstein, S., Wernegreen, J.J., and Werren, J.H. 2006."Widespread recombination throughout *Wolbachia* genomes." **Mol. Biol. Evol**. 23, 437- 449.

Banziger, H. 1992. "Remarkable new cases of moths drinking human tears in Thailand (Lepidoptera: Thyatritridae, Sphingidae, Notodontidae)." **Nat Hist Bull Siam Soc.** 40(1) : 91– 102.

Biodiversity Research Center Her Majesty the Queen's 72nd Birthday Anniversary. 2553. **Moths in Lam Phaya Valley**. Songkhla: YaLa Rajaphat University. 112 p.

Bipinchandra, K., Salunke, R.C., Salunkhe, D.P., Dhotre, S.A., Walujkar, A.B., Khandagale, R.C., Rakesh, K., Chandode, H.V., Ghate, M.S., Patole, J.H., and Werren, Y.S. 2012. "Determination of *Wolbachia* Diversity in Butterflies from Western Ghats, India, by a Multigene Approach." **Appl. Environ. Microbiol**. 12 : 4458-4467.

Bing, X. L., Xia, W. O., Gui, J. D., Yan, G. H., Wang, X. W. and Liu, S. S. 2014. "Diversity and evolution of the *Wolbachia* endosymbionts of *Bemisia* (Hemiptera: Aleyrodidae) whiteflies". **Ecol. Evol.** 4(13): 2714-2737.

Braig, H.R., Zhou, W., Dobson, S.L., and O'Neill, S.L. 1998. "Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*." **J. Bacteriol**. 180 : 2373-2378.

Breeuwer, J. A. J., Stouthamer, R., Barns, S. M., Pelletier, D. A., Weisburg, W. G. and Werren, J. H. 1992. "Phylogeny of cytoplasmic incompatibility microorganisms

in the parasitoid wasp genus Nasonia (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences". Insect. **Mol. Biol**. 1(1): 25-36.

Brownstein, J.S., Hett, E., and O'Neill, S.L. 2003. "The potential of virulent *Wolbachia* to modulate disease transmission by insects." **J. Invertebr. Pathol.** 84n : 24–29.

Bordenstein, S. R., and Wernegreen, J. J. 2004. "Bacteriophage in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates". **Mol. Biol. Evol.** 21: 1981-1991.

Clancy, D. J., and Hoffmann, A. A. 1998. "Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia* infected *Drosophila simulans*." **Entomol. Exp. Appl.** 86 : 13-24.

Cordaux, R., Michel-Salzat, A., and Bouchon, D., 2001. "*Wolbachia* infection in crustaceans: novel hosts and potential routes for horizontal transmission." **J. Evol. Biol.** 14 : 237–243.

Cordaux, R., Pichon, S., Hatira, H.B., Doublet, V., Grève, P., Marcadé, I., Braquart-Varnier, C., Souty-Grosset, C., Charfi-Cheikhrouha, F., and Bouchon, D. 2012. "Widespread *Wolbachia* infection in terrestrial isopods and other crustaceans." **Zookeys**. 176 : 123-131.

Dale, C. and Welburn, S.C. 2001. "The endosymbionts of tsetse flies: manipulating host-parasite interactions." **Int. J. Parasitol.** 31 : 628-631.

Dao – Hong, Z., and Shao – Gao. 2021. "A prevalence survey of *Wolbachia* in butterflies from southern China." **Entomologia Experimentalis et Applicata.** 169(12) : 148-157.

Derksen C. and Brown R. 2012. "Spring snow cover extent reductions in the 2008-2012 period exceeding climate model projections." **Geophysical Research Letters**. 39(19) : 1-6.

Dionysopoulou, N.K., Papanastasiou, S.A., Kyritsis, G.A, and. Papadopoulos, N.T. 2020. "Effect of host fruit, temperature and *Wolbachia* infection on survival and development of Ceratitis capitate immature stages." **PLoS ONE.** 15(3) : e0229727. https://doi.org/10.1371/journal.pone.0229727.

Ek-Amnuay P. 2012. **Butterflies of Thailand**. 2nd ed. Baan Lae Suan, Amarin Printing and Publishing, Bangkok.

Gabriele C.H. 1996. "Detecting Greenhouse-Gas-Induced Climate Change with an Optimal Fingerprint Method," **Journal of Climate**. 9 : 2281-2306.

Glowska, E., Dragun-Damian, A., Dabert, M. and Gerth, M. 2015. New *Wolbachia* supergroups detected in quill mites (Acari: Syringophilidae). Infect. **Genet. Evol.** 30: 140-146.

Hertig, M. 1936. "The rickettsia, *Wolbachia pipientis* and associated inclusions of the mosquito, Culex pipiens." **Parasitology**. 28 : 453-486.

Hertig, M., and Wolbach, S. B. 1942. "Studies on Rickettsia-like microorganisms in insects." **J. Med.** 44 : 329-374.

Heddi, A., Grenier, A. M., Khatchadourian, C., Charles, H. and Nardon, P. 1999. "Four intracellular genomes direct weevil biology: Nuclear, mitochondrial, principal endosymbiont, and *Wolbachia"*. **Proc. Natl. Acad. Sci. U S A.** 96(12): 6814-6819.

Hiroki, M., Tagami, Y., Miura, K., and Kato, Y. 2004. "Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*." **Proc. Biol. Sci**. 271 : 1751–1755.

Hurst, G. D. D., and Werren, J. H. 2001. The role of selfish genetic elements in eukaryotic evolution. **Nat. Rev. Genet**. 2: 597-606.

Ilinsky, Y., and Kosterin, O. 2017. "Molecular Diversity of *Wolbachia* in Lepidoptera: Prevalent Allelic Content and High Recombination of MLST Genes." **Molecular Phylogenetics and Evolution**. 109 : 10.1016/j.ympev.2016.12.034.

Iturbe-Ormaetxe, I., Walker, T., and O' Neill, SL. 2011. "*Wolbachia* and the biological control of mosquito-borne disease." **EMBO Rep**. 12(6) : 508–518. DOI: 10.1038/embor.2011.84.

Jeong, G., Ahn, J., Jang, Y., Choe, J.C., and Choi, H. 2012. "*Wolbachia* infection in the Loxoblemmus complex (Orthoptera: Gryllidae) in Korea." **J. Asia Pac. Entomol**. 15 : 563-566.

Jiang, W., He, H., Li, Y., Ren, M., Ma, Y., Zheng, L., Zhu, J-Q., and Yu, W. 2016. "Taxonomic status and molecular phylogeography of two sibling species of Polytremis (Lepidoptera: Hesperiidae)." **Scientific Reports**. 6 : 20820. 10.1038/srep20820.

Jiggins, F. M., Hurst, G. D. D., and Majerus, M. E. N. 1998. "Sex ratio distortion in *Acaea*

encedon is caused by a male-killing bacterium". **Heredity**. 81: 87-91.

Kajtoch, L., Kolasa, M., Kubisz, D., Gutowski, J.M., Scibior, R., Mazur, M.A., and Holecová, M. 2019. "Using host species traits to understand the Wolbachia infection distribution across terrestrial beetles." **Sci Rep**. 9 : 847.

Kittayapong, P., Baisley, K.J., Baimai, V., and O'Neill, S.L. 2000. "Distribution and diversity of *Wolbachia* infections in Southeast Asian mosquitoes (Diptera: Culicidae)." **J. Med. Entomol**. 37 : 340–345.

Mike, S., 2023. **Polymerase Chain Reaction**. Avialable online : www.genome.com.

Montllor, C. B., Maxmen, A., and Purcell, A. H. 2002. "Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress." **Ecol. Entomol.** 27 : 189–195.

Muhammad, Z.A., Eli, V. Araujo-Jnr, J.J.W., and Akito, Y.K. 2015. "*Wolbachia* in butterflies and moths: geographic structure in infection frequency." **Front. Zool**. 12(16) : 1-9. https://doi.org/10.1186/s12983-015-0107-z.

Nieukerken, E.J., Kaila, L., Kitching, I.J., Kristensen, N.P., Lees, D.C., Minet, J., *et al*. 2011. Order Lepidoptera Linnaeus, 1758." **Zootaxa.** 3148 : 212–21.

Noda, H. 1984. "Cytoplasmic incompatibility in a rice plant hopper." **Heredity.** 75 : 345-348.

Noda, H., Koizumi, Y., Zhang, Q., and Deng, K. 2001. "Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. Insect Biochem." **Mol. Biol**. 31 : 727–737.

O'Neill, S.L., Giordane, R., Colbert, A.M.E., Timothy, K., and Robertson, H. 1992. "*16S rRNA* phylogenetic analysis of the bacterial endosymbionts associated with CI in insects. Proc. Natl." **Acad. Sci. USA.** 89 : 2699-2702.

Pierce, N.E. 1995. "Predatory and parasitic Lepidoptera: Carnivores living on plants". **Journal of the Lepidopterists' Society**. 49(4) : 412–53.

Plotkin, D., and Goddard, J. 2013. "Blood, sweat, and tears: a review of the hematophagous, *sudophagous*, and *lachryphagous* Lepidoptera." **J Vector Ecol**. 38(2) : 289–94.

Porksakorn C., and Nuchprayoon, S. 2001. "The roles of *Wolbachia* in arthropods and filarial parasites." **Chula Med J.** 45(7) : 603-22.

Ross, P.A., Wiwatanaratanabutr, I., Axford, J.K., White, V.L., Endersby-Harshman, N.M. and Hoffmann, A.A. 2017. "*Wolbachia* infections in *Aedes aegypti* differ markedly in their response to cyclical heat stress." **PLOS Pathogens**. 13(1) : e1006006. doi:10.1371/journal.ppat.1006006.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., and Solignac, M. 1992. "*Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods." **P. Roy. Soc. B-Biol. Sci**. 250 : 91-98.

Saurav, G. K., Daimei, G., Rana, V. S., Popli, S. and Rajagopal, R. 2016. Detection and localization of *Wolbachia* in Thrips *Palmi Karny* (Thysanoptera: Thripidae). Indian. **J. Microbiol**. 56(2): 167-171.

Santer, B.D., Taylor, K.E., Wigley, T. M. L., Johns, T. C., Jones, P. D., Karoly, D. J., Mitchell, J. F. B., Oort, A. H., Penner, J. E., Ramaswamy, V., Schwarzkopf, M. D., Stouffer, R. J., and Tett, S. 1996."A search for human influences on the thermal structure of the atmosphere." **Nature.** 382 : 39-46.

Salunke, B.K., Salunkhe, R.C., Dhotre, D.P., Walujkar, S.A., Khandagale, A.B., Chaudhari, R, Chandode RK, Ghate HV,Patole MS, Werren JH, Shouche, Y.S. 2012. "Determinati on of *Wolbachia* diversity in butterflies from western ghats, India, by a multigene approach". **Appl Environ Microbiol.** 78(12):4458-446.

Schneider, D.I., Garschall, K.I., Parker, A.G., Abd-Alla, A.M.M., and Miller, W.J., 2013. "Global *Wolbachia* prevalence, titer fluctuations and their potential of causing cytoplasmic Incompatibilities in tsetse flies and hybrids of Glossina morsitans subgroup species." **J. Invertebr. Pathol**. 112 : 104–115.

Stouthamer, R., Breeuwer, J. A. J., Luck, R. F. and Werren, J. H. 1993. "Molecular identification of microorganisms associated with parthenogenesis". **Nature**. 361: 66-68.

Stouthamer, R., Breeuwer, J.A.J., and Hurst, G.D.D. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. **Ann. Rev. Microbiol**. 53 : 71–102.

Sumida, Y., Katsuk, M., Okada, K., Okayama, K., and Lewis, Z. 2017. "*Wolbachia* induces costs to life- history and reproductive traits in the moth, *Ephestia kuehniella*." **J. Stored Prod. Res**. 71 : 93-98.

Suwannaphak, K. 2012. **Photographic guide to Moths in Thailand.** Nakhon Nayok : Banna Nature Education Center.

Smith, M. 2023. "**Polymerase chain reaction, National human genome research institute**." Available online: https://www.genome.gov. 7 April 2566.

Tagami, Y., Doi, M., Sugiyama, K., Tatara, A. and Saito, T. 2006. "*Wolbachia*-induced cytoplasmic incompatibility in *Liriomyza trifolii* and its possible use as a tool in insect pest control". **Biol. Control**. 38: 205-209.

Tewaruxsa, P. 2017. **Characterization for *Wolbachia* in leafhopper *Yamatotettix flavovittatus* Matsumura.** Thesis master degree, Thammasat University.

Walker, T., Johnson, P. H., Moreira, L. A., Frentiu, F. D., McMeniman, C. J. andLeong, Y. S. 2011. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. **Nature**. 476: 450-453.

WangKeeree, J., and Hanboonsong, Y. 2016. "Role of Bacterial Symbionts in Insects and its Application for Insect Pests Control. . **Journal of Science and Technology**. 24(4) : 614 -623.

Wechprasit., N. 2015. "Determination of food pathogen in food products." **Ramkhamhaeng university.** 12-16.

Werren Lab Department of Biology University of Rochester. 2011. "**Werren Lab**." Available. Online: http://www.sas.rochester.edu/bio/labs/WerrenLab/Werren_Lab_Home. html.

Werren, J.H., Baldo, L., and Clark, M.E. 2008. "*Wolbachia:* master manipulators of invertebrate biology." **Nat. Rev. Microbiol**. 6 : 741–751.

Werren, J.H., Windsor, D., and Guo, L. 1995. "Distribution of *Wolbachia* among neotropical arthropods." **Proc. R. Soc. Lond**. 262 : 197–204.

Wiwatanaratanabutr I. 2013a. "Distribution, diversity and density of *Wolbachia* infections in cladocerans and copepods from Thailand." **J. Invertebr. Pathol.** 114 : 341-345.

Wiwatanaratanabutr, I. 2013. "Geographic distribution of *Wolbachia* infections in mosquitoes from Thailand." **J. Invertebr. Pathol**. 114 : 337–340.

Wiwatanaratanabutr, I. 2013b. "Geographic distribution of Wolbachia infections in mosquitoes from Thailand." **J. Inverteb. Pathol**. 114 : 337-340.

Wiwatanaratanabutr, I. 2015. "*Wolbachia* infection in leafhoppers and planthoppers: Diversity, density and geographic distribution in tropical rice agroecosystems." **J. Asia-Pacific Entomol**. 18(2) : 277-282.

Wiwatanaratanabutr, I., and Grandjean, F. 2016a. "Impacts of temperature and crowding on sex ratio, fecundity and *Wolbachia* infection intensity in the copepod, Mesocyclops thermocyclopoides." **J. Invertebr. Pathol**. 141 : 18-23.

Wiwatanaratanabutr, I., and Kittayapong, P. 2009. "Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of Aedes albopictus." **J. Invertebr. Pathol**. 102(3) : 220–224.

Wiwatanaratanabutr, I., and Zhang, C. 2016b. "*Wolbachia* infections in mosquitoes and their predators inhabiting rice field communities in Thailand and China." **Acta Trop**. 159 : 153- 160.

Wiwatanaratanabutr, I., Kittayapong, P., Caubet, Y., and Bouchon, D. 2009. "Molecular phylogeny of *Wolbachia* strains in arthropod hosts based on groE-homologous gene sequences." **Zool. Sci.** 26 : 171–177.

Wiwatanaratanabutr, S., and Kittayapong, P. 2006. "Effects of tempos and temperature on *Wolbachia* load and life history traits of *Aedes albopictus*." **Med. Vet. Entomol**. 20 : 300–307.

Wright, J. D. 1979. **The etiology and biology of cytoplasmic Incompatibility in the *Aedes scutellaris* Group**. Ph.D. Dissertation, California University.

Yamada, R., Iturbe-Ormaetxe, I., Brownlie, J.C., and O'Neill, S.L. 2011. "Functional test of the influence of *Wolbachia* genes on cytoplasmic incompatibility expression in Drosophila melanogaster." **Insect Mol. Biol**. 20 : 75–85.

Zaspel, J.M., Weller, S.J., and Branham, M.A. 2011. "A comparative survey of proboscis morphology and associated structures in fruit-piercing, tear-feeding, and blood-feeding moths in Calpinae (Lepidoptera: Erebidae)". **Zoo morphology**. 130(3) : 203–25.

Zabalou, S., Riegler, M., Theodorakopoulou, M., Stauffer, C., Savakis, C. and Bourtzis, K. 2004. "*Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control". **P. Natl. A. Sci.** 101: 15042-15045.

Zhang, H., Gao, J., Ma, Z., Liu, Y., Wang, G., Liu, Q., Du, Y., Xing, D., Li, C., Zhao, T., Jiang, Y., Dong, Y., Guo, X., and Zhao, T. 2018. "Influence of *Wolbachia* Infection on Mitochondrial DNA Variation in the Genus *Polytremis* (Lepidoptera: Hesperiidae)." **Molecular Phylogenetics and Evolution**. 12 : 1-12.

Zhao, D. X., Zhang, X. F., Chen, D. S., Zhang, Y. K. and Hong, X. Y. 2013. *Wolbachia*-host interactions: host mating patterns affect *Wolbachia* density dynamics. **PLOS ONE**. 8(6): 1-10.

Zhou, W., Rousset, F., and O'Neil, S. 1998. "Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences". **Proc. Biol. Sci**. 265(1395) : 509–515.

APPENDICES

APPENDIX A

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Infection density, diversity and distribution of *Wolbachia* bacteria in moths (order Lepidoptera): First systematic report from Thailand

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ABSTRACT

Members of the genus *Wolbachia* are a group of Rickettsia-like, intracellular, maternally inherited bacterial endosymbionts that infect a diverse range of insects and cause reproductive changes in their hosts. Although *Wolbachia's* role in many insects has been extensively studied, a little is known about *Wolbachia* in Lepidopteran which is one of the most diverse insects. Here, we present the first survey of the *Wolbachia* infection status in different species of moths from three different geographic regions of Thailand, which was carried out during January to December in 2019 with the screening of 1,235 specimens in total of 58 moth species from 13 families. Specimens were collected from Khao Yai National Park (the Central and the Northeast regions), and Kaeng Krachan National Park (the West region). Infections of *Wolbachia* were screened by using polymerase chain reaction with 16S rRNA, *ftsZ* and *wsp* gene primers which the results indicated high rates of *Wolbachia* infection in moth populations from Thailand. *Wolbachia* was found in all different geographically populations in total of 625 individuals in total of 28 moth species from 9 families, including 144 individuals (46 males and 98 females) from the Central, 156 individuals (49 males and 107 females) from the Northeast, and 325 individuals (114 males and 211 females) from the West. The highest infection rate was 90.47% in the West populations and the average infection rate was 61.90%. The detection of *Wolbachia* in different moth populations from all regions was identical when all primers were used to screen for *Wolbachia*. A bit difference of

detection rate in PCR assay was not a significant difference. The relative densities of *Wolbachia* within each individual were determined using quantitative real-time PCR and the result showed that there was a low *Wolbachia* infection density in these moth populations. These findings indicated that *Wolbachia* are distributed throughout the moth populations from Thailand.

Keywords: Distribution, Infection, Lepidoptera, Moths, *Wolbachia*

INTRODUCTION

Wolbachia, the most common and widespread intracellular bacterium on Earth, is a genus of maternally inherited bacteria that infect a diverse range of arthropods. Around 20-76% of all insect species are estimated to be infected by *Wolbachia* bacteria (Stouthamer et al., 1999; Werren et al., 2008). Some *Wolbachia* strains can sustain themselves in their hosts and decrease adult lifespan. *Wolbachia* can be involved in the manipulation of their host's reproductive system, resulting in different abnormal reproductive phenotypes including cytoplasmic incompatibility, male killing, feminization and parthenogenesis (Bagheri et al., 2019; Kajtoch et al., 2019; Werren et al., 2008). These phenotypes promote the distribution of *Wolbachia* in host populations by increasing the numbers of infected females. The best known of these traits is Cytoplasmic Incompatibility (CI) which its intensity is affected by many factors such as temperature and rearing density (Sumida et al., 2017; Wiwatanaratanabutr and Kittayapong, 2006, 2009). The CI occurs when infected males mate with uninfected females or with females that harbor a different *Wolbachia* strain, and results in the death of the fertilized egg which make a reproductive advantage to infected hosts that enable *Wolbachia* to spread rapidly through a host population (Braig et al., 1998). The detection, distribution, infection density and diversity of *Wolbachia* have been studied in many groups of insects such as root fly, mosquitoes, butterfly, moth, beetles and wasps (Bipinchandra et al., 2012; Wiwatanaratanabutr, 2013; Sumida et al., 2017; Carjaval et al., 2018; Kolasa et al., 2018; Lopez et al., 2018; Bagheri et al., 2019). Sequence analysis of 16S rRNA, *ftsZ* and *wsp* genes shows that members of the genus *Wolbachia* belongs to a monophyletic clade in the alphaproteobacterial group which is closely related to the Ehrlichia assemblage (Zhou et al., 1998). *Wolbachia* can be classified into many supergroups such as A and B that are the most widely distributed in

insects (Werren et al., 2008). Order Lepidoptera constitute one of the most diverse insect orders with more than 157,000 described species of butterflies and moths. Lepidopteran plays an important role in ecosystem function primarily as pollinators and herbivores, though some species feed on blood and other animal secretions (Kajtoch et al., 2019; Plotkin and Goddard, 2011). The order includes many significant agricultural pests, some species serve as models across biological disciplines (Zaspel, 2013) whereas some of them are predatory and parasitic Lepidopteran (Pierce, 1995). Despite the diversity of Lepidoptera and their many associations with other organisms, little is known about the bacterial community and *Wolbachia* infection associated with the order from Thailand (Ek-Amnuay, 2012; Suwannaphak, 2012). The biological, behavioral and ecological diversity of butterflies and moths suggests the need for further characterization of *Wolbachia* to understand the impact of infection on their evolution, speciation and reproduction (Hiroki et al., 2004; Muhammad et al., 2015). The information on molecular biology and phenotypic effects of *Wolbachia* from moth species show the presence of supergroup A and B *Wolbachia* strains (Muhammad et al., 2015). *Wolbachia* strains in butterflies have been implicated in basic biological processes such as sex ratio distortion, sex determination, sperm-egg compatibility, and speciation (Kajtoch et al., 2019). At present, *Wolbachia* is being developed as a new method for biocontrol strategy against many insect pests and disease vectors. While its role in some hosts has been studied extensively, its incidence and distribution among moth species from Thailand is still largely unknown and poorly understood. Here, the diversity and geographic distribution with the infection status and density of *Wolbachia* bacteria in moths from two different tropical forest ecosystems in three different geographic regions of Thailand were first investigated. Our study of the *Wolbachia* infection status would lay the groundwork for further biological investigations of the *Wolbachia* effects on Lepidopteran hosts and has been very helpful in the utilization of insect natural enemies for pest control.

MATERIAL AND METHODS

Insects collection

Moths used in this study were collected during January to December 2019 from two tropical forest ecosystems in three different regions (the Central, the Northeast and the West of Thailand) including Khao Yai National Park (the Central and the Northeast) and Kaeng

Krachan National Park (the West) (Table 1). All specimens under this study were morphologically identified to species (or genus) levels by using the photographic guide to moths in Thailand (Suwannaphak, 2012) at Center of Excellence in Applied Biosciences, King Mongkut's Institute of Technology Ladkrabang in Bangkok, Thailand. Legs or abdomen of the moths were removed and preserved in absolute ethanol at -20 °C until DNA extraction.

DNA preparation

To extract DNA, adult's legs were used for PCR screening, and the rest of the specimen was preserved for identification. In cases that the specimens were small size, their abdomens were used instead of legs. DNA was extracted from tissue using a QIAamp DNA minikit (Qiagen) following the manufacturer's protocol. Briefly, all specimens were lysed around 20 minutes by using optimized buffers and specific enzymes, DNA was stabilized and enhanced selective DNA adsorption to the QIAamp membrane. Alcohol was added and lysates were loaded onto the QIAamp spin column. Wash buffers were used to remove impurities and pure DNA was then eluted in double-distilled water. The extracted DNA was kept at -20 °C for later use. One microliter of the supernatant was used to PCR-screen for *Wolbachia*.

Polymerase Chain reaction (PCR) amplification

The extracted DNA from all collected specimens was checked their quality by PCR targeting moth DNA using arthropod-specific 28S primers (Werren et al., 1995). The DNA from specimens with no amplification from 28S were extracted again. Infection of *Wolbachia* was detected using three sets of primers. All the specimens were screened for *Wolbachia* detection by using *wsp* (Zhou et al., 1998), 16S rRNA (O'Neill et al., 1992) and *ftsZ* (Baldo et al., 2006) genes which amplified the PCR product at around 600 bp, 900 bp and 600 bp, respectively, using previously described primers and protocols. The primers for *Wolbachia wsp* gene were *wsp*-F1: 5′TGGTCCAATAAGTGAGAGAAAC-3′ and *wsp*-R1: 5'-AAAAATTAAACGCTACTCCA-3′. The *Wolbachia ftsZ* primers were *ftsZ*-F1: 5′-TACTGACTGTTGGAGTTGTAACTAAGCCGT-3′ and *ftsZ*-R1: 5′-TGCCAGTTGAAGAAACTCTAACTC-3′. The primers for *Wolbachia* 16S rRNA were 16S-F1: 5'-TTGTAGCCTGCTATGGTATAACT-3′ and 16S-R1: 5'-GAATAGGAGTTTTCATGT-3′. DNA from *Wolbachia*-infected *Aedes*

albopictus was used as a positive control in this study. Contamination was checked by using double-distilled H_2O as a negative control. PCR amplification was done on a thermal cycler using 20 µl reaction mixture volumes containing: 2 µl 25 mM $MgCl_2$, 2 µl 10xPCR buffer, 20 µM of forward primer and reverse primer, 1.5 µl dNTPs (10 mM each) and 1 unit of Taq DNA polymerase. The double distilled H_2O was added to achieve the final volume of 20 µl. The PCR protocol of *wsp* and 16S rRNA primers included an initial denaturation at 94 °C for 3 minutes followed by 35 cycles with a denaturation step at 94 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72° C for 2 minutes and final extension at 72 °C for 10 minutes. The *ftsZ* protocol for PCR included a denaturation at 94° C for 3 minutes followed by 10 cycles of denaturation at 94 °C for 10 seconds, annealing at 65 °C for 30 seconds, and extension at 68 °C for 1 minute followed by 25 cycles with a denaturation step at 94 °C for 10 seconds, annealing at 65 °C for 30 seconds and extension at 68 °C for 1 minute. Ten microliters of each PCR product were run on a 1% agarose gel with a 1-kb ladder to determine the presence and size of amplified DNA. PCR products of the correct size for the region amplified were scored as positive for *Wolbachia*.

Real-time quantitative PCR (qPCR)

An assay of real-time fluorescence detection quantitative PCR (qPCR) was performed to determine the relative *Wolbachia* density in each moth individual using SYBR Green with a LightCycler® 480 System (Roche Diagnostics, Japan). The *Wolbachia ftsZ* primers (*ftsZ*77 Bf1 and *ftsZ*77 Br1) that amplified 111 bp of PCR product were used for the real-time quantitation (Noda et al., 2001). These primers were used specifically to amplify the *ftsZ* gene of *Wolbachia*. The insect host single copy gene, proliferating cell nuclear antigen (PCNA) was quantified to allow normalization for comparing the density among species. A pair of PCNA primers (PCNAF and PCNAR) (Wiwatanaratanabutr and Kittayapong, 2006) was used for the assay. Each run consisted of a series of DNA standard samples for quantitation, the unknown DNA samples (two-fold replication of each sample were performed with similar results) and ddH2O as a negative control to check for contamination. The reaction mixture (25 µl total volume) in each well in a 96-well plate contained a DNA sample, 200 mM of ATP, CTP and GTP, 50 nM SYBR Green, a pair of 300 nM of primers, 0.2 U of AmpErase UNG, 5 mM MgCl2, 0.6 U of Ampli Taq Gold, 400 mM of UTP and 8% glycerol. The

qPCR temperature profile was 50 °C for 2 min to work AmpErase UNG to prevent amplicon carryover contamination and 95 °C for 10 min to activate Ampli Taq Gold, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The absence of non-specific PCR products for all targets was confirmed by electrophoretic separation of the products in agarose gels. For each of the reactions, DNA standard samples including dilution series of PCR products (108,107, 106, 105, 104 and 103 copies/µl) were included to estimate the absolute copy numbers of the target sequence in the DNA samples. To prepare DNA standard samples, the gel of PCR products was excised and purified by Wizard SV (Promega). The DNA standard samples' copy numbers were estimated by the concentration measured with a spectrophotometer and calculated with respect to the molecular weight data of a nucleotide presented. The Absolute Quantification analysis using the Second Derivative Maximum method implemented in the LightCycler® 480 Instrument Operator Software Version 1.5 (Roche) was used to analyze the qPCR data obtained in this study.

Statistical analysis

ANOVA was used to compare and identify the differences in *Wolbachia* infection frequency among different populations from three regions of Thailand. All data was analyzed using DPS software (Tang and Feng, 2002). A $P < 0.05$ was considered significant.

RESULTS

This study was the first survey and first report of the *Wolbachia* infection status in different species of moths from Thailand. A total of 1,235 individuals representing 58 moth species belonging to 13 families in the order Lepidoptera collected during the field surveys in 5 provinces (Saraburi, Nakhon Nayok, Nakhon Ratchasima, Prachinburi and Phetchaburi) from two tropical forests in three different geographic regions including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West) from Thailand were first screened for *Wolbachia* infection status by PCR assay using *Wolbachia*-specific 16S rRNA, *ftsZ* and *wsp* gene primers. In total, the results clearly showed that 625 (male = 209 individuals and female = 416 individuals) of 1,235 individuals (specimens) (50.61%) or 28 of 58 species (48.28%) from all three populations of Thailand were positive for *Wolbachia*

infection. The *wsp* and *ftsZ* PCR products were about 600 bp. while the 16S rRNA PCR products of *Wolbachia* were around 900 bp. Similar PCR results were obtained from all three pairs of primers although there are a few differences but not significant among them. In total of 625 *Wolbachia* positive specimens, they included 28 species from 9 families, i.e., Crambidae (1, 18), Endromidae (2, 52), Erebidae (2, 63), Geometridae (10, 233), Lasiocampidae (1, 11), Lymantriidae (1, 19), Noctuidae (4, 69), Sphingidae (6, 140), Uraniidae (1, 20) (The number in parentheses indicated the number of positive species in each family and the number of positive specimens in each family, respectively). PCR-based distribution of *Wolbachia* in 58 moth species from 13 families in the Lepidoptera in total of 1,235 specimens collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand including species name, family, location (province), geographic regions, no. of samples tested, no. of positive samples (% infection) and no. of positive samples (by sex) was listed in Table 1.

Table 1. PCR-based distribution of *Wolbachia* in 58 moth species from 13 families in the order Lepidoptera in total of 1,235 specimens collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia*.

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of positive samples by sex	
						Male	Femle
Abraxas sp.	**Geometridae**	**Saraburi**	**C**	**11**	**9 (81.81)**	**3**	**6**
		Nakhon Nayok	**C**	**10**	**7 (70.00)**	**4**	**3**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**7**	**9**
Acherontiia styx	Sphingidae	Nakhon Ratchasima	NE	8	0	3	5
		Phetchaburi	W	4	0	1	3
		Prachinburi	C	5	0	3	2
Acosmeryx sericeus	Sphingidae	Prachinburi	C	3	0	1	2
		Phetchaburi	W	7	0	3	4
Acosmeryx shervillii	**Sphingidae**	**Nakhon Ratchasima**	**NE**	**8**	**6 (75.00)**	**2**	**4**
		Phetchaburi	**W**	**15**	**13 (86.67)**	**6**	**7**
		Nakhon Nayok	**C**	**9**	**7 (77.78)**	**3**	**4**
Amata sp.	Erebidae	Prachinburi	C	5	0	2	3
		Nakhon Ratchasima	NE	7	0	1	6
		Saraburi	C	4	0	2	2

Ambulyx sericeipennis	Sphingidae	Nakhon Nayok	C	8	0	3	5
		Phetchaburi	W	11	0	5	6
Ambulyx sp.	**Sphingidae**	**Saraburi**	**C**	**7**	**3 (42.85)**	**1**	**2**
		Nakhon Ratchasima	**NE**	**12**	**8 (66.67)**	**2**	**6**
		Phetchaburi	**W**	**14**	**11 (78.57)**	**4**	**7**
Antheraea assamensis	Saturniidae	Prachinburi	C	6	0	3	3
		Nakhon Nayok	C	9	0	4	5
		Phetchaburi	W	8	0	4	4
Antheraea frithi	Saturniidae	Nakhon Nayok	C	7	0	2	5
		Phetchaburi	W	3	0	0	3
		Nakhon Ratchasima	NE	5	0	1	4
Artena sp.	**Noctuidae**	**Phetchaburi**	**W**	**8**	**6 (75.00)**	**2**	**4**
		Nakhon Nayok	**C**	**3**	**2 (66.67)**	**0**	**2**
		Nakhon Ratchasima	**NE**	**5**	**4 (80.00)**	**1**	**3**
Asota plana	Erebidae	Saraburi	C	7	0	2	5
		Phetchaburi	W	12	0	4	8
		Prachinburi	C	6	0	2	4
Attacus atlas	Saturniidae	Nakhon Ratchasima	NE	8	0	2	6
		Phetchaburi	W	7	0	3	4
		Nakhon Nayok	C	3	0	2	1
Brahmaea hearseyi	Brahmaeidae	Phetchaburi	W	5	0	1	4
		Nakhon Ratchasima	NE	7	0	2	5
		Prachinburi	C	4	0	1	3
Buxura sp.	Geometridae	Nakhon Nayok	C	6	0	2	4
		Phetchaburi	W	4	0	2	2
		Nakhon Ratchasima	NE	8	0	3	5
Buxura inoui	**Geometridae**	**Phetchaburi**	**W**	**13**	**11 (84.61)**	**5**	**6**
		Nakhon Nayok	**C**	**6**	**3 (42.85)**	**1**	**2**
		Prachinburi	**C**	**5**	**4 (80.00)**	**1**	**3**
Carallia brachiata	**Geometridae**	**Nakhon Ratchasima**	**NE**	**7**	**3 (42.85)**	**1**	**2**
		Phetchaburi	**W**	**11**	**7 (63.63)**	**2**	**5**
		Saraburi	**C**	**5**	**2 (40.00)**	**0**	**2**
Celenna festivaria	**Geometridae**	**Phetchaburi**	**W**	**15**	**13 (86.67)**	**5**	**8**
		Nakhon Ratchasima	**NE**	**10**	**6 (60.00)**	**2**	**4**
		Nakhon Nayok	**C**	**6**	**4 (66.67)**	**1**	**3**
Cleora tenebrata	Geometridae	Saraburi	C	5	0	2	3
		Phetchaburi	W	8	0	3	5
		Nakhon Ratchasima	NE	4	0	2	2
Comibaena sp.	**Geometridae**	**Nakhon Nayok**	**C**	**7**	**4 (57.14)**	**1**	**3**
		Nakhon Ratchasima	**NE**	**9**	**6 (66.67)**	**2**	**4**
		Phetchaburi	**W**	**13**	**11 (84.61)**	**4**	**7**
Creatonotos transiens	**Erebidae**	**Saraburi**	**C**	**8**	**6 (75.00)**	**1**	**5**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**6**	**10**
		Nakhon Ratchasima	**NE**	**11**	**9 (81.81)**	**3**	**6**
Daphis hypothous	**Sphingidae**	**Phetchaburi**	**W**	**21**	**19 (90.47)**	**7**	**12**

		Nakhon Nayok	**C**	**9**	**6 (66.67)**	**2**	**4**
		Nakhon Ratchasima	**NE**	**13**	**11 (84.61)**	**4**	**7**
Dudusa vethi	Notodontidae	Phetchaburi	W	6	0	2	4
		Nakhon Nayok	C	2	0	2	0
		Prachinburi	C	4	0	1	3
Dysphania militaris	Geometridae	Nakhon Ratchasima	NE	2	0	0	2
		Prachinburi	C	5	0	3	2
		Phetchaburi	W	7	0	2	5
Euhampsonia roepkei	Notodontidae	Saraburi	C	3	0	1	2
		Nakhon Ratchasima	NE	6	0	3	3
		Nakhon Nayok	C	4	0	3	1
Euplocia membliaria	Erebidae	Phetchaburi	W	5	0	2	3
		Prachinburi	C	3	0	1	2
		Nakhon Nayok	C	5	0	2	3
Eudocima phalonia	Noctuidae	Nakhon Ratchasima	NE	4	0	2	2
		Saraburi	C	3	0	2	1
		Prachinburi	C	2	0	2	0
Fodina contigua	Erebidae	Nakhon Nayok	C	4	0	0	4
		Phetchaburi	W	7	0	4	3
		Nakhon Ratchasima	NE	5	0	2	3
Herochroma flavibasalis	**Geometridae**	**Phetchaburi**	**W**	**19**	**17 (89.47)**	**6**	**11**
		Prachinburi	**C**	**6**	**4 (66.67)**	**2**	**2**
		Nakhon Ratchasima	**NE**	**15**	**13 (86.67)**	**4**	**9**
Hippotion sp.	**Sphingidae**	**Prachinburi**	**C**	**8**	**5 (62.50)**	**2**	**3**
		Nakhon Ratchasima	**NE**	**11**	**7 (63.63)**	**3**	**4**
		Phetchaburi	**W**	**7**	**4 (57.14)**	**1**	**3**
Ischyja marapok	**Noctuidae**	**Nakhon Nayok**	**C**	**5**	**4 (80.00)**	**2**	**2**
		Saraburi	**C**	**9**	**6 (66.67)**	**1**	**5**
		Phetchaburi	**W**	**20**	**17 (85.00)**	**4**	**13**
Lyssa zampa	**Uraniidae**	**Nakhon Ratchasima**	**NE**	**8**	**5 (62.50)**	**2**	**3**
		Phetchaburi	**W**	**14**	**11 (78.57)**	**5**	**6**
		Nakhon Nayok	**C**	**7**	**4 (57.14)**	**1**	**3**
Marumba cristata	Sphingidae	Prachinburi	C	7	0	3	4
		Nakhon Nayok	C	4	0	1	3
		Nakhon Ratchasima	NE	6	0	2	4
Mustilizans dierli	**Endromidae**	**Phetchaburi**	**W**	**20**	**18 (90.00)**	**6**	**12**
		Nakhon Ratchasima	**NE**	**9**	**7 (77.78)**	**3**	**4**
		Prachinburi	**C**	**8**	**6 (75.00)**	**2**	**4**
Mustilia sphingiformis	**Endromidae**	**Nakhon Ratchasima**	**NE**	**10**	**7 (70.00)**	**1**	**6**
		Phetchaburi	**W**	**12**	**9 (75.00)**	**2**	**7**
		Nakhon Nayok	**C**	**7**	**5 (71.42)**	**1**	**4**
Neochera inops	Erebidae	Saraburi	C	4	0	3	1
		Prachinburi	C	2	0	0	2
		Phetchaburi	W	5	0	3	2
Neochera dominia	Erebidae	Nakhon Ratchasima	NE	3	0	1	2

		Nakhon Nayok	C	1	0	0	1
Olene mendosa	**Lymantriidae**	**Nakhon Nayok**	**C**	**6**	**3 (50.00)**	**1**	**2**
		Phetchaburi	**W**	**14**	**12 (85.71)**	**5**	**7**
		Prachinburi	**C**	**5**	**4 (80.00)**	**0**	**4**
Olepa sp.	**Erebidae**	**Nakhon Ratchasima**	**NE**	**12**	**10 (83.33)**	**3**	**7**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**6**	**10**
		Nakhon Nayok	**C**	**8**	**6 (75.00)**	**1**	**5**
Ourapteryx sp.	**Geometridae**	**Nakhon Nayok**	**C**	**9**	**6 (66.67)**	**2**	**4**
		Prachinburi	**C**	**5**	**3 (60.00)**	**1**	**2**
		Phetchaburi	**W**	**16**	**14 (87.50)**	**3**	**11**
Oxydes scrobiculata	**Noctuidae**	**Nakhon Ratchasima**	**NE**	**8**	**6 (75.00)**	**1**	**5**
		Phetchaburi	**W**	**10**	**8 (80.00)**	**2**	**6**
		Saraburi	**C**	**5**	**2 (40.00)**	**0**	**2**
Parasa bicolor	Limacodidae	Nakhon Nayok	C	2	0	2	0
		Prachinburi	C	4	0	3	1
Pergesa acteus	**Sphingidae**	**Phetchaburi**	**W**	**13**	**11 (84.61)**	**4**	**7**
		Nakhon Ratchasima	**NE**	**10**	**8 (80.00)**	**3**	**5**
		Nakhon Nayok	**C**	**7**	**4 (57.14)**	**1**	**3**
Pareuchaetes pseudoinsulata	Erebidae	Saraburi	C	2	0	0	2
		Phetchaburi	W	4	0	3	1
Parotis marginata	**Crambidae**	**Phetchaburi**	**W**	**11**	**7 (63.63)**	**3**	**4**
		Nakhon Ratchasima	**NE**	**9**	**8 (88.89)**	**2**	**6**
		Prachinburi	**C**	**4**	**3 (75.00)**	**1**	**2**
Prooedema inscisala	Crambidae	Nakhon Nayok	C	2	0	0	2
		Prachinburi	C	1	0	1	0
		Phetchaburi	W	5	0	2	3
Plutodes flavescens	**Geometridae**	**Nakhon Nayok**	**C**	**7**	**4 (57.14)**	**2**	**2**
		Nakhon Ratchasima	**NE**	**15**	**12 (80.00)**	**4**	**8**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**5**	**11**
Ramadasa pavo	Noctuidae	Saraburi	C	6	0	2	4
		Phetchaburi	W	10	0	2	8
Ruttellerona pallicostaria	**Geometridae**	**Nakhon Ratchasima**	**NE**	**11**	**9 (81.81)**	**3**	**6**
		Phetchaburi	**W**	**15**	**13 (86.67)**	**5**	**8**
		Prachinburi	**C**	**7**	**5 (71.42)**	**3**	**2**
Semiothisa eleonora	Geometridae	Nakhon Nayok	C	4	0	1	3
		Nakhon Ratchasima	NE	8	0	3	5
		Prachinburi	C	5	0	2	3
Spodoptera litura	Noctuidae	Nakhon Ratchasima	NE	7	0	2	5
		Saraburi	C	3	0	0	3
		Phetchaburi	W	8	0	2	6
Sympis rufibasis	Noctuidae	Nakhon Nayok	C	4	0	2	2
		Phetchaburi	W	5	0	1	4
Thyas honesta	**Noctuidae**	**Nakhon Ratchasima**	**NE**	**6**	**3 (50.00)**	**1**	**2**
		Phetchaburi	**W**	**11**	**9 (81.81)**	**4**	**5**
		Prachinburi	**C**	**5**	**2 (40.00)**	**0**	**2**
Theretra sp.	**Sphingidae**	**Phetchaburi**	**W**	**12**	**9 (75.00)**	**3**	**6**
		Nakhon Nayok	**C**	**5**	**2 (40.00)**	**0**	**2**
		Nakhon Ratchasima	**NE**	**9**	**6 (66.67)**	**2**	**4**

Testa montana	**Geometridae**	**Prachinburi**	**C**	**4**	**2 (50.00)**	**2**	**0**
		Phetchaburi	**W**	**8**	**6 (75.00)**	**1**	**5**
		Saraburi	**C**	**6**	**4 (66.67)**	**1**	**3**
Thosea siamica	Limacodidae	Nakhon Nayok	C	1	0	1	0
		Phetchaburi	W	3	0	2	1
Trabala sp.	**Lasiocampidae**	**Phetchaburi**	**W**	**7**	**5 (71.42)**	**1**	**4**
		Nakhon Nayok	**C**	**5**	**3 (60.00)**	**2**	**1**
		Nakhon Ratchasima	**NE**	**6**	**2 (33.33)**	**0**	**2**
Tarsolepis elephantorum	Notodontidae	Prachinburi	C	3	0	1	2
		Phetchaburi	W	5	0	2	3
		Nakhon Ratchasima	NE	4	0	2	2
Xanthomelaena schematias	Crambidae	Saraburi	C	2	0	0	2
		Phetchaburi	W	4	0	1	3
		Nakhon Nayok	C	1	0	1	0

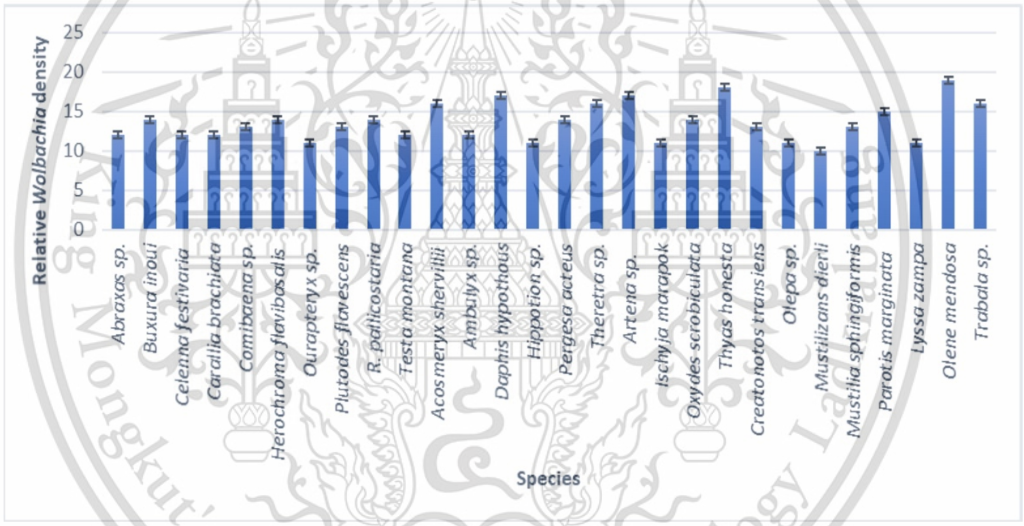

Fig. 1. The relative *Wolbachia* densities in 28 *Wolbachia*-infected moths from Thailand. The error bars represent standard error.

Most of the *Wolbachia* infected individuals were collected from Kaeng Krachan National Park where locates at Phetchaburi province in the West region of Thailand although Khao Yai National Park covers four provinces in two regions of Thailand, i.e., Saraburi, Nakhon Nayok, Prachinburi in the Central region, and Nakhon Ratchasima which is the largest city in Thailand in the Northeast region. The infection frequency of *Wolbachia* in different moth populations which collected from different three geographical regions of Thailand (the Central, the Northeast and the West) varied significantly ($F = 2.3650$, $p < 0.01$, df =

18), ranging from 33.33 % - 90.47 % with an average of 61.90% of infection based on the number of positive samples (Table 1). The highest infection frequency was found in Phetchaburi (the Western) populations (90.47%), while the lowest frequency was recorded in Nakhon Ratchasima (the Northeast) populations (33.33%) despite each region (West and Northeast) includes only 1 province in this study, i.e., Phetchaburi and Nakhon Ratchasima, respectively. The 28 *Wolbachia*-infected moth species included *Abraxas sp.*, *Acosmeryx shervillii*, *Ambulyx sp.*, *Artena sp.*, *Buxura inoui*, *Carallia brachiata*, *Celenna festivaria*, *Comibaena sp.*, *Creatonotos transiens*, *Daphnis hypothous*, *Herochroma flavibasalis*, *Hippotion sp.*, *Ischyja marapok*, *Lyssa zampa*, *Mustilizans dierli*, *Mustilia sphingiformis*, *Olene mendosa*, *Olepa sp.*, *Ourapteryx sp.*, *Oxydes scrobiculata*, *Pergesa acteus*, *Parotis marginate*, *Plutodes flavescens*, *Ruttellerona pallicostaria*, *Thyas honesta* , *Theretra sp.*, *Testa montana* and *Trabala sp.* In a total of 28 *Wolbachia*-infected species of moth (625 individuals), 144 individuals (Males = 46, Females = 98), 156 individuals (Males = 49, Females = 107) and 325 individuals (Males = 114, Females = 211) could be found in the Central region, the Northeast region, and the West region, respectively. All these 28 species could be found at all three regions above. The number of all moths collected in the West region was significantly higher than those moths collected in the other two regions ($F = 2.1750$, $p < 0.01$, $df = 13$). Regarding the *Wolbachia*-infected specimens, *D. hypothous*, the jade hawkmoth in the family Sphingidae, had the highest number of infected specimens collected in this study (36 individuals) whereas *Trabala sp.* in the family Lasiocampidae had the lowest number of infected specimens collected here (10 individuals). For *Wolbachia*-uninfected species, *A. plana* had the highest number of specimens collected in this study (25 individuals) whereas *P. bicolor*, the green rice moth in the family Limacodidae, had the lowest number of collected specimens (6 individuals). The infections of *Wolbachia* were not detected from all collected species of the four families including Brahmaeidae, Limacodidae, Notodontidae and Saturniidae although these families include some important species such as the family Saturniidae which contains some of the largest species of moths in the world. Comparisons among moth species indicated that the frequency of *Wolbachia*-infected species differed significantly ($F = 2.4830$, $p < 0.01$, $df = 14$). The conclusion regarding the number of infected specimens was shown as follow (In parentheses is the family name): *D. hypothous* (Sphingidae) 36 individuals, *H. flavibasalis* (Geometridae) 34 individuals, *Abraxas sp.*

(Geometridae) 32 individuals, *P. flavescens* (Geometridae) 32 individuals, *Olepa sp.* (Erebidae) 32 individuals, *M. dierli* (Endromidae) 31 individuals, *C. transiens* (Erebidae) 31 individuals, *I. marapok* (Noctuidae) 27 individuals, *R. pallicostaria* (Geometridae) 27 individuals, *A. shervillii* (Sphingidae) 26 individuals, *C. festivaria* (Geometridae) 23 individuals, *P. acteus* (Sphingidae) 23 individuals, *Ourapteryx sp.* (Geometridae) 23 individuals, *Ambulyx sp.* (Sphingidae) 22 individuals, *Comibaena sp.* (Geometridae) 21 individuals, *M. sphingiformis* (Endromidae) 21 individuals, *L. zampa* (Uraniidae) 20 individuals, *O. mendosa* (Lymantriidae) 19 individuals, *B. inoui* (Geometridae) 18 individuals, *P. marginata* (Crambidae) 18 individuals, *Theretra sp.* (Sphingidae) 17 individuals, *O. scrobiculata* (Noctuidae) 16 individuals, *Hippotion sp.* (Sphingidae) 16 individuals, *T. honesta* (Noctuidae) 14 individuals, *T. montana* (Geometridae) 12 individuals, *Artena sp.* (Noctuidae) 12 individuals, *C. brachiata* (Geometridae) 12 individuals and *Trabala sp.* (Lasiocampidae) 12 individuals in total of 28 species in 625 individuals.The relative *Wolbachia* densities from 28 infected moth species were determined by quantitative real-time PCR assay using the *ftsZ* gene as the target sequence (Fig. 1). The relative *Wolbachia* densities within each species were different from each other. *Olene mendosa*, the brown tussock moth or hairy tussock moth of the family Erebidae, had the highest relative *Wolbachia* densities compared with the other 27 infected species while *M. dierli* showed the lowest density among all infected species. The sequences of relative *Wolbachia* densities among these 28 species from the highest to the lowest are as follows: *O. mendosa*, *T. honesta*, *Artena sp.*, *D. hypothous*, *Trabala sp.*, *Theretra sp.*, *A. shervillii*, *P. marginata*, *B. inoui*, *H. flavibasalis*, *R. pallicostaria*, *P. acteus*, *O. scrobiculata*, *M. sphingiformis*, *C. transiens*, *P. flavescens*, *Comibaena sp.*, *Abraxas sp.*, *C. festivaria*, *C. brachiate*, *T. montana*, *Ambulyx sp.*, *L. zampa*, *Olepa sp.*, *I. marapok*, *Hippotion sp.*, *Ourapteryx sp.* and *M. dierli*. Interestingly, at least ten species from these infected species are agricultural insect pests including *O. mendosa*, *O. scrobiculata*, *T. honesta*, *D. hypothous*, *A. shervillii*, *P. acteus*, *C. transiens*, *T. montana*, *I. marapok* and *Hippotion sp.* The *O. mendosa*, which had the highest relative *Wolbachia* densities, was an important agricultural pest of Durian in Thailand. The jade hawkmoth, *D. hypothous* that had the highest number of infected specimens in total of 36 individuals of 43 collected individuals, had the relative *Wolbachia* densities in the fourth rank.

DISCUSSION

This report was the first discovery of *Wolbachia* infection and their relative density in different moth species from Thailand. The percentage reported here might undoubtedly increase if more specimens were examined or different methods of DNA extraction or PCR were applied. This study implies that the widespread distribution of *Wolbachia* was unequal among moths' genera and geographic regions of Thailand. Species of moth which positive for *Wolbachia* included many important pest species that involved in the agriculture of Thailand. Some moth species from this study have been previously reported to be infected with *Wolbachia* but not from Thailand (Muhammad et al., 2015; Sumida et al., 2017). The distribution of all species in this study was mostly collected in the Western region where is the main source of forest ecosystem in Thailand. However, all species could be found in all three regions of Thailand. Most of *Wolbachia*-infected individuals were also collected from the Western region. In this region, habitats and temperature are quite different from the other regions of Thailand. These results may imply that climate differences might influence survival of moths as well as the infections of *Wolbachia*. Infected individuals were found in many species including major pests in agriculture. So far, no previous study has been reported on *Wolbachia* infection in moths from Thailand. Therefore, this is the first report on the findings of *Wolbachia* infection in those moth species from Thailand. The field investigation has indicated that 50.61 % (625 of 1,235 collected specimens) or 48.28 % (28 of 58 collected species) of moths from Thailand were infected with *Wolbachia*. *Wolbachia* infections have been reported in many insect species such as mosquitoes, planthoppers, leafhoppers, etc. from Thailand but they have never been reported in moths. Most *Wolbachia*-infected moth species collected in this study were in the family Geometridae that has been known to be important pests in Thai agriculture. All individuals were collected from the two tropical forests in five provinces of three regions from Thailand which have been reported on the abundance of these Lepidopteran throughout Thailand. Many of collected moth species are commonly found in several areas of Thailand although some of them are rare to find in the present situation under the global warming and climate changes all over the world. The differences in the abundance of all collected moth species from Thailand in the present study and the previous study during 2010-2012 (around 10 years ago) by Suwannaphak

(2012) that reported about moth species from Thailand (specimens collected before the year 2012) might have resulted primarily from the changes in global climate, habitats, local temperature, ecosystem, time as well as other relevant factors. In 2013, Schneider and others have reported a VNTR-based (Variable-Number-Tandem-Repeat) molecular screening tool for detecting *Wolbachia* infections in tsetse flies and showed that their infections in *Glossina* spp. can escape the standard PCR screening methods by hiding as low-titer infections below the detection threshold. Therefore, it is possible that not all infections were detected in the current work due to the low titre infections of *Wolbachia* if we used only a standard PCR screening. In this study, another method using real-time quantitative PCR was applied to this study for confirmation of *Wolbachia* infections. A quantitative real-time PCR requires the absolute quantification of *fisZ* gene that is a single copy per genome in *Wolbachia*, and thus DNA standard with known concentration was required and the host single copy gene was also quantified in order to allow normalization for making a comparison among species. The real-time PCR does not detect PCR products but measures fluorescence that is released from a reporter dye. So, threshold cycles, in which the fluorescence begins to increase from the background level, are measured. The serial dilution of standards produced a reliable standard curve that decreases in any differences in the binding efficiencies of primers with DNA samples. The relative *Wolbachia* densities within each host species of all three regions from Thailand were different from each other but not significant difference as shown in Fig. 1 ($P \geq 0.01$). According to the Fig. 1, there are more than 10 species of collected moths that are agricultural insect pests of Thailand. Those moths had a *Wolbachia* densities in the similar level, not significant difference among each other. The low density in the infected moths probably implied that *Wolbachia* might not spread through moth populations in Thailand due to some climate change effects or temperature difference. However, another possibility of the detection of this low density or the absence of *Wolbachia* in other moth species is that the *Wolbachia* load might be below the threshold level of a real-time PCR detection system. Therefore, we need to continue and further study about the diversity and distribution of *Wolbachia* infection as well as their infection density in Lepidopteran from Thailand in the future. Our findings are relevant to ongoing efforts to manage pollinators like moths and to understand the distribution of moth populations and their *Wolbachia* infections from Thailand. In addition, this certain result invites further thought on and investigation into our understanding of the

evolutionary dynamics of *Wolbachia* infections, their density in hosts and insects across ecosystems. We found that geographical variation with closer locations tending to display the similar infection levels, and no evidence closely related to taxonomic groups indicate similar infection levels. This observational study explains the intensive and substantial undertakings required to address this finding with well-designed research studies. We also found that latitudinal gradient might be an important factor in infection level, i.e., lower frequencies towards higher latitudes. This study shows a latitudinal gradient in *Wolbachia* infection at a broad taxonomic and geographic scales in different regions as well as implies that *Wolbachia* infection might be predictable from ecological variables. Therefore, the study of the *Wolbachia* infection density would be the fundamental research for further biological investigations of the *Wolbachia* effects on moths and has been very helpful in the application of insect natural enemies for pest managements such as caterpillars' control in the future.

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REFERENCES

Bagheri, Z., Talebi, A.A., Asgari, S., Mehrabadi, M., 2019. *Wolbachia* induce cytoplasmic incompatibility and affect mate preference in *Habrobracon hebetor* to increase the chance of its transmission to the next generation. J. Invert Pathol. 163, 1-7.

Baldo, L., Bordenstein, S., Wernegreen, J.J., Werren, J.H., 2006. Widespread recombination throughout *Wolbachia* genomes. Mol. Biol. Evol. 23, 437- 449.

Bipinchandra, K., Salunke, R.C., Salunkhe, D.P., Dhotre, S.A., Walujkar, A.B., Khandagale, R.C., Rakesh, K., Chandode, H.V., Ghate, M.S., Patole, J.H., Werren, Y.S., 2012. Determination of *Wolbachia* Diversity in Butterflies from Western Ghats, India, by a Multigene

Approach. Appl. Environ. Microbiol. 12, 4458-4467. DOI: 10.1128/AEM.07298-11

Braig, H.R., Zhou, W., Dobson, S.L., O'Neill, S.L., 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180, 2373-2378.

Carjaval, T.M., Capistrano, J.D.R., Hashimoto, K., Go, K.J.D., Cruz, M.A.I.J., Martinez, M.J.L. B., Tiopianco, V.S.P., Amalin, D.M., Watanabe, K., 2018. Detection and distribution of *Wolbachia* endobacteria in *Culex quinquefasciatus* populations (Diptera: Culicidae) from Metropolitan Manila, Philippines. J. Vector Borne Dis. 55, 265-270.

Hiroki, M., Tagami, Y., Miura, K., Kato, Y., 2004. Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*. Proc. Biol. Sci. 271, 1751–1755.

Kajtoch, L., Kolasa, M., Kubisz, D., Gutowski, J.M., Ścibior, R., Mazur, M.A., Holecová, M. 2019. Using host species traits to understand the *Wolbachia* infection distribution across terrestrial beetles. Sci Rep. 9, 847.

Kolasa, M., Kubisz, D., Mazur, M.A., Scibior, R., Kajtoch, L., 2018. *Wolbachia* prevalence and diversity in selected riverine predatory beetles (Bembidiini and Paederini). B. Insectol. 71, 193-200.

Lopez, V., Cortesero, A.M., Poinsot, D., 2018. Influence of the symbiont *Wolbachia* on life history traits of the cabbage root fly (*Delia radicum*). J. Invert Pathol. 158, 24-31.

Muhammad, Z.A., Eli, V. Araujo-Jnr, J.J.W., Akito, Y.K., 2015. *Wolbachia* in butterflies and moths: geographic structure in infection frequency. Front. Zool. 12, 16 https://doi.org/10.1186/s12983-015-0107-z.

Noda, H., Koizumi, Y., Zhang, Q., Deng, K., 2001. Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. Insect Biochem. Mol. Biol. 31, 727–737.

O'Neill, S.L., Giordane, R., Colbert, A.M.E., Timothy, K., Robertson, H., 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with CI in insects. Proc. Natl. Acad. Sci. USA. 89, 2699-2702.

Pierce, NE., 1995. Predatory and parasitic Lepidoptera: Carnivores living on plants. Journal of the Lepidopterists' Society. 49(4), 412–53.

Plotkin, D., Goddard, J., 2013. Blood, sweat, and tears: a review of the hematophagous, sudophagous, and lachryphagous Lepidoptera. J Vector Ecol. 38(2), 289–94.

Schneider, D.I., Garschall, K.I., Parker, A.G., Abd-Alla, A.M.M., Miller, W.J., 2013. Global *Wolbachia* prevalence, titer fluctuations and their potential of causing cytoplasmic Incompatibilities in tsetse flies and hybrids of *Glossina morsitans* subgroup species. J. Invertebr. Pathol. 112, 104–115.

Stouthamer, R. Breeuwer, J.A.J., Hurst, G.D.D., 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Ann. Rev. Microbiol. 53, 71–102.

Sumida, Y., Katsuk, M., Okada, K., Okayama, K., Lewis, Z., 2017. *Wolbachia* induces costs to life- history and reproductive traits in the moth, *Ephestia kuehniella*. J. Stored Prod. Res. 71, 93-98.

Suwannaphak, K., 2012. Photographic guide to Moths in Thailand. Published by BNEC (Banna Nature Education Center, Nakhon Nayok, Thailand.

Tang, Q.Y., Feng, M.G., 2002. DPS data processing system for practical statistics. Science Press.

Werren, J.H., Baldo, L., Clark, M.E., 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751.

Werren, J.H., Windsor, D., Guo, L., 1995. Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. B 262, 197–204.

Wiwatanaratanabutr, S., Kittayapong, P., 2006. Effects of temephos and temperature on *Wolbachia* load and life history traits of *Aedes albopictus*. Med. Vet. Entomol. 20, 300–307.

Wiwatanaratanabutr, I., Kittayapong, P., 2009. Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. J. Invertebr. Pathol. 102, 220–224.

Wiwatanaratanabutr, I., 2013. Geographic distribution of *Wolbachia* infections in mosquitoes from Thailand. J. Invertebr. Pathol. 114, 337–340.

Zaspel, J.M., Weller, S.J., Branham, M.A., 2011. A comparative survey of proboscis morphology and associated structures in fruit-piercing, tear-feeding, and blood-feeding moths in Calpinae (Lepidoptera: Erebidae). Zoomorphology. 130(3), 203–25.

Zhou, W., Rousset, F., O'Neil, S., 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. Biol. Sci. 265, 509–515.


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First systematic survey of *Wolbachia* infection in butterflies (Order Lepidoptera) from Thailand: Distribution and diversity in tropical ecosystem

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ABSTRACT

The genus of maternally inherited bacteria, *Rickettsia*-like named *Wolbachia* infects a wide scale of insects. It is reported as a reproductive manipulator which causes cytoplasmic incompatibility and many phenotypes, although their biology and ecology have not been surveyed in detail for Thai butterflies. In this study, the first systematic survey of the *Wolbachia* infection status which was conducted between January to December 2019 in different species of butterflies (Order Lepidoptera) from tropical forests in 3 geographic regions of Thailand was reported. The 623 specimens from 46 butterfly species in 5 families were checked for *Wolbachia* infection. All samples were collected from two tropical forests consist of Khao Yai National Park (the Northeast and the Central regions) and Kaeng Krachan National Park (the West region) of Thailand. The *Wolbachia* infections were checked by using PCR method with three gene primers including *16S rRNA*, *ftsZ* and *wsp* which the results clearly showed the evidence of *Wolbachia* infection in these butterflies. *Wolbachia* was detected in 291 individuals from 17 butterfly species of 2 families, comprising 86 individuals from the Central (32 males and 54 females), 72 individuals from the Northeast (25 males and 47 females), and 133 individuals from the West region (46 males and 87 females). This report is potentially useful to understand the *Wolbachia* diversity in butterflies, especially in Asia and other tropical areas.

Keywords: Distribution, Infection, Lepidoptera, Butterfly, *Wolbachia*

INTRODUCTION

Members of the intracellular-inherited bacteria in the genus *Wolbachia* frequently infect a wide range of insects. Approximately 20-76% of all insects are estimated to be infected by *Wolbachia* (Bagheri et al. 2019). The *Wolbachia* strains can decrease adult lifespan and sustain themselves in their hosts. *Wolbachia* can be involved in the host's reproductive manipulation, resulting in various abnormal reproductive changes including feminization, parthenogenesis, male killing and cytoplasmic incompatibility (Kajtoch et al. 2019). These changes support *Wolbachia*'s distribution in their hosts by increasing the infected female individuals. The most well-known of these alterations is Cytoplasmic Incompatibility (CI) which takes place when *Wolbachia*-infected males mate with uninfected females / infected females harbor a different *Wolbachia* strain, and causes unfertilized egg.

The Order Lepidoptera constitutes around 157,000 butterfly and moth species which has already been identified at present. The Lepidoptera play an important role in ecosystem as they are beneficial insects, however, some species also feed on animal secretions and its blood. They consist of many agricultural pests whereas some species are predators and parasites. Nowadays, *Wolbachia* is being applied as a novel biocontrol method to control many disease vectors and insect pests (Cardona-Salgado et al. 2020). In this study, the geographic distribution and diversity, together with the infection status of *Wolbachia* infection in butterflies from three different geographic regions in two different tropical forest ecosystems of Thailand were first investigated. The exploration of *Wolbachia* infection status will lay the basis for further biological study of *Wolbachia* infection in Lepidopteran which has been very useful in the application of insect natural enemies for pest management.

MATERIAL AND METHODS

Materials

Collection of insects

Butterflies were collected during January - December 2019 in two tropical forest ecosystems from three different regions (the Central, the Northeast and the West of Thailand) including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West). All butterfly specimens under this study were collected by netting a butterfly in an aerial net and then morphologically identified to species (or genus) at King Mongkut's Institute of Technology Ladkrabang in Bangkok, Thailand. Legs were removed and kept in ethanol at -20 °C until use.

DNA Extraction

Legs of butterflies were used for PCR detection, and their body was kept for identification. DNA was isolated using a QIAamp DNA minikit (Qiagen) according to the manufacturer's recommendation. The tissues were lysed about 20 minutes by using specific enzymes and buffers. Absolute ethanol was added, and lysates were loaded onto the QIAamp spin column. Buffers were used to wash and remove contamination, and then DNA was eluted in distilled water. The extracted DNA was maintained at -20 °C for later processes. The supernatant 1 µl was used for PCR detection.

Polymerase chain reaction

The extracted DNA's quality from all collected specimens was confirmed by PCR using arthropod-specific 28S primers. The DNA from specimens with no amplification from 28S were extracted again. Infection of *Wolbachia* was detected using three sets of primers. The specimens were checked for *Wolbachia* infection using *16S rRNA*, *wsp* and *ftsZ* gene primers which amplified the PCR product around 900 bp, 600 bp and 600 bp, respectively. The *wsp* primers were *wsp*-F1: 5′TGGTCCAA TAAGTGAGAGAAAC-3′ and *wsp*-R1: 5'-AAAAATTAAACGCTACTCCA-3′. The *ftsZ* primers for *Wolbachia* were *ftsZ*-F1: 5′-TACTGACTGTTGGAGTTGTAACTAAGCCGT-3′ and *ftsZ*-R1: 5′-TGCCAGTTGAAGAAAC TCTAACTC-3′. The *Wolbachia* 16S rRNA primers were 16S-F1: 5'-TTGTAGCCTGCTATGGTATAA CT-3′ and 16S-R1: 5'-GAATAGGAGTTTTCATGT-3′. DNA of *Aedes albopictus* (*Wolbachia* positive) was selected as a positive control in this study. The double-distilled H_2O (ddH_2O) was used as a negative control for contamination check. PCR assay was conducted using a reaction mixture of 20 µl comprising $MgCl_2$ 25 mM 2 µl, 10xPCR buffer 2 µl, forward and reverse primers 20 µM, dNTPs 10 mM 1.5 µl, and *Taq* polymerase. The ddH_2O was added to make the final volume of 20 µl. The PCR assay of *16S rRNA* and *wsp* primers was an

initial step 3 minutes at 94 °C followed by 35 cycles of a denaturation 1 minute at 94 °C, annealing 1 minute at 55 °C, extension 2 minutes at 72° C, and final extension 10 minutes at 72 °C. The protocol for *ftsZ* PCR included 3 minutes at 94° C for denaturation followed by 25 cycles of denaturation 10 seconds at 94 °C, annealing 30 seconds at 65 °C, and extension 1 minute at 68 °C. PCR product 10 µl was applied on a 1% agarose gel to examine the size and presence of amplified DNA. PCR products with the correct size were defined as positive for *Wolbachia* infection.

Statistical analysis

ANOVA was used to compare and identify the *Wolbachia* infection frequency in different populations from three parts of Thailand. All data were analyzed using DPS software. A $P < 0.05$ was considered significant.

RESULTS

This investigation was the first survey and first report of the *Wolbachia* infection status in different species of butterfly from Thailand. The 46 butterfly species in total of 623 individuals from 5 families in the Order Lepidoptera collected during the field surveys in 5 provinces (Saraburi, Nakhon Nayok, Nakhon Ratchasima, Prachinburi and Phetchaburi) from two tropical forests in three different geographic regions including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West) from Thailand were first checked for *Wolbachia* infection status using PCR method with *16S rRNA*, *ftsZ* and *wsp* primers. In total, the results showed that 291 (male = 103 individuals and female = 188 individuals) of 623 individuals (specimens) (46.71%) or 17 of 46 species (36.96%) from all three populations of Thailand were positive for *Wolbachia* infection.

The *ftsZ* and *wsp* PCR products were about 600 bp. while the *16S rRNA* PCR products of *Wolbachia* were around 900 bp. Similar PCR results were obtained from all three pairs of primers. In total of 291 *Wolbachia* positive specimens, they included 17 species from 2 families including Nymphalidae (16, 273) and Papilionidae (1, 18) (The number in parentheses indicated the number of positive species in each family and the number of positive specimens in each family, respectively). PCR-based distribution of *Wolbachia* in 46 butterfly species from 5 families in the Lepidoptera in total of 623 specimens collected from two tropical forests in three different parts (C = the Central, NE = the Northeast and W = the West) of Thailand.

Many of *Wolbachia*-infected butterflies were collected from Kaeng Krachan National Park which were in Phetchaburi province (the West region of Thailand) whereas some of the *Wolbachia*-infected butterflies were found from Khao Yai National Park which covers four provinces including Saraburi, Nakhon Nayok, Prachinburi and Nakhon Ratchasima in the Northeast and the Central regions. The infection frequency of *Wolbachia* in different butterfly populations which were collected from different three geographical regions of Thailand (the West, the Northeast and the Central) differed significantly ($F = 1.8340$, df = 14, $p < 0.01$) from 40.00 % - 87.50 % with the average of 63.75 % of infection based on the number of positive samples. The highest infection frequency was found in Nakhon Nayok (the Central) populations (87.50%), while the lowest frequency was recorded in Phetchaburi (the West) populations (40.00%). Each region (the West and the Northeast) includes only 1 province in this study, i.e., Phetchaburi and Nakhon Ratchasima, respectively. The 17 *Wolbachia*-infected butterfly species included *Acraea violae*, *Cethosia cyane*, *Cynthia lepidea*, *Danaus chrysippus*, *Danaus genutia*, *Danaus melanippus*, *Doleschallia bisaltide*, *Junonia atlites*, *Junonia lemonias*, *Junonia orithya*, *Melanitis leda*, *Mycalesis mineus*, *Mycalesis perseoides*, *Mycalesis perseus*, *Papilio polytes*, *Parantica aglea* and *Parantica agleoides*.

In a total of 17 *Wolbachia*-infected species of butterfly (291 individuals), 86 individuals (Males = 32, Females = 54), 72 individuals (Males = 25, Females = 47) and 133 individuals (Males

= 46, Females = 87) could be found in the Central region, the Northeast region, and the West region, respectively. All these 17 species could be found at all three regions above. Interestingly, the number of positive females appeared to be higher than males from all populations. In addition, the butterflies collected in the West region was significantly higher than butterflies collected from the other two areas ($F = 1.7460$, df = 18, $p < 0.01$). Regarding the *Wolbachia*-infected specimens, *M. leda*, the common evening brown butterfly, in the family Nymphalidae, had the highest number of infected individuals collected (25 from 30 individuals) whereas *C. lepidea* and *D. melanippus* in the family Nymphalidae had the lowest infected samples collected (12 from 17 and 18 individuals, respectively). For *Wolbachia*-uninfected species, *C. pomona* had the highest specimens number collected (26 individuals) whereas *H. bolina, L. athymmus, N. hylas, P. helenus, P. nephelus* in the family Nymphalidae, Lymphalidae, Nymphalidae, Papilionidae and Papilionidae, respectively had the lowest number of collected specimens (3 individuals).

The infections of *Wolbachia* were not detected from all collected species of these three families including Hesperiidae, Lymphalidae and Pieridae, although these families include some important species of the world. The comparisons in these butterflies showed that the frequency of *Wolbachia* infection differed significantly ($F = 1.5960$, $p < 0.01$, df = 12). The conclusion regarding the number of infected specimens was shown as follow (In parentheses is the family name): *M. leda* (Nymphalidae) 25 individuals, *M. perseus* (Nymphalidae) 24 individuals, *P. aglea* (Nymphalidae) 23 individuals, *M. mineus* (Nymphalidae) 21 individuals, *C. cyane* (Nymphalidae) 19 individuals, *M. perseoides* (Nymphalidae) 19 individuals, *P. polytes* (Papilionidae) 18 individuals, *P. agleoides* (Nymphalidae) 18 individuals, *A. violae* (Nymphalidae) 15 individuals, *D. chrysippus* (Nymphalidae) 15 individuals, *D. bisaltide* (Nymphalidae) 15 individuals, *J. atlites* (Nymphalidae) 15 individuals, *J. orithya* (Nymphalidae) 14 individuals, *D. genutia* (Nymphalidae) 13 individuals, *J. lemonias* (Nymphalidae) 13 individuals, *C. lepidea* (Nymphalidae) 12 individuals and *D. melanippus* (Nymphalidae) 12 individuals in total of 17 species from 291 individuals.

DISCUSSION

This survey was the first report of *Wolbachia* infection in different butterfly species from Thailand. The report implied that the *Wolbachia* distribution was unbalanced in butterfly species and in different geographic parts of Thailand. Many of butterflies which were infected by *Wolbachia* are the significant agricultural pests of Thailand. Some butterflies have previously been reported for *Wolbachia* infection but not from Thailand (Muhammad et al. 2015). Mostly, the species distribution including infected individuals was collected in the West region where is the major area of tropical forest ecosystem in Thailand. Nevertheless, all collected species could be found in all three regions. The temperature and habitats in each region are also different from each other. The results could suggest that the temperature differences might affect the survival of butterfly and *Wolbachia* infections. The multiple *Wolbachia* infections inducing different reproductive traits were found in some butterfly species, for example, *Eurema hecabe*. So far, there has never been reported on the infection of *Wolbachia* in butterfly from Thailand, therefore, this is the first report regarding this finding.

This field survey has shown that 46.71 % (291 of 623 collected samples) or 36.96 % (17 of 46 collected species) in butterflies from Thailand were infected with *Wolbachia*. These findings were not much different from the previous report of Salunke et al. (2012) regarding the determination of *Wolbachia* diversity in butterflies from India. The infections of *Wolbachia* have been studied in several insect species such as planthoppers, leafhoppers and mosquitoes from Thailand (Wiwatanaratanabutr, 2015) but they have never been reported in butterfly. Most *Wolbachia*-infected butterflies were in the family Nymphalidae that are the important pests in

Thailand. All butterfly species were collected from the two tropical forests in five provinces of three regions from Thailand which have been reported on the abundance of these Lepidopteran throughout Thailand.

Our findings are relevant to ongoing efforts to manage pollinators like butterflies and to understand the distribution of butterfly populations and their *Wolbachia* infections from Thailand. The results suggested further investigation on the evolutionary dynamics of *Wolbachia* infections in insect hosts across tropical ecosystems. In addition, the geographical variation with nearer locations might show the similar level of infections without any evidence relative to taxonomic groups. This report could explain the intensive and substantial undertakings needed to address this finding with our well-designed research studies. Moreover, the latitude might be another factor affecting the infection level. This report also displays the effect of latitudinal gradient on the infection of *Wolbachia* at a wide geographic scale and various taxonomic groups in different regions of Thailand. Therefore, the study of the *Wolbachia* infection status would be the fundamental research for further biological studies about the effects of *Wolbachia* in butterflies and has been much helpful for the application used in pest managements such as caterpillar control in the future. This work is potentially useful to understand the diversity of *Wolbachia* in insects, especially in tropical ecosystems.

CONCLUSION

The conclusion regarding the number of infected specimens was shown as follow (In parentheses is the family name): *M. leda* (Nymphalidae) 25 individuals, *M. perseus* (Nymphalidae) 24 individuals, *P. aglea* (Nymphalidae) 23 individuals, *M. mineus* (Nymphalidae) 21 individuals, *C. cyane* (Nymphalidae) 19 individuals, *M. perseoides* (Nymphalidae) 19 individuals, *P. polytes* (Papilionidae) 18 individuals, *P. agleoides* (Nymphalidae) 18 individuals, *A. violae* (Nymphalidae) 15 individuals, *D. chrysippus* (Nymphalidae) 15 individuals, *D. bisaltide* (Nymphalidae) 15 individuals, *J. atlites* (Nymphalidae) 15 individuals, *J. orithya* (Nymphalidae) 14 individuals, *D. genutia* (Nymphalidae) 13 individuals, *J. lemonias* (Nymphalidae) 13 individuals, *C. lepidea* (Nymphalidae) 12 individuals and *D. melanippus* (Nymphalidae) 12 individuals in total of 17 species from 291 individuals.

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REFERENCES

[1] Bagheri Z, Talebi AA, Asgari S, Mehrabadi M (2019). *Wolbachia* induce cytoplasmic incompatibility and affect mate preference in *Habrobracon hebetor* to increase the chance of its transmission to the next generation. *J. Invertebr Pathol* 163:1-7.

[2] Cardona-Salgado D, Campo-Duarte DE, Sepulveda-Salcedo LS, Vasilieva O (2020). *Wolbachia*-based biocontrol for dengue reduction using dynamic optimization approach. *Appl Math Model* 82:125-149.

[3] Kajtoch L, Kolasa M, Kubisz D, Gutowski JM, Ścibior R, Mazur MA, Holecová M (2019). Using host species traits to understand the *Wolbachia* infection distribution across terrestrial beetles. Sci Rep 9:847 https://doi.org/10.1038/s41598-018-38155-5.

[4] Muhammad ZA, Eli V, Araujo-Jnr JJW, Akito YK (2015). *Wolbachia* in butterflies and moths: geographic structure in infection frequency. *Front Zool* 12:16 https://doi.org/10.1186/ s12983-015-0107-Z

[5] Salunke BK, Salunkhe RC, Dhotre DP, Walujkar SA, Khandagale AB, Chaudhari R, Chandode RK, Ghate HV, Patole MS, Werren JH, Shouche YS (2012). Determination of *Wolbachia* diversity in butterflies from western ghats, India, by a multigene approach. *Appl Environ Microbiol* 78(12):4458-4467.

[6] Wiwatanaratanabutr I (2015). *Wolbachia* infection in leafhoppers and planthoppers: Diversity, density and geographic distribution in tropical rice agroecosystems. *J Asia-Pacific Entomol* 18:277-282.


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Infection density, diversity, and distribution of *Wolbachia* bacteria in moths (Order Lepidoptera): First systematic report from Thailand

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ABSTRACT

Members of the genus *Wolbachia* are a group of *Rickettsia*-like, intracellular, maternally inherited bacterial endosymbionts that infect a diverse range of insects and cause reproductive changes in their hosts. Although *Wolbachia*'s role in many insects has been extensively studied, only a little of their effects on host's reproduction and their infection frequencies were reported in Lepidopteran which is one of the most diverse insects. Here, we present the first systematic survey of the *Wolbachia* infection status in different species of moths from three different geographic regions of Thailand, which was carried out during January to December in 2019 with the screening of 1,235 specimens in total of 58 moth species from 13 families. Specimens were collected from Khao Yai National Park (the Central and the Northeast regions), and Kaeng Krachan National Park (the West region). Infections of *Wolbachia* were screened by using polymerase chain reaction with 16S rRNA, *ftsZ* and *wsp* gene primers which the results indicated high rates of *Wolbachia* infection in moth populations from Thailand. *Wolbachia* was found in all different geographically populations in total of 625 individuals in total of 28 moth species from 9 families, including 144 individuals (46 males and 98 females) from the Central, 156 individuals (49 males and 107 females) from the Northeast, and 325 individuals (114 males and 211 females) from the West. The highest infection rate was 90.47% in the West populations and the average infection rate was 61.90%. The detection of *Wolbachia* in different moth populations from all regions was identical when all primers were used to screen for *Wolbachia*. The relative densities of *Wolbachia* within each individual were determined using quantitative real-time PCR and the result showed that there was a low *Wolbachia* infection density in these moth populations. These findings indicated that *Wolbachia* are distributed throughout the moth populations from Thailand.

Introduction

Wolbachia, the most common and widespread intracellular bacterium on Earth, is a genus of maternally inherited bacteria that infect a diverse range of arthropods. Around 20–76% of all insect species are estimated to be infected by *Wolbachia* bacteria (Stouthamer et al., 1999; Werren et al., 2008). Some *Wolbachia* strains can sustain themselves in their hosts and decrease adult lifespan (Jturbe-Ormaetxe et al., 2011). *Wolbachia* can be involved in the manipulation of their host's reproductive system, resulting in different abnormal reproductive phenotypes including cytoplasmic incompatibility, male killing, feminization and parthenogenesis (Werren et al., 2008; Bagheri et al., 2019; Kajtoch et al., 2019). These phenotypes promote the distribution of *Wolbachia* in host populations by increasing the numbers of infected females. The best known of these traits is Cytoplasmic Incompatibility (CI) which its intensity is affected by many factors such as temperature and rearing density (Wiwatanaratanabutr and Kittayapong, 2006, 2009; Sumida et al., 2017). The CI occurs when infected males mate with uninfected females or with females that harbor a different *Wolbachia* strain, and results in the death of the fertilized egg which make a reproductive advantage to infected hosts that enable *Wolbachia* to spread rapidly through a host population (Braig et al., 1998).

The detection, distribution, infection density and diversity of *Wolbachia* have been studied in many groups of insects such as root fly, mosquitoes, leafhoppers, planthoppers, butterfly, moth, beetles and wasps (Salunke et al., 2012; Wiwatanaratanabutr, 2013, 2015; Sumida

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et al., 2017; Carvajal et al., 2018; Kolasa et al., 2018; Lopez et al., 2018; Bagheri et al., 2019). Sequence analysis of 16S rRNA, *ftsZ* and *wsp* genes shows that members of the genus *Wolbachia* belongs to a monophyletic clade in the alphaproteobacterial group which is closely related to the *Ehrlichia* assemblage (Zhou et al., 1998). *Wolbachia* can be classified into many supergroups such as A and B that are the most widely distributed in insects (Werren et al., 2008).

Order Lepidoptera constitute one of the most diverse insect orders with more than 157,000 described species of butterflies and moths. Lepidopteran plays an important role in ecosystem function primarily as pollinators and herbivores, though some species feed on blood and other animal secretions (Plotkin and Goddard, 2013; Kajtoch et al., 2019). The order includes many significant agricultural pests, some species serve as models across biological disciplines (Zaspel et al., 2011) whereas some of them are predatory and parasitic Lepidopteran (Pierce, 1995). In Thailand, the diversity of Lepidopteran species has not been much studied, especially those associated with *Wolbachia* infection which has never been reported from Thailand. The biological, behavioral and ecological diversity of butterflies and moths suggests the need for further characterization of *Wolbachia* to understand the impact of infection on their evolution, speciation and reproduction (Hiroki et al., 2004; Muhammad et al., 2015). The information on molecular biology and phenotypic effects of *Wolbachia* from moth species show the presence of supergroup A and B *Wolbachia* strains (Muhammad et al., 2015). *Wolbachia* strains in butterflies have been implicated in basic biological processes such as sex ratio distortion, sex determination, sperm-egg compatibility, and speciation (Kajtoch et al., 2019).

While its role in some hosts has been studied extensively, its incidence and distribution among moth species from Thailand is still largely unknown and poorly understood. Here, the diversity and geographic distribution with the infection status and density of *Wolbachia* bacteria in moths from two different tropical forest ecosystems in three different geographic regions of Thailand were first investigated. Our study of the *Wolbachia* infection status would lay the groundwork for further biological investigations of the *Wolbachia* effects on Lepidopteran hosts and has been very helpful in the utilization of insect natural enemies for pest control.

Materials and methods

Insects collection

Moths used in this study were collected during January to December 2019 from two tropical forest ecosystems in three different regions (the Central, the Northeast and the West of Thailand) including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West) (Table 1). All specimens under this study were morphologically identified to species (or genus) levels by using the photographic guide to moths in Thailand (Suwannaphak, 2012) at Center of Excellence in Applied Biosciences, King Mongkut's Institute of Technology Ladkrabang in Bangkok, Thailand. Legs or abdomen of the moths were removed and preserved in absolute ethanol at −20 °C until DNA extraction.

DNA preparation

To extract DNA, adult's legs were used for PCR screening, and the rest of the specimen was preserved for identification. In cases that the specimens were small size, their abdomens were used instead of legs. DNA was extracted from tissue using a QIAamp DNA minikit (Qiagen) following the manufacturer's protocol. Briefly, all specimens were lysed around 20 min by using optimized buffers and specific enzymes, DNA was stabilized and enhanced selective DNA adsorption to the QIAamp membrane. Alcohol was added and lysates were loaded onto the QIAamp spin column. Wash buffers were used to remove impurities and pure DNA was then eluted in double-distilled water. The extracted DNA was kept at −20 °C for later use. One microliter of the supernatant was used to PCR-screen for *Wolbachia*.

Polymerase chain reaction (PCR) amplification

The extracted DNA from all collected specimens was checked their quality by PCR targeting moth DNA using arthropod-specific 28S primers (Werren et al., 1995). The DNA from specimens with no amplification from 28S were extracted again. Infection of *Wolbachia* was detected using three sets of primers. All the specimens were screened for *Wolbachia* detection by using *wsp* (Zhou et al., 1998), 16S rRNA (O'Neill et al., 1992) and *ftsZ* (Baldo et al., 2006) genes which amplified the PCR product at around 600 bp, 900 bp and 600 bp, respectively, using previously described primers and protocols. The primers for *Wolbachia wsp* gene were *wsp*-F1: 5′-TGGTCCAATAAGTGAGAGAAAC-3′ and *wsp*-R1: 5′-AAAAATTAAACGCTACTCCA-3′. The *Wolbachia ftsZ* primers were *ftsZ*-F1: 5′-TACTGACTGTTGGAGTTGTAACTAAGCCGT-3′ and *ftsZ*-R1: 5′-TGCCAGTTGAAGAAACTCTAACTC-3′. The primers for *Wolbachia* 16S rRNA were 16S-F1: 5′-TTGTAGCCTGCTATGGTATAACT-3′ and 16S-R1: 5′-GAATAGGAGTTTTCATGT-3′.

DNA from *Wolbachia*-infected *Aedes albopictus* was used as a positive control in this study. Contamination was checked by using double-distilled H_2O as a negative control. PCR amplification was done on a thermal cycler using 20 µl reaction mixture volumes containing: 2 µl 25 mM $MgCl_2$, 2 µl 10xPCR buffer, 20 µM of forward primer and reverse primer, 1.5 µl dNTPs (10 mM each) and 1 unit of *Taq* DNA polymerase. The double distilled H_2O was added to achieve the final volume of 20 µl. The PCR protocol of *wsp* and 16S rRNA primers included an initial denaturation at 94 °C for 3 min followed by 35 cycles with a denaturation step at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72° C for 2 min and final extension at 72 °C for 10 min. The *ftsZ* protocol for PCR included a denaturation at 94° C for 3 min followed by 10 cycles of denaturation at 94 °C for 10 s, annealing at 65 °C for 30 s, and extension at 68 °C for 1 min followed by 25 cycles with a denaturation step at 94 °C for 10 s, annealing at 65 °C for 30 s and extension at 68 °C for 1 min. Ten microliters of each PCR product were run on a 1% agarose gel with a 1-kb ladder to determine the presence and size of amplified DNA. PCR products of the correct size for the region amplified were scored as positive for *Wolbachia*.

Real-time quantitative PCR (qPCR)

A method of quantitative real-time PCR (qPCR) was conducted to determine the density of *Wolbachia* in each moth individual using SYBR Green for fluorescence detection in a LightCycler 480 System (Roche Diagnostics, Switzerland). The *Wolbachia ftsZ* primers (*ftsZ77* Bf1 and *ftsZ77* Br1) that amplified 111 bp of the *ftsZ* gene in PCR products were used for the quantitation (Noda et al., 2001). The insect host single copy gene, proliferating cell nuclear antigen (PCNA) was quantified to allow normalization for comparing the density among species. A pair of PCNA primers (PCNAF and PCNAR) (Wiwatanaratanabutr and Kittayapong, 2006) was used for the assay. Each run consisted of a series of DNA standard samples for quantitation, the unknown DNA samples (two-fold replication of each sample) and ddH_2O as a negative control to check for contamination. The reaction mixture in each well was 25 µl total volume contained a DNA sample, 200 mM of dNTP, 50 nM SYBR Green, 300 nM of primers, 0.2 U of AmpErase UNG, 5 mM $MgCl_2$, 0.6 U of Ampli *Taq* Gold and 8% glycerol. The qPCR temperature profile was 50 °C for 2 min to work AmpErase UNG and 95 °C for 10 min to activate Ampli *Taq* Gold, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The absence of non-specific PCR products was confirmed by electrophoretic separation of the products in agarose gels.

For each of the reactions, DNA standard including dilution series of PCR products (10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies/µl) were included to estimate the absolute copy numbers of the target sequence in the DNA samples. To prepare DNA standard samples, the gel of PCR products was

Table 1
PCR-based distribution of *Wolbachia* in 58 moth species from 13 families in the order Lepidoptera in total of 1,235 specimens collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 28 species (625 specimens) from 9 families in bold are those found positive for *Wolbachia*. * For positive species, the number of positive samples by sex was shown.

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of samples by sex*	
						Male	Female
Abraxas sp.	**Geometridae**	**Saraburi**	**C**	**11**	**9 (81.81)**	**3**	**6**
		Nakhon Nayok	**C**	**10**	**7 (70.00)**	**4**	**3**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**7**	**9**
Acherontiia styx	Sphingidae	Nakhon Ratchasima	NE	8	0	3	5
		Phetchaburi	W	4	0	1	3
		Prachinburi	C	5	0	3	2
Acosmeryx sericeus	Sphingidae	Prachinburi	C	3	0	1	2
		Phetchaburi	W	7	0	3	4
Acosmeryx shervillii	**Sphingidae**	**Nakhon Ratchasima**	**NE**	**8**	**6 (75.00)**	**2**	**4**
		Phetchaburi	**W**	**15**	**13 (86.67)**	**6**	**7**
		Nakhon Nayok	**C**	**9**	**7 (77.78)**	**3**	**4**
Amata sp.	Erebidae	Prachinburi	C	5	0	2	3
		Nakhon Ratchasima	NE	7	0	1	6
		Saraburi	C	4	0	2	2
Ambulyx sericeipennis	Sphingidae	Nakhon Nayok	C	8	0	3	5
		Phetchaburi	W	11	0	5	6
Ambulyx sp.	**Sphingidae**	**Saraburi**	**C**	**7**	**3 (42.85)**	**1**	**2**
		Nakhon Ratchasima	**NE**	**12**	**8 (66.67)**	**2**	**6**
		Phetchaburi	**W**	**14**	**11 (78.57)**	**4**	**7**
Antheraea assamensis	Saturniidae	Prachinburi	C	6	0	3	3
		Nakhon Nayok	C	9	0	4	5
		Phetchaburi	W	8	0	4	4
Antheraea frithi	Saturniidae	Nakhon Nayok	C	7	0	2	5
		Phetchaburi	W	3	0	0	3
		Nakhon Ratchasima	NE	5	0	1	4
Artena sp.	**Noctuidae**	**Phetchaburi**	**W**	**8**	**6 (75.00)**	**2**	**4**
		Nakhon Nayok	**C**	**3**	**2 (66.67)**	**0**	**2**
		Nakhon Ratchasima	**NE**	**5**	**4 (80.00)**	**1**	**3**
Asota plana	Erebidae	Saraburi	C	7	0	2	5
		Phetchaburi	W	12	0	4	8
		Prachinburi	C	6	0	2	4
Attacus atlas	Saturniidae	Nakhon Ratchasima	NE	8	0	2	6
		Phetchaburi	W	7	0	3	4
		Nakhon Nayok	C	3	0	2	1
Brahmaea hearseyi	Brahmaeidae	Phetchaburi	W	5	0	1	4
		Nakhon Ratchasima	NE	7	0	2	5
		Prachinburi	C	4	0	1	3
Buxura sp.	Geometridae	Nakhon Nayok	C	6	0	2	4
		Phetchaburi	W	4	0	2	2
		Nakhon Ratchasima	NE	8	0	3	5
Buxura inoui	**Geometridae**	**Phetchaburi**	**W**	**13**	**11 (84.61)**	**5**	**6**
		Nakhon Nayok	**C**	**6**	**3 (42.85)**	**1**	**2**
		Prachinburi	**C**	**5**	**4 (80.00)**	**1**	**3**
Celenna festivaria	**Geometridae**	**Phetchaburi**	**W**	**15**	**13 (86.67)**	**5**	**8**
		Nakhon Ratchasima	**NE**	**10**	**6 (60.00)**	**2**	**4**
		Nakhon Nayok	**C**	**6**	**4 (66.67)**	**1**	**3**
Cleora determinata	**Geometridae**	**Nakhon Ratchasima**	**NE**	**7**	**3 (42.85)**	**1**	**2**
		Phetchaburi	**W**	**11**	**7 (63.63)**	**2**	**5**
		Saraburi	**C**	**5**	**2 (40.00)**	**0**	**2**
Cleora tenebrata	Geometridae	Saraburi	C	5	0	2	3
		Phetchaburi	W	8	0	3	5
		Nakhon Ratchasima	NE	4	0	2	2
Comibaena sp.	**Geometridae**	**Nakhon Nayok**	**C**	**7**	**4 (57.14)**	**1**	**3**
		Nakhon Ratchasima	**NE**	**9**	**6 (66.67)**	**2**	**4**
		Phetchaburi	**W**	**13**	**11 (84.61)**	**4**	**7**
Creatonotos transiens	**Erebidae**	**Saraburi**	**C**	**8**	**6 (75.00)**	**1**	**5**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**6**	**10**
		Nakhon Ratchasima	**NE**	**11**	**9 (81.81)**	**3**	**6**
Daphis hypothous	**Sphingidae**	**Phetchaburi**	**W**	**21**	**19 (90.47)**	**7**	**12**
		Nakhon Nayok	**C**	**9**	**6 (66.67)**	**2**	**4**
		Nakhon Ratchasima	**NE**	**13**	**11 (84.61)**	**4**	**7**
Dudusa vethi	Notodontidae	Phetchaburi	W	6	0	2	4

(*continued on next page*)

Table 1 (*continued*)

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of samples by sex*	
						Male	Female
		Nakhon Nayok	C	2	0	2	0
		Prachinburi	C	4	0	1	3
Dysphania militaris	Geometridae	Nakhon Ratchasima	NE	2	0	0	2
		Prachinburi	C	5	0	3	2
		Phetchaburi	W	7	0	2	5
Euhampsonia roepkei	Notodontidae	Saraburi	C	3	0	1	2
		Nakhon Ratchasima	NE	6	0	3	3
		Nakhon Nayok	C	4	0	3	1
Euplocia membliaria	Erebidae	Phetchaburi	W	5	0	2	3
		Prachinburi	C	3	0	1	2
		Nakhon Nayok	C	5	0	2	3
Eudocima phalonia	Noctuidae	Nakhon Ratchasima	NE	4	0	2	2
		Saraburi	C	3	0	2	1
		Prachinburi	C	2	0	2	0
Fodina contigua	Erebidae	Nakhon Nayok	C	4	0	0	4
		Phetchaburi	W	7	0	4	3
		Nakhon Ratchasima	NE	5	0	2	3
Herochroma flavibasalis	**Geometridae**	**Phetchaburi**	**W**	**19**	**17 (89.47)**	**6**	**11**
		Prachinburi	**C**	**6**	**4 (66.67)**	**2**	**2**
		Nakhon Ratchasima	**NE**	**15**	**13 (86.67)**	**4**	**9**
Hippotion sp.	**Sphingidae**	**Prachinburi**	**C**	**8**	**5 (62.50)**	**2**	**3**
		Nakhon Ratchasima	**NE**	**11**	**7 (63.63)**	**3**	**4**
		Phetchaburi	**W**	**7**	**4 (57.14)**	**1**	**3**
Ischyja marapok	**Noctuidae**	**Nakhon Nayok**	**C**	**5**	**4 (80.00)**	**2**	**2**
		Saraburi	**C**	**9**	**6 (66.67)**	**1**	**5**
		Phetchaburi	**W**	**20**	**17 (85.00)**	**4**	**13**
Lyssa zampa	**Uraniidae**	**Nakhon Ratchasima**	**NE**	**8**	**5 (62.50)**	**2**	**3**
		Phetchaburi	**W**	**14**	**11 (78.57)**	**5**	**6**
		Nakhon Nayok	**C**	**7**	**4 (57.14)**	**1**	**3**
Marumba cristata	Sphingidae	Prachinburi	C	7	0	3	4
		Nakhon Nayok	C	4	0	1	3
		Nakhon Ratchasima	NE	6	0	2	4
Mustilizans dierli	**Endromidae**	**Phetchaburi**	**W**	**20**	**18 (90.00)**	**6**	**12**
		Nakhon Ratchasima	**NE**	**9**	**7 (77.78)**	**3**	**4**
		Prachinburi	**C**	**8**	**6 (75.00)**	**2**	**4**
Mustilia sphingiformis	**Endromidae**	**Nakhon Ratchasima**	**NE**	**10**	**7 (70.00)**	**1**	**6**
		Phetchaburi	**W**	**12**	**9 (75.00)**	**2**	**7**
		Nakhon Nayok	**C**	**7**	**5 (71.42)**	**1**	**4**
Neochera inops	Erebidae	Saraburi	C	4	0	3	1
		Prachinburi	C	2	0	0	2
		Phetchaburi	W	5	0	3	2
Neochera dominia	Erebidae	Nakhon Ratchasima	NE	3	0	1	2
		Nakhon Nayok	C	1	0	0	1
Olene mendosa	**Lymantriidae**	**Nakhon Nayok**	**C**	**6**	**3 (50.00)**	**1**	**2**
		Phetchaburi	**W**	**14**	**12 (85.71)**	**5**	**7**
		Prachinburi	**C**	**5**	**4 (80.00)**	**0**	**4**
Olepa sp.	**Erebidae**	**Nakhon Ratchasima**	**NE**	**12**	**10 (83.33)**	**3**	**7**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**6**	**10**
		Nakhon Nayok	**C**	**8**	**6 (75.00)**	**1**	**5**
Ourapteryx sp.	**Geometridae**	**Nakhon Nayok**	**C**	**9**	**6 (66.67)**	**2**	**4**
		Prachinburi	**C**	**5**	**3 (60.00)**	**1**	**2**
		Phetchaburi	**W**	**16**	**14 (87.50)**	**3**	**11**
Oxyodes scrobiculata	**Noctuidae**	**Nakhon Ratchasima**	**NE**	**8**	**6 (75.00)**	**1**	**5**
		Phetchaburi	**W**	**10**	**8 (80.00)**	**2**	**6**
		Saraburi	**C**	**5**	**2 (40.00)**	**0**	**2**
Parasa bicolor	Limacodidae	Nakhon Nayok	C	2	0	2	0
		Prachinburi	C	4	0	3	1
Pergesa acteus	**Sphingidae**	**Phetchaburi**	**W**	**13**	**11 (84.61)**	**4**	**7**
		Nakhon Ratchasima	**NE**	**10**	**8 (80.00)**	**3**	**5**
		Nakhon Nayok	**C**	**7**	**4 (57.14)**	**1**	**3**
Pareuchaetes pseudoinsulata	Erebidae	Saraburi	C	2	0	0	2
		Phetchaburi	W	4	0	3	1
Parotis marginata	**Crambidae**	**Phetchaburi**	**W**	**11**	**7 (63.63)**	**3**	**4**
			NE	**9**	**8 (88.89)**	**2**	**6**

(*continued on next page*)

Table 1 (*continued*)

Species name	Family	Location (Province)	Geographic regions	No. of samples tested	No. of positive samples (% infection)	No. of samples by sex*	
						Male	Female
		Nakhon Ratchasima Prachinburi	**C**	**4**	**3 (75.00)**	**1**	**2**
Prooedema inscisala	Crambidae	Nakhon Nayok	C	2	0	0	2
		Prachinburi	C	1	0	1	0
		Phetchaburi	W	5	0	2	3
Plutodes flavescens	**Geometridae**	**Nakhon Nayok**	**C**	**7**	**4 (57.14)**	**2**	**2**
		Nakhon Ratchasima	**NE**	**15**	**12 (80.00)**	**4**	**8**
		Phetchaburi	**W**	**18**	**16 (88.89)**	**5**	**11**
Ramadasa pavo	Noctuidae	Saraburi	C	6	0	2	4
		Phetchaburi	W	10	0	2	8
Ruttellerona pallicostaria	**Geometridae**	**Nakhon Ratchasima**	**NE**	**11**	**9 (81.81)**	**3**	**6**
		Phetchaburi	**W**	**15**	**13 (86.67)**	**5**	**8**
		Prachinburi	**C**	**7**	**5 (71.42)**	**3**	**2**
Semiothisa eleonora	Geometridae	Nakhon Nayok	C	4	0	1	3
		Nakhon Ratchasima	NE	8	0	3	5
		Prachinburi	C	5	0	2	3
Spodoptera litura	Noctuidae	Nakhon Ratchasima	NE	7	0	2	5
		Saraburi	C	3	0	0	3
		Phetchaburi	W	8	0	2	6
Sympis rufibasis	Noctuidae	Nakhon Nayok	C	4	0	2	2
		Phetchaburi	W	5	0	1	4
Thyas honesta	**Noctuidae**	**Nakhon Ratchasima**	**NE**	**6**	**3 (50.00)**	**1**	**2**
		Phetchaburi	**W**	**11**	**9 (81.81)**	**4**	**5**
		Prachinburi	**C**	**5**	**2 (40.00)**	**0**	**2**
Theretra sp.	**Sphingidae**	**Phetchaburi**	**W**	**12**	**9 (75.00)**	**3**	**6**
		Nakhon Nayok	**C**	**5**	**2 (40.00)**	**0**	**2**
		Nakhon Ratchasima	**NE**	**9**	**6 (66.67)**	**2**	**4**
Testa montana	**Geometridae**	**Prachinburi**	**C**	**4**	**2 (50.00)**	**2**	**0**
		Phetchaburi	**W**	**8**	**6 (75.00)**	**1**	**5**
		Saraburi	**C**	**6**	**4 (66.67)**	**1**	**3**
Thosea siamica	Limacodidae	Nakhon Nayok	C	1	0	1	0
		Phetchaburi	W	3	0	2	1
Trabala sp.	**Lasiocampidae**	**Phetchaburi**	**W**	**7**	**5 (71.42)**	**1**	**4**
		Nakhon Nayok	**C**	**5**	**3 (60.00)**	**2**	**1**
		Nakhon Ratchasima	**NE**	**6**	**2 (33.33)**	**0**	**2**
Tarsolepis elephantorum	Notodontidae	Prachinburi	C	3	0	1	2
		Phetchaburi	W	5	0	2	3
		Nakhon Ratchasima	NE	4	0	2	2
Xanthomelaena schematias	Crambidae	Saraburi	C	2	0	0	2
		Phetchaburi	W	4	0	1	3
		Nakhon Nayok	C	1	0	1	0

excised and purified by Wizard SV (Promega). The DNA standard samples' copy numbers were estimated by the concentration measured with a spectrophotometer and calculated with respect to the molecular weight data of a nucleotide presented. The Absolute Quantification analysis using the Second Derivative Maximum method implemented in the LightCycler 480 Instrument Operator Software Version 1.5 (Roche) was used to analyze the qPCR data obtained in this study.

Statistical analysis

ANOVA was used to compare and identify the differences in *Wolbachia* infection frequency among different populations from three regions of Thailand. All data was analyzed using DPS software (Tang and Feng, 2002). A $P < 0.05$ was considered significant.

Results

This study was the first survey and first report of the *Wolbachia* infection status in different species of moths from Thailand. A total of 1235 individuals representing 58 moth species belonging to 13 families in the order Lepidoptera collected during the field surveys in 5 provinces (Saraburi, Nakhon Nayok, Nakhon Ratchasima, Prachinburi and Phetchaburi) from two tropical forests in three different geographic regions including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West) from Thailand (Fig. 1) were first screened for *Wolbachia* infection status by PCR assay using *Wolbachia*-specific 16S rRNA, *ftsZ* and *wsp* gene primers. To display the differences in prevalence among these three regions, the three pie-charts for each location indicating % infected and % uninfected were prepared in Fig. 2. In total, the results clearly showed that 625 (male = 209 individuals and female = 416 individuals) of 1235 individuals (specimens) (50.61%) or 28 of 58 species (48.28%) from all three populations of Thailand were positive for *Wolbachia* infection. Similar PCR results were obtained from all three pairs of primers although there are a few differences but not significant among them. In total of 625 *Wolbachia* positive specimens, they included 28 species from 9 families, i.e., Crambidae, Endromidae, Erebidae, Geometridae, Lasiocampidae, Lymantriidae, Noctuidae, Sphingidae, Uraniidae. PCR-based

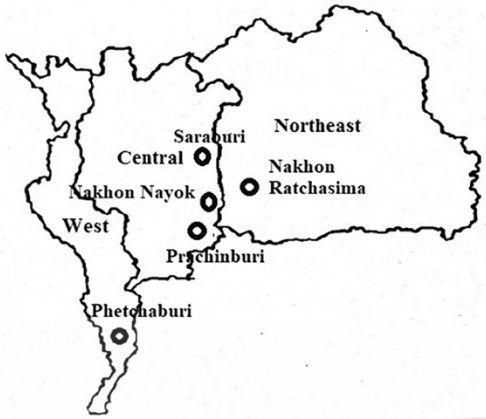

Fig. 1. Geographic distribution of the five field sites surveyed in two tropical forests from three different geographic regions including Khao Yai National Park (the Central and the Northeast regions including four provinces - Saraburi, Nakhon Nayok, Nakhon Ratchasima and Prachinburi) and Kaeng Krachan National Park (the West region including 1 province - Phetchaburi) of Thailand.

distribution of *Wolbachia* in 58 moth species from 13 families in the Lepidoptera in total of 1235 specimens collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand including species name, family, location (province), geographic regions, no. of samples tested, no. of positive samples (% infection) and no. of positive samples (by sex) was listed in Table 1.

Most of the *Wolbachia* infected individuals were collected from Kaeng Krachan National Park where locates at Phetchaburi province in the West region of Thailand although Khao Yai National Park covers four provinces in two regions of Thailand, i.e., Saraburi, Nakhon Nayok, Prachinburi in the Central region, and Nakhon Ratchasima which is the largest city in Thailand in the Northeast region. The infection frequency of *Wolbachia* in different moth populations which collected from different three geographical regions of Thailand (the Central, the Northeast and the West) varied significantly ($F = 2.3650$, $p < 0.01$, df = 18), ranging from 33.33% (*Trabala* sp.) to 90.47% (*Daphis hypothous*) with an average of 61.90% of infection based on the number of positive samples (Table 1). The highest infection frequency was found in *D. hypothous* collected from Phetchaburi (the Western) populations (90.47%), while the lowest frequency was recorded in *Trabala* sp. collected from Nakhon Ratchasima (the Northeast) populations (33.33%) despite each region (West and Northeast) includes only 1 province in this study, i.e., Phetchaburi and Nakhon Ratchasima, respectively.

In a total of 28 *Wolbachia*-infected species of moth (625 individuals), 144 individuals (Males = 46, Females = 98), 156 individuals (Males = 49, Females = 107) and 325 individuals (Males = 114, Females = 211) could be found in the Central region, the Northeast region, and the West region, respectively. Interestingly, the number of positive females appeared to be higher than males from all populations. In addition, the number of all moths collected in the West region was significantly higher than those moths collected in the other two regions ($F = 2.1750$, $p < 0.01$, df = 13). Regarding the *Wolbachia*-infected specimens, *D. hypothous*, the jade hawkmoth in the family Sphingidae, had the highest number of infected specimens collected in this study (36 of 43 individuals) whereas *Trabala* sp. in the family Lasiocampidae had the lowest number of infected specimens collected here (10 of 18 individuals). Regarding *Wolbachia*-uninfected species, *A. plana* had the highest number of specimens collected (25 individuals) whereas *P. bicolor*, the green rice moth, had the lowest number of collected specimens (6 individuals).

The infections of *Wolbachia* were not detected from all collected species of the four families including Brahmaeidae, Limacodidae, Notodontidae and Saturniidae. Comparisons among moth species indicated that the frequency of *Wolbachia*-infected species differed significantly ($F = 2.4830$, $p < 0.01$, df = 14). The conclusion regarding the average infection ratio (the average percentage of *Wolbachia* infections in each species) of infected specimens was shown as follow (In parentheses is the family name): *D. hypothous* (Sphingidae) 80.58%, *H. flavibasalis* (Geometridae) 80.93%, *Abraxas* sp. (Geometridae) 80.23%, *P. flavescens* (Geometridae) 75.34%, *Olepa* sp. (Erebidae) 82.40%, *M. dierli* (Endromidae) 80.92%, *C. transiens* (Erebidae) 81.90%, *I. marapok* (Noctuidae) 77.22%, *R. pallicostaria* (Geometridae) 79.96%, *A. shervillii* (Sphingidae) 79.81%, *C. festivaria* (Geometridae) 71.11%, *P. acteus* (Sphingidae) 73.91%, *Ourapteryx sp*. (Geometridae) 71.39%, *Ambulyx* sp. (Sphingidae) 62.69%, *Comibaena* sp. (Geometridae) 69.47%, *M. sphingiformis* (Endromidae) 72.14%, *L. zampa* (Uraniidae) 66.07%, *O. mendosa* (Lymantriidae) 71.90%, *B. inoui* (Geometridae) 69.15%, *P. marginata* (Crambidae) 75.84%, *Theretra* sp. (Sphingidae) 60.56%, *O. scrobiculata* (Noctuidae) 65.00%, *Hippotion* sp. (Sphingidae) 61.09%, *T. honesta* (Noctuidae) 57.27%, *T. montana* (Geometridae) 63.89%, *Artena* sp. (Noctuidae) 73.89%, *C. determinata* (Geometridae) 48.82% and *Trabala* sp. (Lasiocampidae) 54.91% in total of 28 species in 625 individuals.

The relative *Wolbachia* densities from 28 infected moth species were determined by quantitative real-time PCR assay using the *ftsZ* gene as the target sequence (Fig. 3). The relative *Wolbachia* densities within each species were different from each other. In Fig. 3, the relative *Wolbachia* densities in 28 *Wolbachia*-infected moths from Thailand were shown in which the relative values were obtained by comparing the ratio between the *Wolbachia ftsZ* gene copy number of each sample and the proliferating cell nuclear antigen gene (PCNA) of each host. *Olene mendosa*, the brown tussock moth or hairy tussock moth of the family Erebidae, had the highest relative *Wolbachia* densities compared with the other 27 infected species while *M. dierli* showed the lowest density among all infected species. The order of relative *Wolbachia* densities among these 28 species from the highest to the lowest are as follows: *O. mendosa, T. honesta, Artena* sp., *D. hypothous, Trabala* sp., *Theretra* sp., *A. shervillii, P. marginata, B. inoui, H. flavibasalis, R. pallicostaria, P. acteus, O. scrobiculata, M. sphingiformis, C. transiens, P. flavescens, Comibaena* sp., *Abraxas* sp., *C. festivaria, C. determinata, T. montana, Ambulyx* sp., *L. zampa, Olepa*

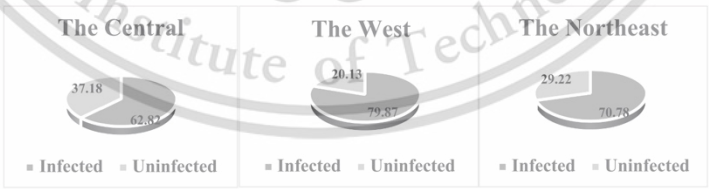

Fig. 2. The percent of *Wolbachia* infection frequency (Infected/Uninfected) from each region.

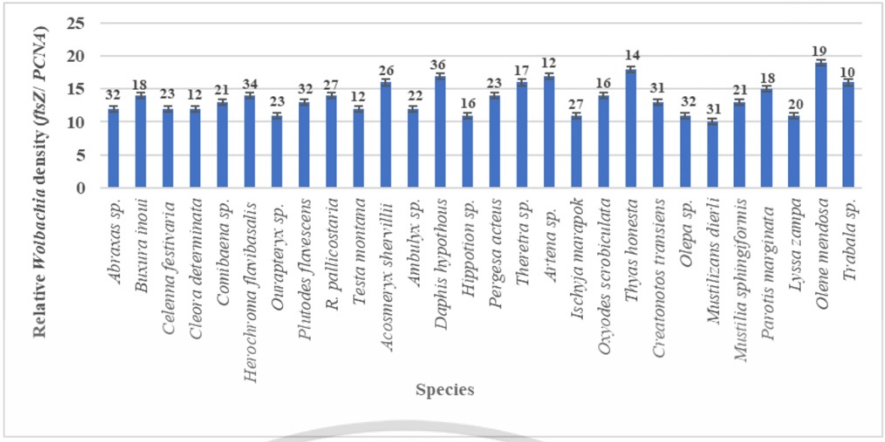

Fig. 3. The relative *Wolbachia* densities in 28 *Wolbachia*-infected moths from Thailand. The error bars represent standard error. The number of samples was added on the bar of each species. Two replications of each sample were done. The relative values (Y-axis) were obtained by comparing the ratio between the *Wolbachia ftsZ* gene copy number of each sample and the proliferating cell nuclear antigen gene (PCNA) of each host.

sp., *I. marapok*, *Hippotion* sp., *Ourapteryx* sp. and *M. dierli*. Interestingly, at least ten species from these infected species are agricultural insect pests including *O. mendosa*, *O. scrobiculata*, *T. honesta*, *D. hypothous*, *A. shervillii*, *P. acteus*, *C. transiens*, *T. montana*, *I. marapok* and *Hippotion* sp. The *O. mendosa*, which had the highest relative *Wolbachia* densities, was an important agricultural pest of Durian in Thailand. The jade hawk-moth, *D. hypothous* that had the highest number of infected specimens in total of 36 individuals of 43 collected individuals, had the relative *Wolbachia* densities in the fourth rank.

Discussion

This report was the first discovery of *Wolbachia* infection and their relative density in different moth species from Thailand. The percentage reported here might undoubtedly increase if more specimens were examined and/or different methods of DNA extraction or PCR were applied. This study implies that the widespread distribution of *Wolbachia* was unequal among moths' genera and geographic regions of Thailand. Species of moth which positive for *Wolbachia* included many important pest species that involved in the agriculture of Thailand. Some moth species from this study have been previously reported to be infected with *Wolbachia* but not from Thailand (Muhammad et al., 2015; Sumida et al., 2017). The distribution of all species in this study was mostly collected in the Western region where is the main source of forest ecosystem in Thailand. However, all species could be found in all three regions of Thailand. Most of *Wolbachia*-infected individuals were also collected from the Western region. In this region, habitats and temperature are quite different from the other regions of Thailand in terms of high humidity and temperature. These results may imply that climate differences might influence survival of moths and other insects as well as the infections of *Wolbachia* (Toju and Fukatsu, 2010; Muhammad et al., 2015). Infected individuals were found in many species including major pests in agriculture.

The number of Lepidopteran species in Thailand are estimated over 1,291 species, and as far as our survey, 28 out of 58 (48.28%) moth species collected in this study were positive for *Wolbachia*. This is in line with the estimation of *Wolbachia* infection in insects from Thailand by Wiwatanaratanabutr (2015). *Wolbachia* infections have been reported in many insect species such as mosquitoes, planthoppers, leafhoppers, etc. from Thailand but they have never been reported in moths (Wiwatanaratanabutr, 2013, 2015). Most *Wolbachia*-infected moth species collected in this study were in the family Geometridae. All individuals were collected from the two tropical forests in five provinces of three regions from Thailand which have been reported on the abundance of these Lepidopteran throughout Thailand. Many of collected moth species are commonly found in several areas of Thailand (Pierce, 1995; Suwannaphak, 2012) although some of them are rare to find in the present situation under the global warming and climate changes all over the world. The differences in the abundance of all collected moth species from Thailand in the present study and the previous study during 2010–2012 (around 10 years ago) by Suwannaphak (2012) that reported about moth species from Thailand (specimens collected before the year 2012) might have resulted primarily from the changes in global climate, habitats, local temperature, ecosystem, time as well as other relevant factors. Schneider et al. (2013) have reported a Variable-Number-Tandem-Repeat molecular screening tool for detecting *Wolbachia* infections in tsetse flies and showed that their infections in *Glossina* spp. can escape the standard PCR screening methods by hiding as low-density infections below the detection threshold.

In this study, another method using real-time quantitative PCR was applied to this study for confirmation of *Wolbachia* infections. A quantitative real-time PCR requires the absolute quantification of *ftsZ* gene that is a single copy per genome in *Wolbachia*, and thus DNA standard with known concentration was required and the host single copy gene was also quantified to allow normalization for making a comparison among species. The relative *Wolbachia* densities within each host species of all three regions from Thailand were different from each other but not significant difference as shown in Fig. 3 ($P > 0.01$). According to the Fig. 3, there are more than 10 species of collected moths that are agricultural insect pests of Thailand. The low density in the infected moths probably implied that *Wolbachia* might not spread through moth populations in Thailand due to some climate change effects or temperature difference. However, another possibility of the detection of this low density or the absence of *Wolbachia* in other moth species is that the *Wolbachia* load might be below the threshold level of a real-time PCR detection system. Therefore, we may need to continue and conduct a further study in larger scale on the diversity and distribution of *Wolbachia* infection as well as their infection density in Lepidopteran species and in other insects from Thailand in the future. However, the information on *Wolbachia* infections status and their densities from this study

can assist our understandings of *Wolbachia* diversity in moths from both Thailand and other Asian countries.

Wolbachia have attracted the interest of biologists as the basis for gene driving systems for the distribution of disease-blocking transgenes through insect populations. Our findings are relevant to ongoing efforts to manage pollinators like moths and to understand the distribution of moth populations and their *Wolbachia* infections from Thailand. In addition, this certain result invites further thought on and investigation into our understanding of the evolutionary dynamics of *Wolbachia* infections, their density in hosts and insects across ecosystems. We found that geographical variation with closer locations tending to display the similar infection levels, and no evidence closely related to taxonomic groups indicate similar infection levels. This observational study explains the intensive and substantial undertakings required to address this finding with well-designed research studies. We also found that latitudinal gradient might be an important factor in infection level, i.e., lower frequencies towards higher latitudes. This study shows a latitudinal gradient in *Wolbachia* infection at a broad taxonomic and geographic scales in different regions, as well as implies that *Wolbachia* infection might be predictable from ecological variables. Therefore, the study of the *Wolbachia* infection density would be the fundamental research for further biological investigations of the *Wolbachia* effects on moths. By manipulating *Wolbachia*, these bacteria may prove useful as a control system for reducing insect pest populations and has been very helpful in the application of insect natural enemies for pest management in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Bagheri, Z., Talebi, A.A., Asgari, S., Mehrabadi, M., 2019. *Wolbachia* induce cytoplasmic incompatibility and affect mate preference in *Habrobracon hebetor* to increase the chance of its transmission to the next generation. J. Invert Pathol. 163, 1–7.

Baldo, L., Bordenstein, S., Wernegreen, J.J., Werren, J.H., 2006. Widespread recombination throughout Wolbachia genomes. Mol. Biol. Evol. 23, 437–449.

Braig, H.R., Zhou, W., Dobson, S.L., O'Neill, S.L., 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180 (9), 2373–2378.

Carvajal, ThaddeusM, Capistrano, J.D., Hashimoto, K., Go, K.J., Cruz, M.A.I., Martinez, M.J.L., Tiopianco, V.S., Amalin, DivinaM, Watanabe, K., 2018. Detection and distribution of *Wolbachia* endobacteria in *Culex quinquefasciatus* populations (Diptera: Culicidae) from Metropolitan Manila, Philippines. J. Vector Borne Dis. 55 (4), 265. https://doi.org/10.4103/0972-9062.256561.

Hiroki, M., Tagami, Y., Miura, K., Kato, Y., 2004. Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*. Proc. Biol. Sci. 271 (1549), 1751–1755.

Iturbe-Ormaetxe, I., Walker, T., O' Neill, SL., 2011. Wolbachia and the biological control of mosquito-borne disease. EMBO Rep. 12(6), 508–518. Doi: 10.1038/embor.2011.84.

Kajtoch, L., Kolasa, M., Kubisz, D., Gutowski, J.M., Ścibior, R., Mazur, M.A., Holecová, M., 2019. Using host species traits to understand the *Wolbachia* infection distribution across terrestrial beetles. Sci. Rep. 9, 847.

Kolasa, M., Kubisz, D., Mazur, M.A., Scibior, R., Kajtoch, L., 2018. *Wolbachia* prevalence and diversity in selected riverine predatory beetles (Bembidiini and Paederini). B. Insectol. 71, 193–200.

Lopez, Valérie, Cortesero, Anne Marie, Poinsot, Denis, 2018. Influence of the symbiont *Wolbachia* on life history traits of the cabbage root fly (*Delia radicum*). J. Invert Pathol. 158, 24–31.

Muhammad, Z.A., Eli, V., Araujo-Jnr, J.J.W., Akito, Y.K., 2015. *Wolbachia* in butterflies and moths: geographic structure in infection frequency. Front. Zool. 12, 16. https://doi.org/10.1186/s12983-015-0107-z.

Noda, Hiroaki, Koizumi, Yoko, Zhang, Qiang, Deng, Kejing, 2001. Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. Insect. Biochem. Mol. Biol. 31 (6-7), 727–737.

O'Neill, S.L., Giordane, R., Colbert, A.M.E., Timothy, K., Robertson, H., 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with CI in insects. Proc. Natl. Acad. Sci. U.S.A. 89, 2699–2702.

Pierce, N.E., 1995. Predatory and parasitic lepidoptera: carnivores living on plants. J. Lepidopterists' Soc. 49 (4), 412–453.

Plotkin, David, Goddard, Jerome, 2013. Blood, sweat, and tears: a review of the hematophagous, sudophagous, and lachryphagous Lepidoptera. J. Vector Ecol. 38 (2), 289–294.

Salunke, B.K., Salunkhe, R.C., Dhotre, D.P., Walujkar, S.A., Khandagale, A.B., Chaudhari, R., Chandode, R.K., Ghate, H.V., Patole, M.S., Werren, J.H., Shouche, Y. S., 2012. Determination of *Wolbachia* diversity in butterflies from western ghats, India, by a multigene approach. Appl. Environ. Microbiol. 78 (12), 4458–4467. https://doi.org/10.1128/AEM.07298-11.

Schneider, Daniela I., Garschall, Kathrin I., Parker, Andrew G., Abd-Alla, Adly M.M., Miller, Wolfgang J., 2013. Global *Wolbachia* prevalence, titer fluctuations and their potential of causing cytoplasmic Incompatibilities in tsetse flies and hybrids of *Glossina morsitans* subgroup species. J. Invertebr. Pathol. 112, S104–S115.

Stouthamer, R., Breeuwer, J.A.J., Hurst, G.D.D., 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Ann. Rev. Microbiol. 53 (1), 71–102.

Sunida, Yn, Katsuki, Masako, Okada, Kensuke, Okayama, Keisuke, Lewis, Zenobia, 2017. *Wolbachia* induces costs to life- history and reproductive traits in the moth, *Ephestia kuehniella*. J. Stored Prod. Res. 71, 93–98.

Suwannaphak, K., 2012. Photographic guide to Moths in Thailand. Published by BNEC (Banna Nature Education Center, Nakhon Nayok, Thailand.

Tang, Q.Y., Feng, M.G., 2002. DPS Data Processing System for Practical Statistics. Science Press.

Toju, H., Fukatsu, T., 2010. Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. Mol. Ecol. https://doi.org/10.1111/j.1365-294X.2010.04980.x.

Werren, John H., Baldo, Laura, Clark, Michael E., 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6 (10), 741–751.

Werren, J.H., Windsor, D., Guo, L., 1995. Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. B 262, 197–204.

Wiwatanaratanabutr, S., Kittayapong, P., 2006. Effects of temephos and temperature on *Wolbachia* load and life history traits of *Aedes albopictus*. Med. Vet. Entomol. 20 (3), 300–307.

Wiwatanaratanabutr, Itsanun, Kittayapong, Pattamaporn, 2009. Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of Aedes albopictus. J. Invertebr. Pathol. 102 (3), 220–224.

Wiwatanaratanabutr, I., 2013. Geographic distribution of *Wolbachia* infections in mosquitoes from Thailand. J. Invertebr. Pathol. 114, 337–340.

Wiwatanaratanabutr, Itsanun, 2015. *Wolbachia* infection in leafhoppers and planthoppers: Diversity, density and geographic distribution in tropical rice agroecosystems. J. Asia-Pacific Entomol. 18 (2), 277–282.

Zaspel, Jennifer M., Weller, S.J., Branham, M.A., 2011. A comparative survey of proboscis morphology and associated structures in fruit-piercing, tear-feeding, and blood-feeding moths in Calpinae (Lepidoptera: Erebidae). Zoomorphology 130 (3), 203–225.

Zhou, Weiguo, Rousset, François, O'Neill, Scott, 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. Biol. Sci. 265 (1395), 509–515.

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Distribution of *Wolbachia* infection in butterflies (Lepidoptera): First systematic report from Thailand

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ABSTRACT

Wolbachia is a genus of *Rickettsia*-like bacteria that infects a broad range of insect species throughout the world. It often influences host reproduction to facilitate its transmission to their offspring and spread through populations. The occurrence and ecology of *Wolbachia* has not been surveyed in detail in Thai butterflies. In this study, we conduct the first systematic survey of *Wolbachia* infection by sampling 623 specimens from 46 butterfly species from 5 families obtained from tropical forests in 3 geographic regions of Thailand. *Wolbachia* infections were detected using three PCR primer sets: 16S rRNA, *ftsZ* and *wsp*. The results showed evidence for widespread *Wolbachia* infection in Thai butterflies. The *Wolbachia* presence was confirmed in 291 individuals from 17 butterfly species of 2 families, comprising 86 individuals from the Central Region (32 males and 54 females), 72 individuals from the Northeast Region (25 males and 47 females), and 133 individuals from the West Region (46 males and 87 females). This report will be useful for understanding the distribution of *Wolbachia* in butterflies from Thailand.

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Distribution; infection; Lepidoptera; butterfly; *Wolbachia*

Introduction

Members of the genus *Wolbachia* are bacteria that are endosymbionts of many arthropod and nematode species and are known to influence the reproduction of their host to facilitate vertical transmission from mother to offspring (Werren et al. 2008). The genus *Wolbachia* can infect a wide diversity of insects, however the exact rate of infection is difficult to indicate, expanding from 20% to 76% of all insect species (Stouthamer et al. 1999; Werren et al. 2008). Analyses of *ftsZ*, *wsp*, and 16S rRNA gene regions

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suggest that *Wolbachia* are a monophyletic group in the alphaproteobacteria (Wang et al. 2020). *Wolbachia* infections provide direct benefits to their hosts, such as through nutrient provisioning (Brownlie et al. 2009), upregulate hosts' immune genes (Hedges et al. 2008), protect insects from natural enemies and influence thermal preference (Truitt et al. 2019), and influence susceptibility/resistance to insecticides (Liu and Guo 2019), but can also significantly alter host reproductive modes leading to feminisation, parthenogenesis, male mortality, or cytoplasmic incompatibility (Gotoh et al. 2007; Werren et al. 2008; Carjaval et al. 2018; Kajtoch et al. 2019). All these listed phenomena (except parthenogenesis) have already been reported in Lepidoptera (Duplouy and Hornett 2018). This can result in an increase in the frequency of *Wolbachia* infection in a population and increase the proportion of females that can transmit *Wolbachia*. The detection, infection status, distribution, and diversity of *Wolbachia* have been surveyed in many species of arthropods, for instance, mosquitoes (Wiwatanaratanabutr 2013), moths (Sumida et al. 2017), mites (Sakamoto et al. 2019), root flies (Lopez et al. 2018), butterflies (Salunke et al. 2012), wasps (Bagheri et al. 2019), planthoppers (Wiwatanaratanabutr 2015), beetles (Kolasa et al. 2018), and terrestrial isopods (Bouchon et al. 1998).

The Order Lepidoptera comprises approximately 20,000 butterfly species in the world (Plotkin and Goddard 2013; Muhammad et al. 2015). Although the Lepidoptera diversity and their symbionts have been studied in many countries, the investigations of *Wolbachia* infection associated with butterflies from Thailand have never been reported (Ek-Amnuay 2012). The biological diversity of butterflies makes this group important for determining the occurrence of *Wolbachia* and their associated impact on the biology and evolution of infected species (Hiroki et al. 2004; Muhammad et al. 2015). Studies of *Wolbachia*-infected butterflies have found associations with fundamental changes in biological processes including speciation, sex ratio distortion and sex determination (Jiggins et al. 2001; Zaspel 2011; Kageyama et al. 2017). *Wolbachia* infections are also being applied as a novel biological control for disease vectors and insect pests (Ross et al. 2019). Although the role of *Wolbachia* in many insects has been extensively reported, their incidence and distribution among butterfly species from Thailand is still poorly studied and largely unknown.

In this study, we investigated the geographic distribution of *Wolbachia* infections in butterflies from three geographic regions in two tropical forest ecosystems of Thailand. This survey of *Wolbachia* infections will provide a basis for further studies of *Wolbachia* infections in lepidopterans, which may be useful for the application of *Wolbachia* for insect pest management.

Materials and methods

Insect collection

Butterflies were collected during January and December 2019 in two tropical forest ecosystems from three different regions (the Central, the Northeast and the West of Thailand) including Khao Yai National Park (the Central and the Northeast) and Kaeng Krachan National Park (the West) (Fig. 1, Table 2). All butterfly specimens were collected by netting and morphologically identified to species (or genus) levels by using the photographic guide to butterflies of Thailand (Ek-Amnuay 2012) at King Mongkut's Institute of Technology Ladkrabang in Bangkok, Thailand. Abdomens were removed and kept in ethanol at −20°C for molecular genetic analyses.

DNA extraction

DNA was isolated from abdominal tissue using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendation. The tissues

Figure 1. The geographic distribution of the five field sites in two tropical forests from three different geographic regions including Khao Yai National Park (the Central and the Northeast regions including four provinces – Saraburi, Nakhon Nayok, Nakhon Ratchasima and Prachinburi) and Kaeng Krachan National Park (the West region including one province – Phetchaburi) of Thailand.

were lysed for 20 minutes before adding absolute ethanol. Lysates were then loaded onto the QIAamp spin column, and the DNA was eluted in distilled water. The extracted DNA was maintained at −20°C for later processing.

Polymerase chain reaction

The quality of DNA extracted from all collected specimens was first confirmed by PCR using arthropod-specific 28S primers (Werren et al. 1995). The DNA from specimens with no amplification from 28S were re-extracted using the same abdomen that was used and the process repeated. The presence of *Wolbachia* was tested using three sets of primers comprising 16S rRNA (O'Neill et al. 1992), *wsp* (Zhou et al. 1998), and *ftsZ* (Baldo et al. 2006) which amplify a PCR product size around 900 bp, 600 bp and 600 bp, respectively. DNA from *Aedes albopictus* (*Wolbachia* positive) was selected as a positive control. Double-distilled H_2O (ddH_2O) and *Aedes aegypti* DNA (*Wolbachia* negative) was used as a negative control for contamination. PCR assays were conducted using a reaction mixture of 20 µl comprising $MgCl_2$ 25 mM 2 µl, 10xPCR buffer (Promega) 2 µl, forward and reverse primers 20 µM 0.5 µl each, dNTPs 10 mM 1.5 µl, and *Taq* polymerase (Promega) 0.125 µl. The ddH_2O was added to make the final volume of 20 µl. The PCR assay of 16S rRNA and *wsp* primers was an initial step 3 minutes at 94°C followed by 35 cycles of a denaturation 1 minute at 94°C, annealing 1 minute at 55°C, extension 2 minutes at 72°C, and final extension 10 minutes at 72°C. The protocol for *ftsZ* PCR included 3 minutes at 94°C for denaturation followed by 25 cycles of denaturation 10 seconds at 94°C, annealing 30 seconds at 65°C, and extension 1 minute at 68°C. To examine the presence and size of amplified DNA, 10 µl of PCR product and 1 kb DNA ladder (Bio-Helix) were applied on a 1% agarose gel. PCR products of the anticipated size were considered as positive for *Wolbachia* infection.

Statistical analysis

Chi-square tests were used to compare *Wolbachia* infection frequencies between the sexes. All data were analysed using DPS software (Tang and Feng 2002). A $P < 0.05$ was considered significant.

Results

The 623 individuals representing 46 butterfly species from 5 families collected in 5 provinces including Saraburi (14°27'10.98"N, 101°12'54.68"E), Nakhon Nayok (14°21'58.56"N, 101°24'10.72"E), Nakhon Ratchasima (14°26'04.02"N, 101°23'45.41"E), Prachinburi (14°10'54.75"N, 101°35'33.58"E) and Phetchaburi (14°10'54.75"N, 101°35'33.58"E) from Thailand (Fig. 1, Table 1) were screened

Table 1. PCR-based distribution of *Wolbachia* in 46 butterfly species from 5 families in the order Lepidoptera across geographic regions in Thailand. The 17 species (291 specimens) in bold are those found positive for *Wolbachia*.

Species name	Family	No. of specimens tested	No. of positive specimens (% infection)		No. of positive males (% infection)		No. of positive females (% infection)	
Acraea violae	**Nymphalidae**	26	15	(57.69)	6	(40.00)	9	(60.00)
Appias libythea	Pieridae	11	0		0		0	
Arhopala centaurus	Lycaenidae	4	0		0		0	
Catopsilia pomona	Pieridae	26	0		0		0	
Cepora nerissa	Pieridae	8	0		0		0	
Cethosia cyane	**Nymphalidae**	25	19	(76.00)	7	(36.84)	12	(63.15)
Cynithia lepidea	**Nymphalidae**	17	12	(70.58)	3	(25.00)	9	(75.00)
Danaus chrysippus	**Nymphalidae**	21	15	(71.42)	6	(40.00)	9	(60.00)
Danaus genutia	**Nymphalidae**	18	13	(72.22)	5	(38.46)	8	(61.53)
Danaus melanippus	**Nymphalidae**	18	12	(66.67)	4	(33.33)	8	(66.67)
Delias hyparete	Pieridae	13	0		0		0	
Doleschallia bisaltide	**Nymphalidae**	20	15	(75.00)	7	(46.67)	8	(53.33)
Euploea algea	Nymphalidae	6	0		0		0	
Euploea camaralzeman	Nymphalidae	10	0		0		0	
Euploea mulciber	Nymphalidae	6	0		0		0	
Euploea sylvester	Nymphalidae	9	0		0		0	
Eurema simulatrix	Pieridae	5	0		0		0	
Euthalia evelina	Nymphalidae	11	0		0		0	
Graphium agamemnon	Papilionidae	11	0		0		0	
Graphium antiphates	Papilionidae	9	0		0		0	
Graphium doson	Papilionidae	6	0		0		0	
Hypolimnas bolina	Nymphalidae	3	0		0		0	
Junonia atlites	**Nymphalidae**	20	15	(75.00)	4	(26.67)	11	(73.33)
Junonia lemonias	**Nymphalidae**	21	13	(61.90)	4	(30.76)	9	(69.23)
Junonia orithya	**Nymphalidae**	20	14	(70.00)	6	(42.85)	8	(57.14)
Lexias albopunctata	Nymphalidae	7	0		0		0	
Loxura athymnus	Lycaenidae	3	0		0		0	
Melanitis leda	**Nymphalidae**	30	25	(83.33)	8	(32.00)	17	(68.00)
Mycalesis mineus	**Nymphalidae**	28	21	(75.00)	8	(38.09)	13	(61.90)
Mycalesis perseoides	**Nymphalidae**	25	19	(76.00)	4	(21.05)	15	(78.94)
Mycalesis perseus	**Nymphalidae**	29	24	(82.75)	11	(45.83)	13	(54.16)
Neomyrina nivea	Lycaenidae	5	0		0		0	
Neptis hylas	Nymphalidae	3	0		0		0	
Pachliopta aristolochiae	Papilionidae	8	0		0		0	
Papilio demoleus	Papilionidae	7	0		0		0	
Papilio helenus	Papilionidae	3	0		0		0	
Papilio memnon	Papilionidae	4	0		0		0	
Papilio nephelus	Papilionidae	3	0		0		0	
Papilio polytes	**Papilionidae**	24	18	(75.00)	7	(38.88)	11	(61.11)
Parantica aglea	**Nymphalidae**	30	23	(76.67)	6	(26.08)	17	(73.91)
Parantica agleoides	**Nymphalidae**	24	18	(75.00)	7	(38.88)	11	(61.11)
Parthenos sylvia	Nymphalidae	10	0		0		0	
Pieris rapae	Pieridae	6	0		0		0	
Suastus gremius	Hesperiidae	5	0		0		0	
Tanaecia julii	Nymphalidae	10	0		0		0	
Ypthima huebneri	Nymphalidae	15	0		0		0	

for *Wolbachia* infection. A total of 291 out of the 623 individuals (46.7%) including 103 males and 188 females and 17 of the 46 species (36.96%) were found to be positive for *Wolbachia* infection. Each positive sample showed a PCR product of the expected size for all three genes (16S rRNA, *wsp* and *ftsZ*). The pie chart in Fig. 2 shows the *Wolbachia* infection frequency from each surveyed region in Thailand. The percentage of the total species diversity

Table 2. PCR-based distribution of *Wolbachia* in 46 butterfly species from 5 families in the order Lepidoptera from a total of 623 specimens collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast and W = the West) from Thailand. The 17 species (291 specimens) in bold are those found positive for *Wolbachia*.

Species name	Family	Location (Province)	Geographic regions	No. of specimens tested	No. of positive specimens (%infection)	No. of positive specimens by sex		No. of negative specimens by sex	
						Male	Female	Male	Female
Acraea violae	**Nymphalidae**	**Phetchaburi**	**W**	**10**	**4 (40.00)**	**2**	**2**	**1**	**5**
		Nakhon Nayok	**C**	**9**	**6 (66.67)**	**2**	**4**	**1**	**3**
		Nakhon Ratchasima	**NE**	**7**	**5 (71.42)**	**2**	**3**	**1**	**1**
Appias libythea	Pieridae	Nakhon Nayok	C	2	0	0	0	0	2
		Phetchaburi	W	8	0	0	0	3	5
		Saraburi	C	1	0	0	0	0	1
Arhopala centaurus	Lycaenidae	Phetchaburi	W	2	0	0	0	0	2
		Nakhon Nayok	C	2	0	0	0	0	2
Catopsilia pomona	Pieridae	Nakhon Ratchasima	NE	8	0	0	0	3	5
		Prachinburi	C	6	0	0	0	2	4
		Phetchaburi	W	12	0	0	0	5	7
Cepora nerissa	Pieridae	Saraburi	C	2	0	0	0	1	1
		Phetchaburi	W	4	0	0	0	1	3
		Nakhon Ratchasima	NE	2	0	0	0	0	2
Cethosia cyane	**Nymphalidae**	**Nakhon Nayok**	**C**	**5**	**3 (60.00)**	**1**	**2**	**1**	**1**
		Phetchaburi	**W**	**11**	**9 (81.81)**	**3**	**6**	**0**	**2**
		Nakhon Ratchasima	**NE**	**9**	**7 (77.78)**	**3**	**4**	**1**	**1**
Cynithia lepidea	**Nymphalidae**	**Saraburi**	**C**	**6**	**4 (66.67)**	**1**	**3**	**2**	**0**
		Nakhon Nayok	**C**	**3**	**2 (66.67)**	**0**	**2**	**0**	**1**
		Phetchaburi	**W**	**8**	**6 (75.00)**	**2**	**4**	**0**	**2**
Danaus chrysippus	**Nymphalidae**	**Nakhon Ratchasima**	**NE**	**10**	**8 (80.00)**	**3**	**5**	**1**	**1**
		Nakhon Nayok	**C**	**6**	**4 (66.67)**	**1**	**3**	**0**	**2**
		Saraburi	**C**	**5**	**3 (60.00)**	**2**	**1**	**1**	**1**
Danaus genutia	**Nymphalidae**	**Phetchaburi**	**W**	**9**	**7 (77.78)**	**2**	**5**	**2**	**0**
		Prachinburi	**C**	**3**	**2 (66.67)**	**0**	**2**	**1**	**0**
		Nakhon Ratchasima	**NE**	**6**	**4 (66.67)**	**3**	**1**	**1**	**1**
Danaus melanippus	**Nymphalidae**	**Prachinburi**	**C**	**4**	**2 (50.00)**	**0**	**2**	**0**	**2**
		Nakhon Nayok	**C**	**5**	**3 (60.00)**	**1**	**2**	**1**	**1**
		Phetchaburi	**W**	**9**	**7 (77.78)**	**3**	**4**	**0**	**2**

(*Continued*)

Table 2. (Continued).

Species name	Family	Location (Province)	Geographic regions	No. of specimens tested	No. of positive specimens (%infection)	No. of positive specimens by sex		No. of negative specimens by sex	
						Male	Female	Male	Female
Delias hyparete	Pieridae	Saraburi	C	3	0	0	0	1	2
		Phetchaburi	W	8	0	0	0	2	6
		Prachinburi	C	2	0	0	0	1	1
Doleschallia bisaltide	**Nymphalidae**	**Nakhon Ratchasima**	**NE**	**8**	**6 (75.00)**	**2**	**4**	**0**	**2**
		Nakhon Nayok	**C**	**5**	**4 (80.00)**	**3**	**1**	**1**	**0**
		Phetchaburi	**W**	**7**	**5 (71.42)**	**2**	**3**	**1**	**1**
Euploea algea	Nymphalidae	Nakhon Ratchasima	NE	3	0	0	0	1	2
		Prachinburi	C	1	0	0	0	0	1
		Saraburi	C	2	0	0	0	0	2
Euploea camaralzeman	Nymphalidae	Nakhon Nayok	C	2	0	0	0	0	2
		Phetchaburi	W	5	0	0	0	1	4
		Prachinburi	C	3	0	0	0	1	2
Euploea mulciber	Nymphalidae	Nakhon Ratchasima	NE	3	0	0	0	2	1
		Prachinburi	C	1	0	0	0	0	1
		Nakhon Nayok	C	2	0	0	0	0	2
Euploea sylvester	Nymphalidae	Nakhon Nayok	C	3	0	0	0	2	1
		Phetchaburi	W	5	0	0	0	1	4
		Saraburi	C	1	0	0	0	0	1
Eurema simulatrix	Pieridae	Nakhon Ratchasima	NE	3	0	0	0	2	1
		Prachinburi	C	1	0	0	0	1	0
		Nakhon Nayok	C	1	0	0	0	0	1
Euthalia evelina	Nymphalidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	7	0	0	0	2	5
		Nakhon Ratchasima	NE	3	0	0	0	1	2
Graphium agamemnon	Papilionidae	Phetchaburi	W	6	0	0	0	2	4
		Nakhon Ratchasima	NE	4	0	0	0	1	3
		Nakhon Nayok	C	1	0	0	0	1	0
Graphium antiphates	Papilionidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	3	0	0	0	2	1
		Nakhon Ratchasima	NE	5	0	0	0	2	3
Graphium doson	Papilionidae	Saraburi	C	2	0	0	0	1	1
		Phetchaburi	W	3	0	0	0	1	2

(*Continued*)

Table 2. (Continued).

Species name	Family	Location (Province)	Geographic regions	No. of specimens tested	No. of positive specimens (%infection)	No. of positive specimens by sex		No. of negative specimens by sex	
						Male	Female	Male	Female
		Nakhon Ratchasima	NE	1	0	0	0	0	1
Hypolimnas bolina	Nymphalidae	Nakhon Nayok	C	1	0	0	0	0	1
		Phetchaburi	W	2	0	0	0	0	2
Junonia atlites	**Nymphalidae**	**Nakhon Ratchasima**	**NE**	**4**	**3 (75.00)**	**0**	**3**	**1**	**0**
		Phetchaburi	**W**	**9**	**7 (77.78)**	**3**	**4**	**1**	**1**
		Nakhon Nayok	**C**	**7**	**5 (71.42)**	**1**	**4**	**0**	**2**
Junonia lemonias	**Nymphalidae**	**Saraburi**	**C**	**6**	**4 (66.67)**	**2**	**2**	**1**	**1**
		Nakhon Ratchasima	**NE**	**5**	**3 (60.00)**	**0**	**3**	**2**	**0**
		Phetchaburi	**W**	**10**	**6 (60.00)**	**2**	**4**	**1**	**4**
Junonia orithya	**Nymphalidae**	**Prachinburi**	**C**	**6**	**5 (83.33)**	**2**	**3**	**0**	**1**
		Phetchaburi	**W**	**10**	**7 (70.00)**	**2**	**5**	**2**	**1**
		Nakhon Nayok	**C**	**4**	**2 (50.00)**	**2**	**0**	**1**	**1**
Lexias albopunctata	Nymphalidae	Saraburi	C	2	0	0	0	0	2
		Nakhon Ratchasima	NE	4	0	0	0	1	3
		Prachinburi	C	1	0	0	0	0	1
Loxura athymnus	Lycaenidae	Phetchaburi	W	2	0	0	0	0	2
		Nakhon Nayok	C	1	0	0	0	1	0
Melanitis leda	**Nymphalidae**	**Nakhon Ratchasima**	**NE**	**7**	**5 (71.42)**	**2**	**3**	**1**	**1**
		Phetchaburi	**W**	**15**	**13 (86.67)**	**4**	**9**	**2**	**0**
		Nakhon Nayok	**C**	**8**	**7 (87.50)**	**2**	**5**	**0**	**1**
Mycalesis mineus	**Nymphalidae**	**Phetchaburi**	**W**	**14**	**12 (85.71)**	**5**	**7**	**1**	**1**
		Prachinburi	**C**	**9**	**6 (66.67)**	**2**	**4**	**1**	**2**
		Saraburi	**C**	**5**	**3 (60.00)**	**1**	**2**	**1**	**1**
Mycalesis perseoides	**Nymphalidae**	**Nakhon Nayok**	**C**	**7**	**5 (71.42)**	**1**	**4**	**0**	**2**
		Nakhon Ratchasima	**NE**	**10**	**8 (80.00)**	**2**	**6**	**1**	**1**
		Phetchaburi	**W**	**8**	**6 (75.00)**	**1**	**5**	**2**	**0**
Mycalesis perseus	**Nymphalidae**	**Nakhon Ratchasima**	**NE**	**9**	**7 (77.78)**	**4**	**3**	**1**	**1**
		Saraburi	**C**	**5**	**4 (80.00)**	**2**	**2**	**0**	**1**
		Phetchaburi	**W**	**15**	**13 (86.67)**	**5**	**8**	**2**	**0**
Neomyrina nivea	Lycaenidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	3	0	0	0	1	2
		Nakhon Ratchasima	NE	1	0	0	0	0	1

(Continued)

Table 2. (Continued).

Species name	Family	Location (Province)	Geographic regions	No. of specimens tested	No. of positive specimens (%infection)	No. of positive specimens by sex		No. of negative specimens by sex	
						Male	Female	Male	Female
Neptis hylas	Nymphalidae	Nakhon Nayok	C	2	0	0	0	0	2
		Prachinburi	C	1	0	0	0	1	0
Pachliopta aristolochiae	Papilionidae	Nakhon Ratchasima	NE	2	0	0	0	1	1
		Phetchaburi	W	5	0	0	0	1	4
		Nakhon Nayok	C	1	0	0	0	0	1
Papilio demoleus	Papilionidae	Saraburi	C	1	0	0	0	0	1
		Nakhon Ratchasima	NE	2	0	0	0	0	2
		Phetchaburi	W	4	0	0	0	1	3
Papilio helenus	Papilionidae	Phetchaburi	W	2	0	0	0	0	2
		Nakhon Ratchasima	NE	1	0	0	0	0	1
Papilio memnon	Papilionidae	Prachinburi	C	1	0	0	0	1	0
		Nakhon Ratchasima	NE	2	0	0	0	2	0
		Phetchaburi	W	1	0	0	0	1	0
Papilio nephelus	Papilionidae	Prachinburi	C	1	0	0	0	0	1
		Phetchaburi	W	2	0	0	0	0	2
Papilio polytes	**Papilionidae**	**Nakhon Ratchasima**	**NE**	**8**	**5 (62.50)**	**2**	**3**	**2**	**1**
		Nakhon Nayok	**C**	**3**	**2 (66.67)**	**1**	**1**	**0**	**1**
		Phetchaburi	**W**	**13**	**11 (84.61)**	**4**	**7**	**1**	**1**
Parantica aglea	**Nymphalidae**	**Prachinburi**	**C**	**6**	**4 (66.67)**	**2**	**2**	**0**	**2**
		Phetchaburi	**W**	**15**	**12 (80.00)**	**3**	**9**	**2**	**1**
		Nakhon Ratchasima	**NE**	**9**	**7 (77.78)**	**1**	**6**	**1**	**1**
Parantica agleoides	**Nymphalidae**	**Phetchaburi**	**W**	**10**	**8 (80.00)**	**3**	**5**	**2**	**0**
		Nakhon Ratchasima	**NE**	**6**	**4 (66.67)**	**1**	**3**	**1**	**1**
		Nakhon Nayok	**C**	**8**	**6 (75.00)**	**3**	**3**	**1**	**1**
Parthenos sylvia	Nymphalidae	Saraburi	C	2	0	0	0	0	2
		Phetchaburi	W	5	0	0	0	1	4
		Nakhon Ratchasima	NE	3	0	0	0	1	2
Pieris rapae	Pieridae	Prachinburi	C	1	0	0	0	0	1
		Nakhon Nayok	C	2	0	0	0	2	0
		Phetchaburi	W	3	0	0	0	2	1

(*Continued*)

Table 2. (Continued).

Species name	Family	Location (Province)	Geographic regions	No. of specimens tested	No. of positive specimens (%infection)	No. of positive specimens by sex		No. of negative specimens by sex	
						Male	Female	Male	Female
Suastus gremius	Hesperiidae	Nakhon Ratchasima	NE	1	0	0	0	0	1
		Phetchaburi	W	3	0	0	0	1	2
		Nakhon Nayok	C	1	0	0	0	0	1
Tanaecia julii	Nymphalidae	Nakhon Ratchasima	NE	4	0	0	0	2	2
		Saraburi	C	1	0	0	0	0	1
		Phetchaburi	W	5	0	0	0	2	3
Ypthima huebneri	Nymphalidae	Prachinburi	C	7	0	0	0	3	4
		Nakhon Nayok	C	3	0	0	0	1	2
		Nakhon Ratchasima	NE	5	0	0	0	2	3

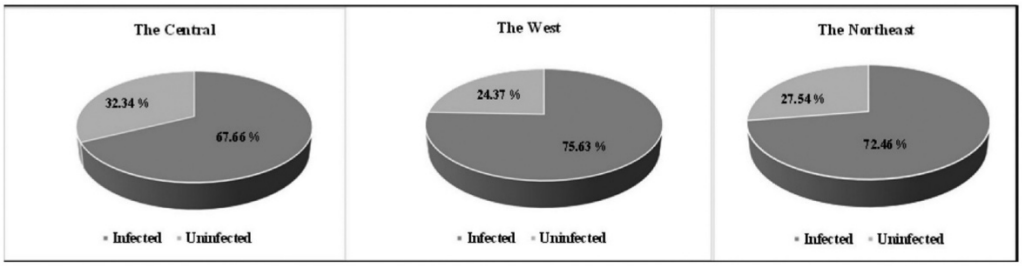

Figure 2. The percent of *Wolbachia* infection frequency (Infected/Uninfected) from each region.

of each of the butterfly families that was included in the study was shown as follows: Nymphalidae 58.69%, Papilionidae 19.56%, Pieridae 13.06%, Lycaenidae 6.52% and Hesperiidae 2.17%. The 291 *Wolbachia* positive specimens were restricted to 2 families (Nymphalidae and Papilionidae). The Nymphalidae had a high infection prevalence, accounting for 273 of the positive samples, representing 16 species. In contrast the Papilionidae returned 18 positive results for a single species (*Papilio polytes*).

Our survey has shown that 46.71% (291 of 623 collected samples) or 36.96% (17 of 46 collected species) in butterflies from Thailand tested positive for *Wolbachia*. The infection frequency of *Wolbachia* in butterflies varied between geographic regions; the highest infection rate (No. of positive specimens) was found in Nakhon Nayok (the Central) populations (87.50%), while the lowest frequency was recorded in Phetchaburi (the West) populations (40.00%). A total of 17 out of 46 butterfly species tested positive for *Wolbachia* (Table 1). These species were found in all three geographic regions (Table 2). Specifically, the 291 individuals representing these species were distributed as follows: 86 individuals (males = 32, females = 54) were found in the Central region, 72 individuals (males = 25, females = 47) in the Northeast region and 133 individuals (males = 46, females = 87) in the West region. Across all species which tested positive for *Wolbachia*, the prevalence of *Wolbachia* did not differ significantly between males (103/149, 69.1%) and females (188/248, 75.8%) according to a Chi-square test ($\chi = 2.122$, df = 1, P = 0.145). For each positive species, there were both females and males testing positive for *Wolbachia*. Of the *Wolbachia*-infected species, *Melanitis leda*, the common evening brown butterfly, had the highest proportion of infected individuals collected (83.33%), whereas *Acraea violae* had the lowest proportion of infected individuals (55.56%).

Discussion

Thailand has a diverse butterflies represented by five families. In the present study, we collected a taxonomically, ecologically, and geographically diverse sample of Thai Lepidopteran (butterflies) species comprising 46 species, 30 genera, and five families from three geographic regions. We found that

46.7% of the specimens have *Wolbachia* infections and were found to be present in similar proportions in the different geographic areas sampled. However, the distribution of infections was taxonomically skewed with almost all *Wolbachia* infections being identified in species of the family Nymphalidae, with several families having no infected individuals, similar to the report of Ahmed et al. (2015) and Salunke et al. (2012).

Wolbachia infections commonly induce different reproductive alterations in their insect hosts, including male-killing, feminisation, and cytoplasmic incompatibility, which can have profound impacts on insect populations (Hornett et al. 2006; Narita et al. 2006). These alterations have been described previously in butterflies (Jiggins et al. 2001; Hiroki et al. 2004), but their prevalence in Thai populations has yet to be studied. While we have identified several species that are likely to be infected with *Wolbachia*, the phenotypic effects of these infections remain unknown and future work is required to determine which reproductive phenotypes they induce. However, the presence of *Wolbachia* in both males and females at similar frequencies and the lack of clear sex bias in each species suggests that male-killing and feminisation are not widespread.

The results from this investigation are similar to broad findings for insects, with *Wolbachia* known to infect around 20–76% of insect species, and similar to the findings of Salunke et al. (2012), regarding *Wolbachia* infection rates of 37.29% in butterflies from India. Most *Wolbachia*-infected butterflies were in the family Nymphalidae which includes species that are important pests in Thailand. No *Wolbachia* infections were detected from the Hesperiidae, Lycaenidae and Pieridae, even though they were presented in the specimens sampling in this study (Table 1).

All species testing positive for *Wolbachia* in our survey showed intermediate infection frequencies, possibly due to incomplete maternal transmission which could be explained by high temperatures or other environmental factors (Wiwatanaratanabutr and Kittayapong 2009; Salunke et al. 2012; Wiwatanaratanabutr 2015). While the absence of *Wolbachia* in other butterfly species may represent a true lack of infection, it is also possible that the *Wolbachia* load might be below the threshold level of a PCR detection system (Wiwatanaratanabutr 2015). Therefore, ongoing monitoring of the diversity and distribution of *Wolbachia* infections as well as their infection density in Lepidoptera from Thailand is required.

Our findings are relevant to understand the distribution of butterfly populations and their *Wolbachia* infections from Thailand. The results provide a foundation for further investigation into the evolutionary dynamics of *Wolbachia* infections in insect hosts across tropical ecosystems. However, more information is needed on the reproductive effects of *Wolbachia* in these species to understand its prevalence in natural populations. This study along

with future experimental work will also provide useful information for potential applications of *Wolbachia* infections for Lepidopteran pest management.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The authors confirm that all relevant data from this report are available in the article.

Ethics approval

The animal ethics of this research project was approved by the Animal Care and Use Committee, King Mongkut's Institute of Technology Ladkrabang (Approval no. ACUC-KMITL-RES/2019/008 and ACUC-KMITL-RES/2019/009).

References

Ahmed MZ, Araujo-Jnr EV, Welch JJ, Kawahara AY. 2015. *Wolbachia* in butterflies and moths: geographic structure in infection frequency. Frontiers in Zoology. 12(16). doi:10.1186/s12983-015-0107-z.

Bagheri Z, Talebi AA, Asgari S, Mehrabadi M. 2019. *Wolbachia* induce cytoplasmic incompatibility and affect mate preference in *Habrobracon hebetor* to increase the chance of its transmission to the next generation. Journal of Invertebrate Pathology. 163:1–7. doi:10.1016/j.jip.2019.02.005.

Baldo L, Bordenstein S, Wernegreen JJ, Werren JH. 2006. Widespread recombination throughout *Wolbachia* genomes. Molecular Biology and Evolution. 23:437–449. doi:10.1093/molbev/msj049.

Bouchon D, Rigaud T, Juchault P. 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. Proceedings of the Royal Society of London Series B, Biological Sciences. 265:1081–1090. doi:10.1098/rspb.1998.0402.

Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL, Holmes EC. 2009. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. PLOS Pathogens. 5(4):e1000368. doi:10.1371/journal.ppat.1000368.

Carjaval TM, Capistrano JDR, Hashimoto K, Go KJD, Cruz MAIJ, Martinez MJL B, Tiopianco VSP, Amalin DM, Watanabe K. 2018. Detection and distribution of *Wolbachia* endobacteria in *Culex quinquefasciatus* populations (Diptera: Culicidae) from Metropolitan Manila, Philippines. Journal of Vector Borne Diseases. 55:265–270. doi:10.4103/0972-9062.256561.

Duplouy A, Hornett EA. 2018. Uncovering the hidden players in Lepidoptera biology: the heritable microbial endosymbionts. Peer J. 6:e4629. doi:10.7717/peerj.4629.

Ek-Amnuay P. 2012. Butterflies of Thailand. 2nd ed. Bangkok: Baan Lae Suan, Amarin Printing and Publishing.

Gotoh T, Sugasawa J, Noda H, Kitashima Y. 2007. *Wolbachia*-induced cytoplasmic incompatibility in Japanese populations of *Tetranychus urticae* (Acari: tetranychidae). Experimental and Applied Acarology. 42:1–16. doi:10.1007/s10493-007-9072-3.

Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. 2008. *Wolbachia* and virus protection in insects. Science. 31:702. 322(5902). doi:10.1126/science.1162418.

Hiroki M, Tagami Y, Miura K, Kato Y. 2004. Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*. Proceedings of the Royal Society of London Series B, Biological Sciences. 271:1751–1755. doi:10.1098/rspb.2004.2769.

Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD, Keller L. 2006. Evolution of male-killer suppression in a natural population. PLOS Biology. 4:e283. doi:10.1371/journal.pbio.0040283.

Jiggins FM, Hurst GDD, Schulenburg JHGVD, Majerus MEN. 2001. Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. Heredity. 86:161–166. doi:10.1046/j.1365-2540.2001.00804.x.

Kageyama D, Ohno M, Sasaki T, Yoshido A, Konagaya T, Jouraku A, Kuwazaki S, Kanamori H, Katayose Y, Narita S, et al. 2017. Feminizing *Wolbachia* endosymbiont disrupts maternal sex chromosome inheritance in a butterfly species. Evolution Letters. 1 (5):232–244. doi:10.1002/evl3.28

Kajtoch L, Kolasa M, Kubisz D, Gutowski JM, Ścibior R, Mazur MA, Holecová M. 2019. Using host species traits to understand the *Wolbachia* infection distribution across terrestrial beetles. Scientific Reports. 9:847. doi:10.1038/s41598-018-38155-5.

Kolasa M, Kubisz D, Mazur MA, Scibior R, Kajtoch L. 2018. *Wolbachia* prevalence and diversity in selected riverine predatory beetles (Bembidiini and Paederini). Bulletin of Insectology. 71:193–200.

Liu XD, Guo HF. 2019. Importance of endosymbionts *Wolbachia* and *Rickettsia* in insect resistance development. Current Opinion in Insect Science. 33:84–90. doi:10.1016/j.cois.2019.05.003.

Lopez V, Cortesero AM, Poinsot D. 2018. Influence of the symbiont *Wolbachia* on life history traits of the cabbage root fly (*Delia radicum*). Journal of Invertebrate Pathology. 158:24–31. doi:10.1016/j.jip.2018.09.002.

Muhammad ZA, Eli V, Araujo-Jnr JJW, Akito YK. 2015. *Wolbachia* in butterflies and moths: geographic structure in infection frequency. Frontiers in Zoology. doi:10.1186/s12983-015-0107-z

Narita S, Nomura M, Kato Y, Fukatsu T. 2006. Genetic structure of sibling butterfly species affected by *Wolbachia* infection sweep: evolutionary and biogeographical implications. Molecular Ecology. 15:1095–1108. doi:10.1111/j.1365-294X.2006.02857.x.

O'Neill SL, Giordane R, Colbert AME, Timothy K, Robertson H. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with CI in insects. Proceedings of

the National Academy of Sciences of the United States of America. 89:2699–2702. doi:10.1073/pnas.89.7.2699.

Plotkin D, Goddard J. 2013. Blood, sweat, and tears: a review of the hematophagous, sudophagous, and lachryphagous Lepidoptera. Journal of Vector Ecology. 38 (2):289–294. doi:10.1111/j.1948-7134.2013.12042.x.

Ross PA, Turelli M, Hoffmann AA. 2019. Evolutionary ecology of *Wolbachia* releases for disease control. Annual Review of Genetics. 53:93–116. doi:10.1146/annurev-genet-112618-043609.

Sakamoto H, Suzuki R, Nishizawa N, Matsuda T, Gotoh T. 2019. Effects of *Wolbachia*/*Cardinium* infection on the mitochondrial phylogeny of *Oligonychus castaneae* (Acari: tetranychidae). Journal of Economic Entomology. 112:883–893. doi:10.1093/jee/toy354.

Salunke BK, Salunkhe RC, Dhotre DP, Walujkar SA, Khandagale AB, Rakesh RC, Chandode K, Ghate HV, Patole MS, Werren JH, et al. 2012. Determination of *Wolbachia* diversity in butterflies from Western Ghats, India, by a multigene approach. Applied and Environmental Microbiology. 12:4458–4467. doi:10.1128/AEM.07298-11

Stouthamer R, Breeuwer JAJ, Hurst GDD. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annual Review of Microbiology. 53:71–102. doi:10.1146/annurev.micro.53.1.71.

Sumida Y, Katsuk M, Okada K, Okayama K, Lewis Z. 2017. *Wolbachia* induces costs to life-history and reproductive traits in the moth, *Ephestia kuehniella*. Journal of Stored Products Research. 71:93–98. doi:10.1016/j.jspr.2017.02.003.

Tang QY, Feng MG. 2002. DPS data processing system for practical statistics. Beijing: Science Press; p. 648.

Truitt AM, Kapun M, Kaur R, Miller WJ. 2019. *Wolbachia* modifies thermal preference in *Drosophila melanogaster*. Environmental Microbiology. 21:3259–3268. doi:10.1111/1462-2920.14347.

Wang X, Xiong X, Cao W, Zhang C, Werren JH, Wang X. 2020. Phylogenomic analysis of *Wolbachia* strains reveals patterns of genome evolution and recombination. Genome Biology and Evolution. 12:2508–2520. doi:10.1093/gbe/evaa219.

Werren JH, Baldo L, Clark ME. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nature Reviews Microbiology. 6:741–751. doi:10.1038/nrmicro1969.

Werren JH, Windsor D, Guo L. 1995. Distribution of *Wolbachia* among neotropical arthropods. Proceedings of the Royal Society of London Series B, Biological Sciences. 262:197–204.

Wiwatanaratanabutr I. 2013. Geographic distribution of *Wolbachia* infections in mosquitoes from Thailand. Journal of Invertebrate Pathology. 114:337–340. doi:10.1016/j.jip.2013.04.011.

Wiwatanaratanabutr I. 2015. *Wolbachia* infection in leafhoppers and planthoppers: diversity, density and geographic distribution in tropical rice agroecosystems. Journal of Asia-Pacific Entomology. 18:277–282. doi:10.1016/j.aspen.2015.03.004.

Wiwatanaratanabutr I, Kittayapong P. 2009. Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. Journal of Invertebrate Pathology. 102:220–224. doi:10.1016/j.jip.2009.08.009.

Zaspel JM, Weller SJ, Branham MA. 2011. A comparative survey of proboscis morphology and associated structures in fruit-piercing, tear-feeding, and blood-feeding moths in Calpinae (Lepidoptera: erebidae). Zoomorphology. 130(3):203–225. doi:10.1007/s00435-011-0132-1.

Zhou W, Rousset F, O'Neil S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proceedings of the Royal Society of London Series B, Biological Sciences. 265:509–515. doi:10.1098/rspb.1998.0324.

APPENDIX B

Samples of butterfly and moth

Table 1 Samples of butterflies in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand.

Acraea violae *Appias libythea*

Arhopala centaurus *Catopsilia pomona*

Cepora nerissa *Cethosia cyane*

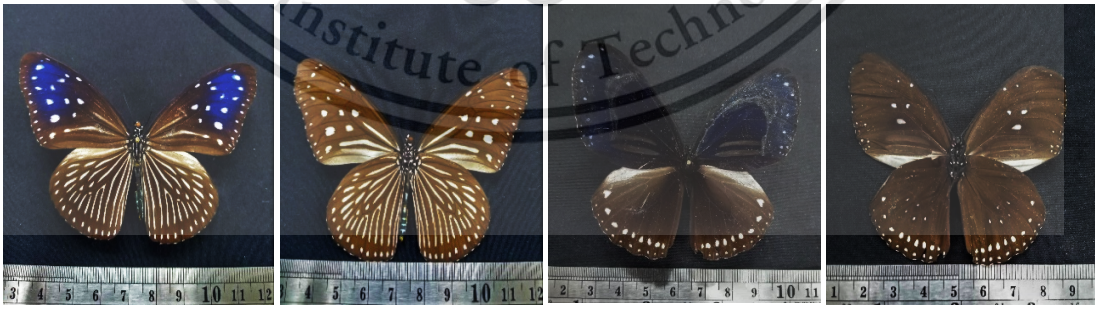
Cynithia lepidea *Danaus chrysippus*

Table 1 Samples of butterflies in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

Danaus genutia *Danaus melanippus*

Delias hyparete *Doleschallia bisaltide*

Euploea algea *Euploea camaralzeman*



Euploea mulciber *Euploea Sylvester*

Table 1 Samples of butterflies in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

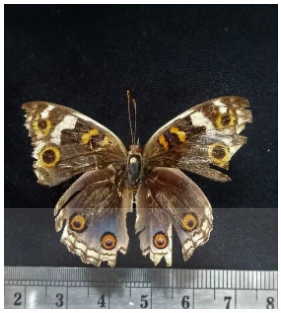
Eurema simulatrix *Euthalia evelina*

Graphium agamemnon *Ypthima huebneri*

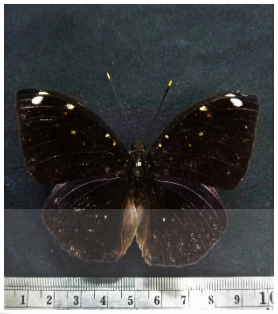
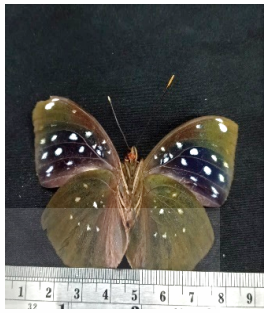
Graphium doson *Hypolimnas bolina*

Junonia atlites *Junonia lemonias*

Table 1 Samples of butterflies in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

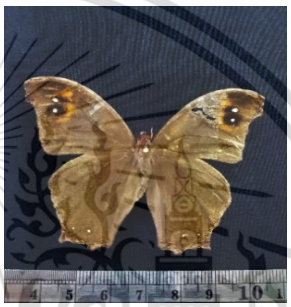



Junonia orithya

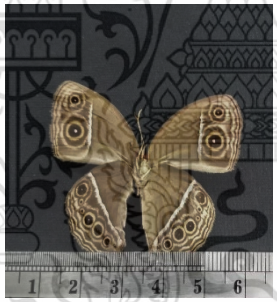



Lexias albopunctata


Loxura athymnus




Melanitis leda

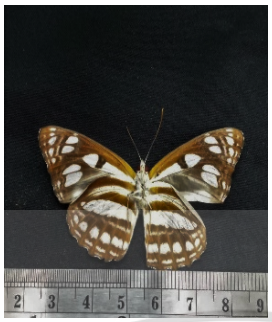
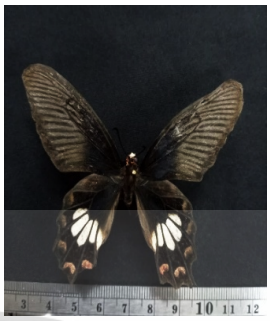
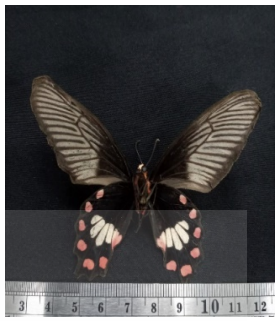
Mycalesis mineus


Mycalesis perseoides

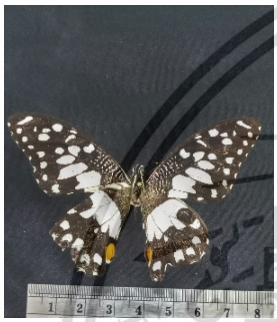
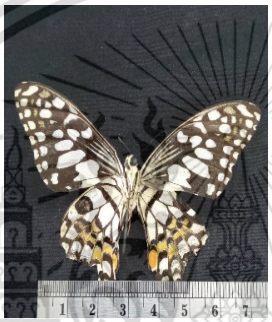

Mycalesis perseus

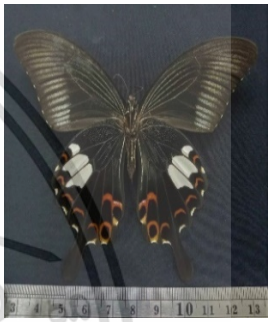

Neomyrina nivea

Table 1 Samples of butterflies in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

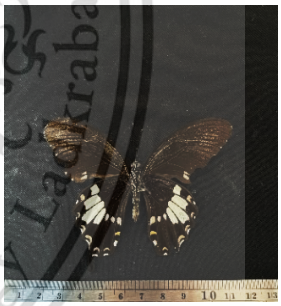
Neptis hylas *Pachliopta aristolochiae*



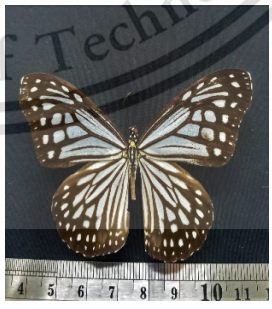
Papilio demoleus *Papilio helenus*



Papilio memnon *Papilio nephelus*






Papilio polytes *Parantica aglea*

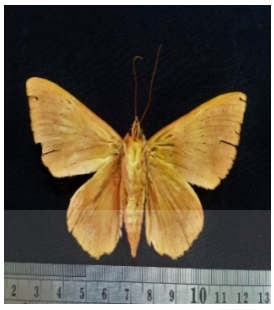
Table 1 Samples of butterflies in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

Parantica agleoides *Parthenos sylvia*

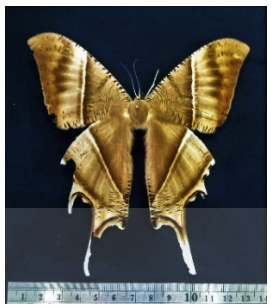
Pieris rapae *Suastus gremius*

Tanaecia julii

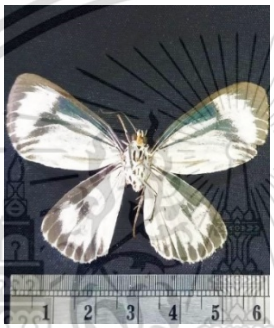
Table 2 Samples of moths in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand.

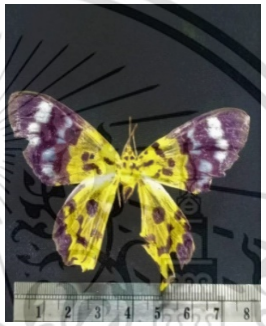
Thyas honesta




Lyssa zampa

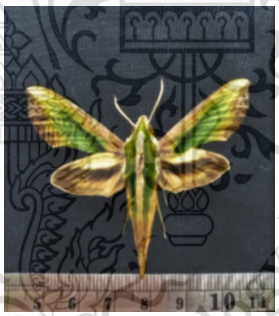



Neochera dominia




Dysphania militaris


Daphnis hypothous




Pergesa acteus


Hippotion sp.


Acherontiia styx

Table 2 Samples of moths in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

Theretra sp.

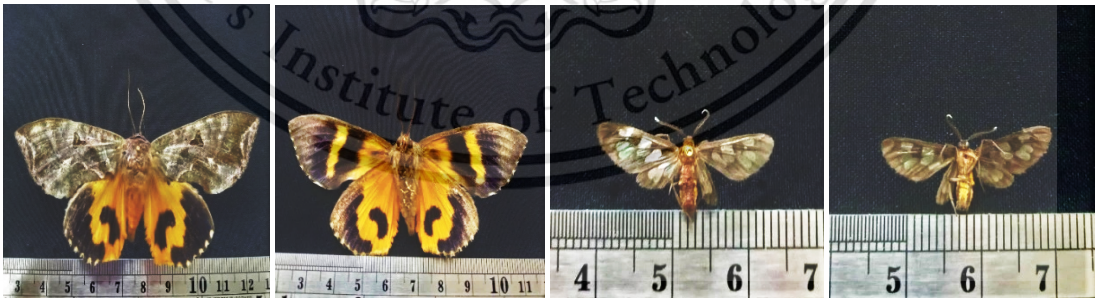
Artena sp.

Olepa sp.

Olene mendosa

Creatonotos transiens

Pareuchaetes pseudoinsulata



Eudocima phalonia

Amata sp.

Table 2 Samples of moths in the order Lepidoptera were collected from two tropical forests in three different geographic regions (C = the Central, NE = the Northeast, and W = the West) from Thailand (Continued).

Spodoptera litura *Antheraea frithi*

Trabala sp. *Attacus atlas*

Ischyja marapok *Thosea siamica*

APPENDIX C

Insect Sampling from Khao Yai and Kaeng Krachan National Parks, Thailand.

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Publications:

Boonsit, P., and Wiwatanaratanabutr, I. (2021). Infection density, diversity, and distribution of *Wolbachia* bacteria in moths (Order Lepidoptera): First systematic report from Thailand. Proceeding Thai Society for Biotechnology International Conference Online, Bangkok, Thailand.

Boonsit, P., and Wiwatanaratanabutr, I. (2021). First systematic survey of *Wolbachia* infection in butterflies (Order Lepidoptera) from Thailand: Distribution and diversity in tropical ecosystem. Proceeding the 33rd Annual Meeting of the Thai Society for Biotechnology and International Conference (TSB 2021), Bangkok, Thailand.

Boonsit, P., and Wiwatanaratanabutr, I. (2021). First report on the integration of copepods and *Wolbachia* to control *Aedes* larvae in Thailand. *International Journal of Mosquito Research*, 8(2): 125-129.

Boonsit, P., and Wiwatanaratanabutr, I. (2021). Diversity and *Wolbachia* infection in rice insect pests from Thailand. *International Journal of Entomology Research, 6* (3), 2455-4758.

Boonsit, P., and Wiwatanaratanabutr, I. (2021). Infection density, diversity, and distribution of *Wolbachia* bacteria in moths (Order Lepidoptera): First systematic report from Thailand. *Journal of Asia-Pacific Entomology, 24* (3) 764 –771.

Boonsit, P., Wiwatanaratanabutr, I., Ross, P.A., Grandjean, F., Austin, C., and Kramchote, S. (2023). Distribution of *Wolbachia* infection in butterflies (Lepidoptera): First systematic report from Thailand. *Oriental Insects, https://doi.org/10.1080/00305316.2023.2167883.*