Vector competence of *Culex tritaeniorhynchus* for duck Tembusu virus

Miss Jitra Sanisuriwong

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ความสามารถในการเป็นพาหะนำเชื้อ duck Tembusu virus ของยุง *Culex tritaeniorhynchus*

น.ส.จิตรา สนิสุริวงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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By	Miss Jitra Sanisuriwong
Field of Study	Veterinary Pathobiology
Thesis Advisor	Associate Professor Sonthaya Tiawsirisup, Ph.D.
Thesis Co Advisor	Assistant Professor AUNYARATANA THONTIRAVONG, Ph.D.

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor ROONGROJE THANAWONGNUWECH, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman

(Associate Professor Theerayuth Kaewamatawong, Ph.D.)

..... Thesis Advisor

(Associate Professor Sonthaya Tiawsirisup, Ph.D.)

..... Thesis Co-Advisor

(Assistant Professor AUNYARATANA THONTIRAVONG, Ph.D.)

..... Examiner

(Assistant Professor Morakot Kaewthamasorn, Ph.D.)

..... Examiner

(Associate Professor Kanisak Oraveerakul, Ph.D.)

..... External Examiner

(Professor Theeraphap Chareonviriyaphap, Ph.D.)

จิตรา สนิสุริวงษ์ : ความสามารถในการเป็นพาหะนำเชื้อ duck Tembusu virus ของยุง *Culex tritaeniorhynchus*. (Vector competence of *Culex tritaeniorhynchus* for duck Tembusu virus) อ.ที่ปรึกษาหลัก : สนธยา เตียวศิริทรัพย์, อ.ที่ปรึกษาร่วม : อัญญรัตน์ ต้นธีรวงศ์

ไวรัสเทมบูซูเป็นไวรัสที่ก่อให้เกิดโรคอุบัติใหม่ในเป็ด จัดอยู่ในกลุ่มนาตายาไวรัส สกุลฟลาวิไวรัสและแฟมิลีฟลาวิวิริดี เชื้อไวรัสนี้พบระบาดในฟาร์มเป็ดเนื้อและเป็ดพันธุ์ในประเทศจีนตั้งแต่ปี พ.ศ. 2553 และในปี พ.ศ. 2556 พบการระบาดในฟาร์มเป็ดเนื้อและเป็ดไข่ของประเทศไทย เป็ดที่ติดเชื้อจะแสดงอาการทางระบบประสาท ได้แก่ ยืนไม่ตรง สั่น และอัมพาต นอกจากนี้ในเป็ดไข่ยังให้ผลผลิตไข่ลดลง ไวรัสเทมบูซูในเป็ดเป็นโรคที่ถ่ายทอดเชื้อโดยอาศัยยุงเป็นพาหะ อย่างไรก็ตามบทบาทของยุงในระบบนิเวศของเชื้อในประเทศไทยยังไม่แน่ชัด ดังนั้นวัตถุประสงค์แรกของงานวิจัยนี้คือการศึกษาถึงความหลากหลายของยุงและการตรวจหาเชื้อไวรัสเทมบูซูในยุงจากฟาร์มเป็ด 4 ฟาร์มในภาคกลางของประเทศไทย โดยเก็บยุงจากฟาร์มเป็ดจำนวน 2 ฟาร์มในจังหวัดสิงห์บุรี และจำนวน 2 ฟาร์มในจังหวัดอ่างทอง ในระหว่างเดือนกันยายน พ.ศ. 2558 และเดือนกรกฎาคม พ.ศ. 2559 โดยจับยุงได้ทั้งหมด 30,841 ตัว และจำแนกชนิดของยุงได้ 7 ชนิด ได้แก่ *Anopheles* (*An.*) *barbirostris*, *An. stephensi*, *Culex* (*Cx.*) *gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus, Mansonia* (*Ma.*) *annulifera* และ *Ma. uniformis* ชนิดของยุงที่เก็บได้จากฟาร์มเป็ดแต่ละฟาร์มส่วนใหญ่จะเป็นยุงรำคาญชนิด *Cx. tritaeniorhynchus* และได้สุ่มตัวอย่างยุงจำนวนทั้งหมด 272 กลุ่มตัวอย่างมาตรวจหาเชื้อไวรัสเทมบูซูโดยใช้วิธีปฏิกิริยาลูกโซ่โพลิเมอร์เรสแบบย้อนกลับ (RT-PCR) ในการศึกษานี้ตรวจพบเชื้อในยุง *Cx. tritaeniorhynchus* จำนวน 1 กลุ่มตัวอย่าง ซึ่งเป็นตัวอย่างยุงที่จับมาจากจังหวัดสิงห์บุรีในระหว่างเดือนพฤศจิกายน พ.ศ. 2558 จากการวิเคราะห์สายวิวัฒนาการของลำดับโพลีโปรตีนยีนแสดงให้เห็นว่า Culex / TH / CU_2015 ถูกจัดอยู่ในกลุ่มย่อย 2.1 จากผลการศึกษาดังกล่าวทำให้มีวัตถุประสงค์ในการศึกษาวิจัยที่ 2 คือ การศึกษาถึงความสามารถของยุงรำคาญชนิด *Cx. tritaeniorhynchus* และ *Cx. quinquefasciatus* ในการเป็นพาหะนำเชื้อไวรัสเทมบูซูในเป็ด ในการศึกษานี้ได้ปล่อยให้ยุง *Cx. tritaeniorhynchus* ดูดเลือดที่มีเชื้อไวรัสที่แตกต่างกัน 4 ระดับ คือ 10^2, 10^3, 10^4และ 10^5 $TCID_{50}$/mL และปล่อยให้ยุง *Cx. quinquefasciatus* ดูดเลือดที่มีเชื้อไวรัสที่แตกต่างกัน 2 ระดับ คือ 10^4 และ 10^5 $TCID_{50}$/mL โดยพบการติดเชื้อในยุง *Cx. tritaeniorhynchus* ทั้ง 4 กลุ่ม แต่พบการแพร่กระจายและการถ่ายทอดเชื้อในยุงที่ได้รับเชื้อในขนาด 10^5 $TCID_{50}$/mL เท่านั้น สำหรับยุง *Cx. quinquefasciatus* นั้นพบการติดเชื้อในยุงที่ได้รับเชื้อขนาด 10^4 และ 10^5 $TCID_{50}$/mL แต่ไม่พบการแพร่กระจายและการถ่ายทอดเชื้อในยุงชนิดนี้ จากผลการศึกษาดังกล่าวทำให้มีวัตถุประสงค์ในการศึกษาวิจัยที่ 3 คือ การศึกษาความสามารถในการถ่ายทอดเชื้อไวรัสเทมบูซูผ่านทางรังไข่ไปสู่รุ่นลูกของยุง *Cx. tritaeniorhynchus* ในการศึกษานี้ได้ปล่อยให้ยุงดูดเลือดที่มีเชื้อไวรัสขนาด 10^5 $TCID_{50}$/mL หลังจากนั้นปล่อยให้ยุงออกไข่และศึกษาการติดเชื้อในรุ่นลูก ในการศึกษาพบว่ามียุงที่ติดเชื้อหลังจากที่ดูดเลือดที่มีเชื้อไวรัสจำนวน 43 ตัว และได้รุ่นลูกจำนวน 182 ตัว แบ่งเป็นยุงเพศผู้จำนวน 75 ตัว และยุงเพศเมียจำนวน 107 ตัว และพบว่ามียุงที่ไม่ติดเชื้อหลังจากที่ดูดเลือดที่มีเชื้อไวรัสจำนวน 37 ตัว และได้รุ่นลูกจำนวน 145 ตัว แบ่งเป็นยุงเพศผู้จำนวน 51 ตัว และยุงเพศเมียจำนวน 94 ตัว และนำยุงรุ่นลูกทั้งหมดมาตรวจหาเชื้อไวรัสเทมบูซูซึ่งไม่พบการติดเชื้อไวรัสในทุกตัวอย่าง การศึกษาทั้งหมดนี้บ่งชี้ว่ายุงรำคาญชนิด *Cx. tritaeniorhynchus* เป็นยุงที่มีศักยภาพในการเป็นพาหะนำเชื้อไวรัสเทมบูซูในเป็ดและน่าจะมีบทบาทสำคัญในวงจรการถ่ายทอดเชื้อไวรัสในเป็ดในประเทศไทย อย่างไรก็ตามการศึกษานี้ไม่พบว่ามีการถ่ายทอดเชื้อจากรุ่นแม่ไปสู่รุ่นลูก

สาขาวิชา	พยาธิชีววิทยาทางสัตวแพทย์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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Jitra Sanisuriwong : Vector competence of *Culex tritaeniorhynchus* for duck Tembusu virus. Advisor: Assoc. Prof. Dr. Sonthaya Tiawsirisup, Ph.D. Co-advisor: Asst. Prof. Dr. AUNYARATANA THONTIRAVONG, Ph.D.

Duck Tembusu virus (DTMUV), an emerging infectious disease in ducks, belongs to the Ntaya virus group of the *Flavivirus* genus in the *Flaviviridae* family. The emergence of DTMUV has been observed in layer and breeder duck farms in China since 2010 and in layer and broiler duck farms in Thailand since 2013. Infected ducks show neurologic signs, including an incapability to stand, ataxia, and paralysis. A significant drop in egg production is usually observed among layer ducks. The transmission of DTUMV involves mosquito vectors, however, the exact role of mosquitoes in the ecology of DTMUV in Thailand remains unclear. The first objective of this dissertation was to examine the mosquito distribution and their DTMUV detection status in four duck farms in central Thailand. Mosquitoes were collected from two duck farms in Sing Buri province and two duck farms in Ang Thong province from September 2015 to July 2016 using four CDC-light traps. A total of 30,841 mosquitoes were collected and identified to seven species (*Anopheles* (*An.*) *barbirostris*, *An. stephensi*, *Culex* (*Cx.*) *gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus, Mansonia* (*Ma.*) *annulifera* and *Ma. uniformis*). The most common collected species from each duck farm and each collection time was *Cx. tritaeniorhynchus*. A total of 273 mosquito pools were examined, with only one pool of *Cx. tritaeniorhynchus* collected from Sing Buri province in November 2015 testing positive for DTMUV by reverse transcription polymerase chain reaction (RT-PCR). Phylogenetic analysis of the polyprotein gene sequence demonstrated that a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) was grouped into subcluster 2.1. Therefore, the second objective of this dissertation was to examine the vector competence of *Cx. tritaeniorhynchus* and *Cx. quinquafasciatus* for DTMUV. Four groups of *Cx. tritaeniorhynchus* were allowed to feed on four levels of DTMUV which were 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL and two groups of *Cx. quinquefasciatus* were allowed to feed on two levels of DTMUV which were 10^4 and 10^5 $TCID_{50}$/mL. The results showed that DTMUV infection in *Cx. tritaeniorhynchus* was found from all groups. While DTMUV dissemination and transmission in *Cx. tritaeniorhynchus* were only found from the mosquitoes that fed on the blood meal with 10^5 $TCID_{50}$/mL of DTMUV. DTMUV infection in *Cx. quinquefasciatus* were found from the mosquitoes that fed on the blood meal with 10^4 and 10^5 $TCID_{50}$/mL of DTMUV; however, there was no virus dissemination and transmission found from all tested mosquitoes. The third objective of this dissertation was to study the transovarial transmission of DTMUV in *Cx. tritaeniorhynchus*. *Cx. tritaeniorhynchus* were allowed to feed on infected blood meal with 10^5 $TCID_{50}$/mL of DTMUV. Each blood-fed mosquito was individually kept in a plastic cup with water to allow the mosquito to lay eggs. After egg-laying, the mosquitoes were tested for DTMUV infection by using RT-PCR. A total of 43 DTMUV infected and 37 non-infected female mosquitoes with eggs were included in this study. A total of 182 (75 male and 107 female) F1 mosquitoes from DTMUV infected mosquitoes and 145 (51 male and 94 female) F1 mosquitoes from non-infected mosquitoes were tested for DTMUV infection; however, all of them were negative for DTMUV. The findings from this dissertation indicated the vector competence of *Cx. tritaeniorhynchus* for DTMUV and possible role as major vectors in the transmission cycle of DTMUV in Thailand. However, there was no transovarial transmission of DTMUV in *Cx. tritaeniorhynchus*.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I

Introduction

1.1 Important and Rationale

Flaviviruses cause hemorrhagic disease, encephalitis, fever, paralysis, and jaundice, which are the typical characterization of diseases in human (Gould and Solomon, 2008). Important flaviviruses include yellow fever, dengue, West Nile, St. Louis encephalitis, Japanese encephalitis, tick borne encephalitis, Kyasaner Forest disease, Omsk hemorrhagic fever, Tembusu, and Zika virus. Japanese encephalitis, Tembusu, and West Nile virus are associated with *Culex* mosquito vectors that are more likely to take blood meals from poultry and cause infections in the nervous system. While yellow fever and dengue virus are associated with *Aedes* mosquito vectors that are more likely to feed on mammals and humans. Both dengue and yellow fever virus cause infections in the vascular system. Moreover, flaviviruses transmitted by ticks cause hemorrhagic or neurological diseases (Gould and Solomon, 2008).

Flavivirus transmission cycle involves arthropod vectors and reservoir vertebrate hosts. Flavivirus life cycle starts from the entry of virus by endocytosis into the host cell by attachment, penetration, uncoating, replication, assembly, and release from host cell by exocytosis. Flaviviruses can be divided into two clades including the vector-borne virus and the others with no known vector (Chambers et al., 1990). The vector-borne virus clade can be subdivided into a mosquito-borne and a tick-borne clade. While the mosquito clade will be divided into *Culex* which are associated with encephalitis diseases and *Aedes* which are associated with

hemorrhagic diseases. Non-vectored flaviviruses are probably maintained in nature by animal to animal transmission via saliva and urinary shedding (Chen and Wilson, 2005).

Duck Tembusu virus (DTMUV) is an emerging infectious disease in ducks. It is a RNA virus belonging to the genus *Flavivirus* of the family *Flaviviridae* with over 70 serotype members (Su et al., 2011). DTMUV is a single stranded positive-sense RNA virus. Other important flaviviruses include dengue, Japanese encephalitis, West Nile, and yellow fever virus.

Tembusu virus (TMUV) was first isolated from *Culex (Cx.) tritaeniorhynchus* and *Cx. gelidus* mosquitoes in 1970s in Malaysia (Platt et al., 1975), but the disease associated with TMUV infection was not known. TMUV were detected from *Cx. tritaeniorhynchus*, *Cx. gelidus*, and *Cx. vishnui* in Kamphaeng Phet and Chiang Mai province, Thailand during 1982-2002. Later in the year 2010, DTMUV were reported in China and central part of Thailand (Su et al., 2011, Chakritbudsabong et al., 2015). In 2013, DTMUV were also detected from Nakhon Ratchasima, Prachinburi, Chonburi, and Suphanburi province in Thailand (O'Guinn et al., 2013b, Su et al., 2011, Chakritbudsabong et al., 2015, Thontiravong et al., 2015a). DTMUV is a mosquito-borne flavivirus, which various species of mosquitoes are important vectors for the transmission cycle of this virus in nature. However, the outbreak of DTMUV has been reported in winter, which the population of mosquitoes were low (Yan et al., 2011c, Teng et al., 2010, Su et al., 2011). Therefore, there are several transmission routes of the DTMUV in nature such as direct contact, horizontal, oral, and aerosol transmission (Tang et al., 2013a, Tang et al., 2012a, Yan et al., 2011b, Cao et al., 2011, Sun et al., 2013, Li et al., 2015).

Cx. tritaeniorhynchus is the most important mosquito vector to transmit several diseases in humans and animals (i.e., Japanese encephalitis virus). It is a primary vector for Japanese encephalitis virus in India and Asia. In Thailand, *Cx.*

tritaeniorhynchus and *Cx. gelidus* are the major population of the mosquito fauna in Bangkok during 1986-1987 (Gingrich et al., 1992). Both mosquitoes were found to be the dominant species and the high population in northern and central Thailand. In 2009-2010, the predominant mosquitoes in Thailand during November though the rest of the period were *Cx. tritaeniorhynchus* (Changbunjong et al., 2013). In 1982, the study of TMUV infection in mosquitoes in Thailand showed the detection of TMUV in *Cx. tritaeniorynchus*, *Cx. vishnui,* and *Cx. gelidus* (Leake et al., 1986). Another study in 2002 also revealed the detection of TMUV in *Cx. vishnui* (O'Guinn et al., 2013a). Studies about mosquito vectors for DTMUV in Thailand are limited. Therefore, the study about the vector competence of *Culex* mosquitoes for DTMUV is important and will be useful. Moreover, the relationship among the virus, host, and vector will explain the biology of DTMUV and the spread of the disease. Finally, this knowledge will be used for disease control and prevention.

1.2 Objectives of the study

1. To investigate the mosquito diversity and distribution in duck farms in Thailand

2. To investigate DTMUV infection in field collected mosquitoes in DTMUV endemic areas

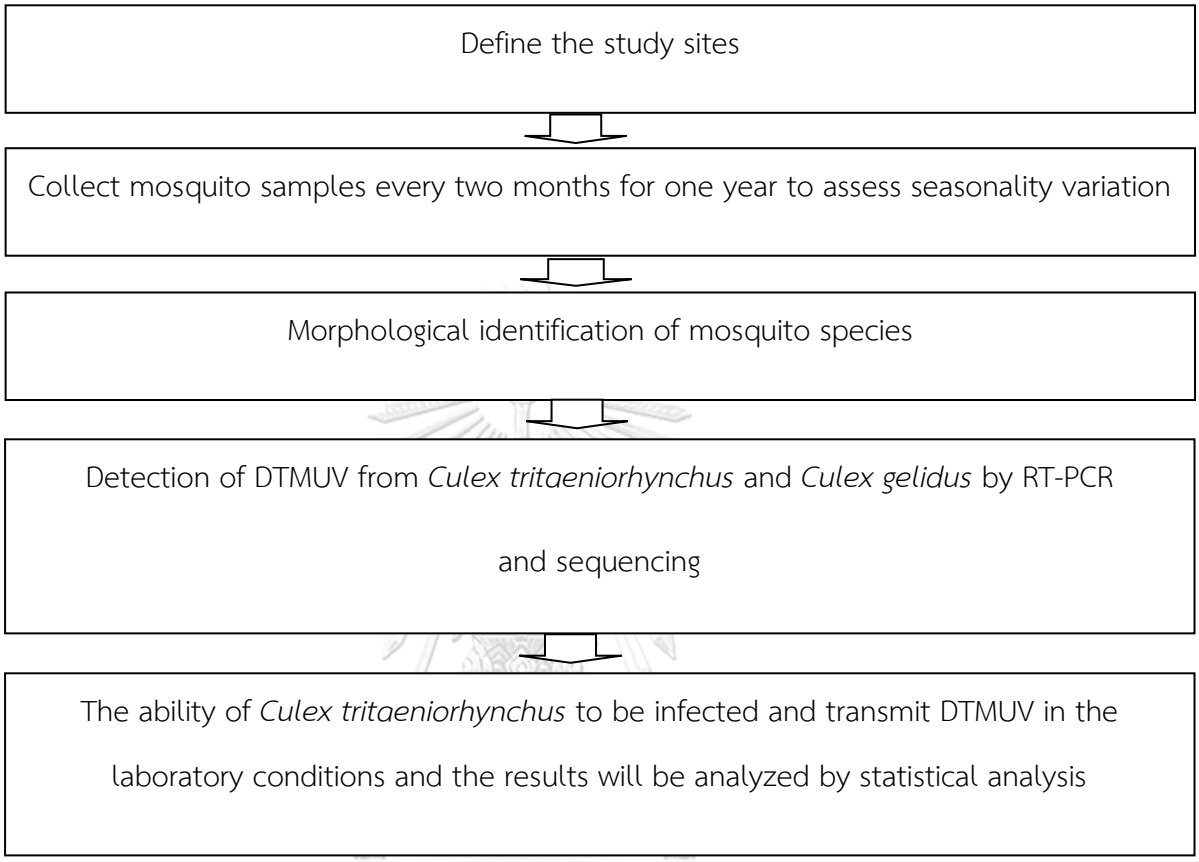
3. To study vector competence of *Culex tritaeniorhynchus* for duck Tembusu virus in the laboratory condition

1.3 Hypothesis

Culex tritaeniorhynchus are competent vectors for duck Tembusu virus

1.4 Conceptual framework

" Vector competence of *Culex tritaeniorhynchus* for duck Tembusu virus "



1.5 Advantages of the study

1. To know the new knowledge on the role of mosquitoes in duck Tembusu virus transmission cycle in Thailand

2. To know duck Tembusu virus infection, dissemination, and transmission in *Culex tritaeniorhynchus*

3. To know the relative among the virus, vector, and host

4. To reduce the incidence of disease in duck farm in Thailand

5. To apply the methodology developed from this research for other mosquito-borne viruses

1.6 Keywords (Thai): ยุงรำคาญ ไวรัสเทมบูซูในเป็ด ความสามารถในการเป็นพาหะนำโรค

1.7 Keywords (English): *Culex tritaeniorhynchus*, duck Tembusu virus, Vector competence

1.8 Literature reviews

1.8.1 Duck Tembusu virus

1.8.1.1 Morphology and characterization of the virus

Duck Tembusu virus (DTMUV) is a member of the Ntaya virus group in the genus *Flavivirus* of the family *Flaviviridae*. The Ntaya virus group is a mosquito-borne flavivirus, which include Bagaza, Ilheus, Israel turkey meningoencephalomyelitis, Ntaya, Tembusu and duck Tembusu virus. Flaviviruses are single stranded positive sense RNA viruses, which are vector-borne viruses. The virus genome of the genus *Flavivirus* is approximately 10.5 kb in length and has a diameter of 45-60 nm, which contains three structural proteins including capsid (C), membrane (M), and envelope (E), and seven nonstructural proteins (NS) (e.g., NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). E protein has an important role for virus receptor-binding, entry, and fusion (McMinn, 1997, Su et al., 2011, Yun et al., 2012). In 2011, the study of a newly emerged Tembusu virus (TMUV) strain in China found that TMUV was a spherical and enveloped particle with a diameter of 45-50 nm, which was observed under an electron microscope and it was a specific RNA virus (Yan et al., 2011a). Another study found that DTMUV had an approximately 11 kb in length. It was positive sense single

stranded RNA genome that comprised a single open reading frame and encoded three structural proteins and seven nonstructural proteins. They flanked by 5' and 3' untranslated regions (UTRs) (Wang et al., 2016). Organization of the duck Tembusu virus genome showed in figure 2. Phylogenetic analyses of the polyproteins of flaviviruses revealed that DTMUV isolates cluster within the clade of mosquito-borne flavivirus (Liu et al., 2012b). Three structural proteins raise the viral particle and seven nonstructural proteins are required for genome replication and polyprotein processing (Heinz and Stiasny, 2012). Electron micrograph of purified TMUV-SDHS from the cell culture supernatants showed in figure 1.

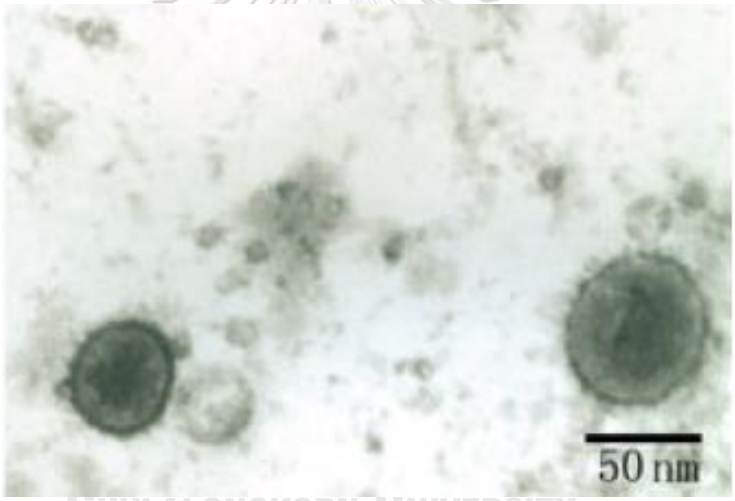


Figure 1: Morphology of Tembusu virus (Tang et al., 2012a).
Electron micrograph of purified TMUV-SDHS from the cell culture supernatants. Spherical virions with a diameter of 50-60 nm are shown (Tang et al., 2012a).

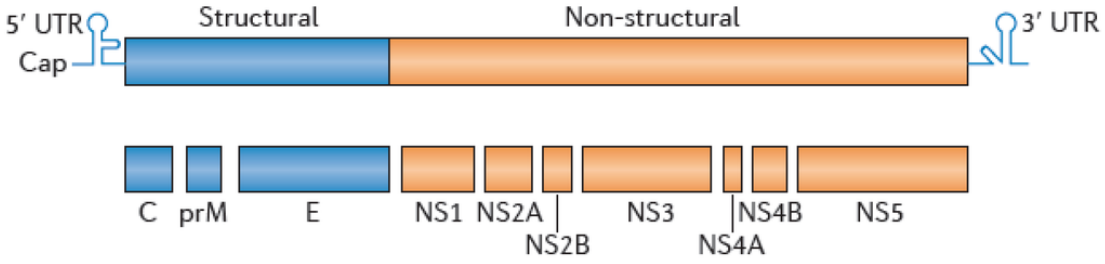


Figure 2: Organization of the duck Tembusu virus genome (Beck et al., 2013).

DTMUV is positive sense single stranded RNA genome that comprises a single open reading frame and encodes three structural proteins, including capsid, premembrane, envelope and seven nonstructural proteins (e.g., NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Beck et al., 2013).

1.8.1.2 Life cycle of duck Tembusu virus

Arthropod-borne flaviviruses can be transmitted to vertebrate hosts by chronically infected mosquito or tick vectors. The four mosquito-borne complexes include Japanese encephalitis, Ntaya, Uganda, and DEN group. Whereas, the tick-borne complexes include TBE and Tyuleniy group, and non-arthropod vector complexes include bat-associated Rio Bravo and rodent-associated Modoc group (Chambers et al., 1990). The life cycle of arthropod-borne flaviviruses relate to complex relationships among insect vectors, vertebrate reservoirs, humans, and environment. Tembusu virus is a mosquito-borne flavivirus and belongs to Ntaya virus group, which includes Bagaza, Ilheus, Israel turkey meningoencephalomyelitis, Ntaya, Tembusu and duck Tembusu virus. The entry of flavivirus into the host cell is achieved by the attachment of the viral envelope protein E to host receptor, which mediates clathrin-mediated endocytosis. Structural proteins are engaged in cellular attachment, membrane fusion, virion assembly, and non-structural proteins are involved in viral replication and counteraction of host immunity. Therefore, the E

protein of DTMUV is the major surface protein of the virion that mediates binding to the cellular receptor and subsequent fusion event between viral and host membranes (Perera et al., 2008). Moreover, E protein of DTMUV is be able to induce protective immune response in target animals (Han et al., 2016), and is a primary and majority target of neutralizing antibodies. DTMUV replication follows the positive stranded RNA virus replication model and the positive stranded RNA virus transcription is the method of transcription. The natural hosts are humans, mammals and mosquitoes. The virus releases secretion, which it is the replication and assembly at cytoplasm. Flavivirus life cycle showed in figure 3.

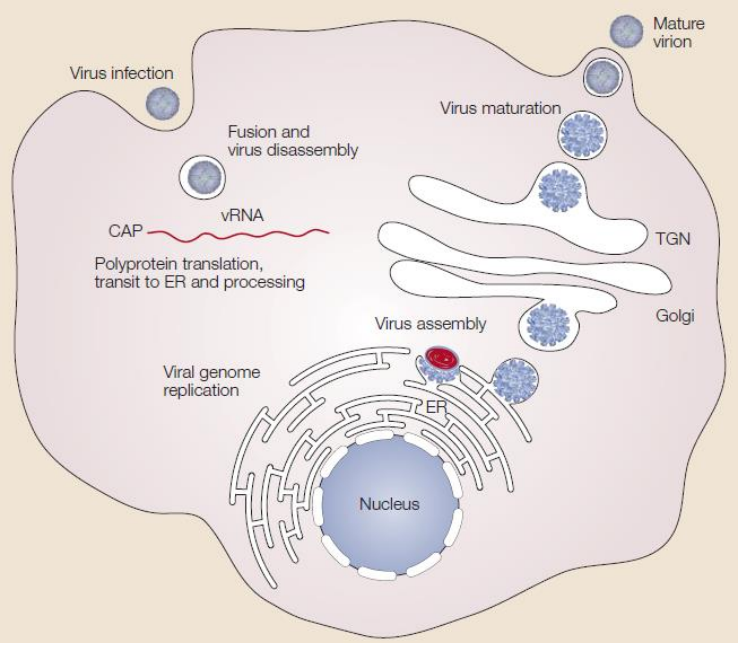


Figure 3: Flavivirus life cycle (Mukhopadhyay et al., 2005).

Virion attaches to the surface of a host cell and subsequently enters the cell by receptor-mediated endocytosis. Several primary receptors and low-affinity co-receptors for flaviviruses have been identified. Acidification of the endosomal vesicle triggers conformational changes in the virion, fusion of viral and cell membranes, and

particle disassembly. Once the genome is released into the cytoplasm, the positive-sense RNA is translated into a single polyprotein that is processed co- and post-translationally by viral and host proteases. Genome replication occurs on intracellular membranes. Virus assembly occurs on the surface of the endoplasmic reticulum when the structural proteins and newly synthesized RNA buds into the lumen of endoplasmic reticulum. The resultant non-infectious, immature viral, and subviral particles are transported through the tras-Golgi network (TGN). The immature virion particle is cleaved by the host protease furin, resulting in mature, infectious particle. Mature virion is subsequenthy released by exocytosis (Mukhopadhyay et al., 2005).

1.8.2 Mosquito vectors

1.8.2.1 *Culex tritaeniorhynchus*

Mosquitoes are insects of the order Diptera and family Culicidae. There are about 4,000 known mosquito species distributed worldwide (who, 2007). Mosquitoes are the most important group of insects concerning public health in tropical and subtropical countries. Mosquito-borne diseases include malaria, Elephantiasis, West Nile encephalitis, dengue fever, chikungunya fever, Japanese encephalitis, and yellow fever (Staples et al., 2009). Mosquitoes are the most important vectors of pathogenic organisms (e.g., parasites and viruses) to humans and animals. The maintenance and transmission of pathogens (e.g., parasites and viruses) are dependent on the availability of competent mosquito vectors. *Cx. tritaeniorhynchus* is the most important mosquito vector to transmit several diseases in humans and animals (i.e., Japanese encephalitis virus) and it is a primary vector of Japanese encephalitis virus in India and Asia.

Cx. tritaeniorhynchus is characterized by no lower mesoepimeral setae, proboscis with pale ring, wing without pattern of pale spots, abdominal terga II-VI

with basal pale bands, seldom with distinct apical pale bands, vertex with erect light brown to dark scales, scutum entirely covered by light brown to dark scales, anterior surfaces of fore and mid femora not speckled with pale scales, vertex with erect dark brown to black scales, scutum with dark brown to black scales, and pale scaling extends proximally from pale ring of proboscis on ventral surface (figure 4). They can be found in rice fields, ground pools, and marshland. Immature stages of these mosquitoes can be found in both dirty and fresh water. The mosquitoes can feed on blood of humans and several animals (i.e., cattle, pigs, goats, deer, chickens, and wild bird) (Sudeep, 2014). They usually take blood meals between evening to night.

Figure 4: Morphology of *Culex tritaeniorhynchus*
Proboscis with pale ring, wing with pattern of pale spots, vertex with erect dark brown to black scales, and scutum with dark brown to black scales.

In 1970, the study of Japanese encephalitis virus (JEV) in Chaingmai valley, Thailand indicated the isolations of JEV from *Cx. tritaeniorhynchus*, *Cx. gelidus*, and *Cx. fuscocephala* pools. Two strains of TMUV were isolated from *Cx. gelidus* and one isolation of TMUV was identified from *Cx. vishnui* complex (Gould et al., 1974). In

Thailand, *Cx. tritaeniorhynchus* and *Cx. gelidus* were the major population of the mosquito fauna in Bangkok during 1986-1987. These mosquitoes were found to be the dominant species and the high population in northern and central Thailand (Gingrich et al., 1992). In 2009-2010, the predominant mosquitoes in Thailand during November though the rest of the period were *Cx. tritaeniorhynchus* (Changbunjong et al., 2013).

1.8.2.2 Factors affecting the transmission of virus in mosquito vector

Mosquitoes are important biological vectors for flaviviruses. The virus can grow or multiply in the mosquito before it can enter the human or animal. There are several factors involved in the relationship between the virus and host (i.e., host susceptibility, vector competence, and virus). Virus infection in the vertebrate host depends on the amount of virus in the mosquito saliva, the frequency of biting, and the duration of feeding. Moreover, the level of viremia in host depends on the rate of clearance by macrophages and humoral antibodies in the host. An enzyme processing may occur in the mosquito's midgut such as trypsin and may exert an impact on the infectivity of the virus (Molina-Cruz et al., 2005). The varies of durations, times of onset, levels of viremia, and subtype or strain of a specific virus have the effects on virus infections in vertebrate hosts (Chen and Wilson, 2005).

1.8.3 Epidemiology of duck Tembusu virus

Tembusu virus was first isolated from *Culex* mosquitoes in Malaysia (Platt et al., 1975). In 2012, the outbreaks of DTMUV were detected in broiler duck farms in Malaysia which occurred after the outbreak of this disease in China in 2010 (Homonnay et al., 2014). In 2010, the severe outbreaks of duck infectious diseases were occurred around the major duck producing farms in China. Infected ducks showed consistently acute anorexia, diarrhea, egg production drop, ovary-oviduct

disease, and severe neurologic disorders. The virus was identified as a new flavivirus (BYD virus) that is closely related to TMUV (Yan et al., 2011b). DTMUV is an emerging infectious disease in ducks. This virus caused a negative effect on the severe economic in China. In Thailand, a severe infectious disease in ducks has emerged since 2013. The outbreaks have been reported from duck farms in Nakhon Ratchasima, Prachinburi, Chonburi, and Suphanburi provinces, Thailand (Thontiravong et al., 2015b, Chakritbudsabong et al., 2015). Phylogenetic analysis of the polyprotein gene sequence of the Thai DTMUV indicated that it is closely related to Chinese DTMUV and belongs to Ntaya virus group of mosquito-borne flavivirus. However, our recent study demonstrated the presence of DTMUV in Thai ducks since 2007 (Ninvilai et al., 2018). Moreover, during 2015 - 2017, DTMUV infected ducks were detected in Lop Buri, Nakhon Pathom, Sara Buri, Sing Buri, Supan Buri, Chachoengsao, Chon Buri, Prachin Buri, Nakhon Ratchasima, and Chumphon provinces, Thailand (Ninvilai et al., 2019). The data indicate the wide distribution of DTMUV in Thailand.

1.8.4 Clinical signs and pathogenesis of duck Tembusu virus

DTMUV causes the disease in ducks and other avian species (i.e., house sparrows, chickens and geese) (Huang et al., 2011, Liu et al., 2012a, Tang et al., 2011). However, DTMUV is not pathogenic for primates (Wang et al., 2016). DTMUV has not been reported to cause illness in humans but DTMUV-specific antibodies and DTMUV-RNA were detected in duck farm workers in China (Tang et al., 2013b). Clinical signs of DTMUV in infected ducks are anorexia, retarded growth, high fever, diarrhea, severe egg production drop, and neurological disorders including inability to stand, ataxia, and paralysis (Su et al., 2011, Tang et al., 2011, Yan et al., 2011c, Thontiravong et al., 2015a). Severe ovarian hemorrhage, ovaritis and regression, and enlarged spleen were found in infected ducks (Su et al., 2011). The experimental

study also showed neurological symptoms (i.e., anorexia, ataxia, and paralysis) at 3 days post infection (dpi) and died at 4-5 dpi by intracerebral (i.c.) inoculation in Pekin duckling. Histopathological studies showed the necrosis in brain and livers of infected ducks (Yun et al., 2012). The previous study of Yan et al. (2011) reported the moderate necrosis of brain tissue, severe nephrosis, pneumorrhagia, and severe necrosis of the spleen of infected ducks. The tissue sections of the infected ducks showed viral antigens in the tissues of brain, kidney, lung, and spleen using immunohistochemistry. Infected ducks showed symptoms after the virus has entered the cell, proliferated, and increased the number of new viruses being released. Clinical outcome of the disease depends on age, breed, and immunity of the host. Age of infected animals is related to pathogenesis, which the younger animal showed severe symptoms including severe neurological disfunction and death (Sun et al., 2013). Severe hemorrhage and regression of ovarian follicles were found in DTMUV infected ducks in Thailand. Histopathologic studies also indicated the moderate multifocal gliosis and perivascular cuffing in cerebellum and spinal cord. Chicken embryos infected with DTMUV strain DK/TH/CU-1 showed severe cutaneous hemorrhage on 3-5 dpi (Thontiravong et al., 2015a). Moreover, additional study of the pathogenesis of a cluster 2.1 Thai DTMUV was investigated in three ages of Cherry Valley ducks (1-, 4- and 27-week-old). Each group of ducks were inoculated with a cluster 2.1 Thai DTMUV and evaluated for clinical signs and pathology. The study revealed that all duck ages were susceptible to Thai DTMUV and Thai DTMUV induced greater disease severity in younger ducks (1- and 4-week-old) by higher morbidity and mortality rates, and higher degree of pathological severity (Ninvilai et al., 2020). Clinical signs and pathogenesis of DTMUV infection showed in figure 5.

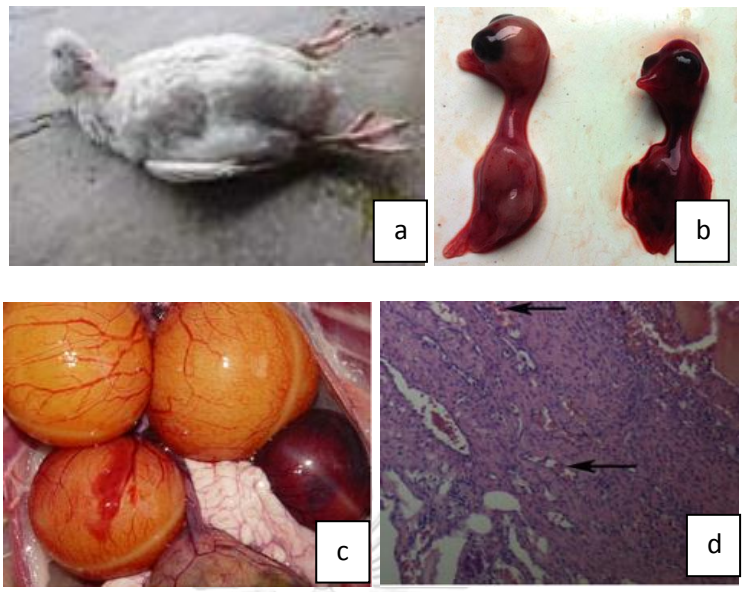


Figure 5: Clinical signs and pathogenesis of DTMUV infection (Tang et al., 2012a, Thontiravong et al., 2015a).

(a) DMTUV-infected ducks showed neurologic signs, including inability to stand, ataxia, and paralysis; (b) The embryos died with sever retardation and cutaneous haemorrhages in Normal embryos, and virus-infected embryos; (c) Ovary with hyperaemia, haemorrhage, and distortion; (d) HE-stained ovary section showed haemorrhage and follicle rupture (Tang et al., 2012a, Thontiravong et al., 2015a).

1.8.5 Duck Tembusu virus transmission

Duck Tembusu virus is a mosquito-borne flavivirus. Therefore, the mosquitoes are important vectors for the transmission cycle of this virus. Some flaviviruses are zoonotic diseases which are transmitted by the biting of arthropod vectors. Flavivirus infection in mosquito occurs when the mosquito ingests a blood meal containing the virus, which infects the midgut epithelial cells and subsequently the salivary gland. For extrinsic incubation period, the virus is secreted in the saliva and reaches a new host when the mosquito takes another blood meal. The infection of salivary gland

leads to lifelong infection in the mosquito (Gea-Banacloche et al., 2004). The several studies have found that DTMUV can be transmitted in a variety of ways, with or without vector. The competent vector mosquitoes in the transmission of DTMUV is *Culex* mosquitoes (O'Guinn et al., 2013b). However, these transmission routes (i.e., direct contact, fecal-oral route, horizontal transmission, and aerosal transmission) can cause DTMUV infection, even without mosquitoes (Yan et al., 2011c, Tang et al., 2012a, Cao et al., 2011, Li et al., 2015).

1.8.6 Diagnosis of duck Tembusu virus

In general, the diagnosis of duck TMUV infections can be divided into 3 methods: direct detection method, virus isolation, and serological tests. The previous studies have shown that these methods are commonly used for the diagnosis of DTMUV. Direct detection method include antigen detection method, molecular-based assays which consists of reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Su et al., 2011, Yan et al., 2011a, Tang et al., 2012b). Virus isolation and propagation could be conducted in cell cultures and embryonated chicken or duck eggs (Su et al., 2011). While the serological methods have been commonly used to detect specific antibodies against flavivirus infection. This method consists of serum neutralization (SN) test, indirect immunofluorescent assay (IFA), hemagglutination inhibition (HI) test, and enzyme-linked immunosorbent assay (ELISA) (Wang et al., 2019, Li et al., 2012).

CHAPTER II

Duck Tembusu virus detection and characterization from mosquitoes in duck farms, Thailand

Transboundary and Emerging Disease. 2020: 1082-1088.

Jitra Sanisuriwong, Nichapat Yurayart, Aunyaratana Thontiravong, Sonthaya Tiawsirisup

2.1 Introduction

Duck Tembusu virus (DTMUV) is an emerging infectious disease in ducks described in China, Malaysia and Thailand. It is a single stranded positive-sense RNA virus belonging to the Ntaya virus group of the genus *Flavivirus* and family *Flaviviridae*, with over 70 known serotype members. It is a spherical and enveloped virus of 45–50 nm in diameter and a genome of approximately 11 kb in length that is comprised of a single open reading frame that encodes for three structural and seven nonstructural proteins (Su et al., 2011). This virus affects various hosts, such as ducks, geese, chickens, sparrows, pigeons, mice, and mosquitoes (Tang et al., 2015, Yu et al., 2018). Tembusu virus (TMUV), the most closely related virus to DTMUV, was first isolated from *Culex* (*Cx.*) *tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia in 1955, while DTMUV was first identified as the causative agent of egg-drop syndrome, neurological signs or death in infected egg-laying and breeder ducks in China in 2010 (Cao et al., 2011, Su et al., 2011). This virus rapidly spread through duck farms and caused economic losses for different types of duck raising.

For DTMUV infection in ducks in Thailand, a severe infectious disease in broiler and layer ducks has emerged since 2013. The outbreaks have been reported from

free-grazing ducks and duck farms in various major duck production areas in Thailand including Sing Buri and Ang Thong Provinces (Ninvilai et al., 2018, Ninvilai et al., 2019, Tunterak et al., 2018). Phylogenetic analysis of the polyprotein gene sequence of the Thai DTMUV isolates indicated that it is closely related to Chinese DTMUV (Thontiravong et al., 2015a). However, an unknown contagious disease associated with severe neurological signs and egg production losses in ducks, resembling DTMUV infection, has been observed in Thailand since 2007 (Ninvilai et al., 2018).

As known, DTMUV is mosquito-borne flavivirus, where various species of mosquitoes are important vectors in the transmission cycle of this virus in nature. It was first detected in *Cx. pipiens* mosquitoes collected during the summer from Shandong Province, China in 2010–2012 using RT-PCR and virus isolation assays (Tang et al., 2015). The epidemiological data, however, indicated that this virus can spread during the winter in China, during which period the mosquitoes are inactive. This suggests the possible involvement of non-vector transmission routes in the spread of the virus, or vectors other than mosquitoes. Subsequent in vivo studies support the former by showing horizontal transmission of the virus among ducks by direct contact and aerosol transmission (Li et al., 2015), and by vertical transmission from breeding ducks to ducklings (Zhang et al., 2015).

In Thailand, TMUV were detected from pools of *Cx. gelidus*, *Cx. tritaeniorynchus*, and *Cx. vishnui* mosquitoes collected from Kamphaengphet Province in 1982 (Leake et al., 1986), *Cx. tritaeniorhynchus* mosquitoes collected from Chiang Mai Province in 1992 (Pandey et al., 1999), and *Cx. quinquefasciatus* mosquitoes collected near a chicken farm in Kanchanaburi Province in 2015 (Nitatpattana et al., 2017). Phylogenetic analysis of TMUV isolated from Kanchanaburi Province indicated that it is closely related to leghorn chicken TMUV, which is different from the DTMUV isolated from ducks in Thailand (Chakritbudsabong et al., 2015, Thontiravong et al., 2015a). However, the exact role of mosquitoes in the

ecology of DTMUV in Thailand remains unclear. Therefore, studies on the mosquito distribution and DTMUV detection on duck farms are needed. This study was conducted to examine the mosquito distribution and DTMUV detection in mosquitoes in major duck production areas in central Thailand. The findings may help to explain the involvement of mosquito vectors in the transmission cycle and ecology of this virus and lead to planning for disease prevention and control in duck farms.

2.2 Materials and Methods

Virus

The DK/TH/CU-1 strain of DTMUV was kindly provided by the Virology Unit, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. It was originally isolated from an infected duck in Nakhon Ratchasima Province, Thailand, and then propagated in 9-day-old specific pathogen-free (SPF) embryonated duck eggs before storage in liquid nitrogen until used. It was used as a positive control for reverse transcription polymerase chain reaction (RT-PCR).

Field mosquito collection

The mosquitoes were collected from two duck farms in Sing Buri Province and two duck farms in Ang Thong Province between September 2015 and July 2016 using four CDC light traps per farm (Figure 6), each operated overnight for 12 h. The collected mosquitoes were then euthanized using dry ice, transferred to the laboratory, and stored at -80 °C. The mosquito genus and species were morphologically identified under a stereomicroscope using morphological

identification keys (Rattanarithikul and Panthusiri, 1994). The identified mosquitoes were then examined for DTMUV. Viral RNA from a pool of collected mosquitoes was extracted and examined by RT-PCR using specific primers for the NS5 gene of the virus. This study was conducted in compliance with the Chulalongkorn University Animal Care and Use Committee, Thailand (Animal Use Protocol No. 1631045).

Detection of DTMUV

Viral nucleic acid extraction

The DTMUV detection status of the collected mosquitoes was examined using RT-PCR. Viral nucleic was extracted from a pool of mosquitoes using the viral nucleic acid extraction kit II (Geneaid, Taiwan) according to the manufacturer's recommendations, where there were 2–50 mosquitoes per pool according to species, location, and collection time. Each mosquito pool was homogenized in 1 mL of 2% (v/v) Eagle's minimal essential medium and centrifuged at 3,000 g for 1 min. The supernatant was then harvested and viral nucleic was extracted and stored at -80 °C until tested.

RT-PCR of the NS5 gene fragment of DTMUV

The DTMUV detection was performed by RT-PCR amplification of the viral NS5 gene using the specific primers forward 5'-GCAGGTTCAGGAAGTGAGAGG-3' and reverse 5'-GGATTGTCTTGGTCATAATGCC-3' to yield a PCR product of 617 bp (Thontiravong et al., 2015b). Total RNA samples were amplified using the SuperScript® III One-Step RT-PCR with Platinum® Taq DNA Polymerase (Invitrogen, USA) in a 25 µL volume comprised of 1.5 µL of RNA, 12.5 µL of 2x reaction mix (0.4 mM dNTP, 3.3 mM $MgSO_4$) (Invitrogen, USA), 1 µL of forward and reverse primer (10 µM), 1 µL of SuperScript III RT/Platinum Taq Mix (Invitrogen, USA), and 8 µL of distilled water

(Invitrogen, USA). The thermal cycling was performed at 48 °C for 45 min, 94 °C for 3 min and then 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and then a final 72 °C for 10 min using a Perkin Elmer Cetus 9600 thermocycler (Perkin Elmer, Waltham, USA). The reaction was then mixed with 6 µL of loading buffer (BlueJuice Gel Loading Buffer, Invitrogen, USA) and resolved by 2% (w/v) agarose gel (UltraPure, Invitrogen, USA) electrophoresis.

After agarose gel electrophoresis, the DNA band of the expected product size was cut out of the gel and purified using the GenepHlowTM Gel/PCR Cleanup Kit (Geneaid, Taiwan) according to the manufacturer's recommendations. The purified amplicon was submitted to a commercial service for DNA sequencing (First Base Laboratories, Kuala Lumpur, Malaysia). The obtained nucleotide sequencing results were aligned and trimmed using the ClustalW multiple alignment in BioEdit (Thompson et al., 1994), and compared with available DNA sequences in the GenBank database to define the pathogen using the Basic Local Alignment Search Tool (Altschul et al., 1990).

Whole genome sequencing and phylogenetic analysis

Viral RNA of Thai DTMUV isolated from *Culex* mosquito in 2015 (*Culex*/TH/CU_2015) was subjected to whole genome sequencing using the primer set for amplification of the polyprotein gene sequence of DTMUV as described previously (Thontiravong et al., 2015a). The nucleotide sequences were assembled using SeqMan software v.5.03 (DNASTAR Inc., Wisconsin, USA). A polyprotein gene sequence of *Culex*/TH/CU_2015 was submitted to GenBank database under the accession number MH460536. Phylogenetic analysis was performed by comparing the polyprotein gene sequence of the *Culex*/TH/CU_2015 with that of the previously

reported Chinese, Malaysian, and Thai DTMUVs and TMUV strains available in the GenBank database. The phylogenetic trees were constructed by neighbor-joining (NJ) and maximum-likelihood (ML) algorithms with 1000 bootstraps using MEGA v.6.0 program (Tamura et al., 2011). The nucleotide and amino acid sequences of the *Culex*/TH/CU_2015 and the selected reference DTMUV and TMUV strains were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA).

Meteorological data

Meteorological data were kindly provided by Thai Meteorological Department. Because there was no weather station in Sing Buri and Ang Thong Provinces, the meteorological data indicated in this study were collected from weather stations in four Provinces (Ayutthaya, Chinat, Lopburi, and Suphanburi Provinces) surrounding Sing Buri and Ang Thong Provinces (Figure 6). The average values of the meteorological data from the four stations, including the minimum temperature, maximum temperature, mean temperature, mean rainfall, and mean relative humidity, are shown in Figure 7.

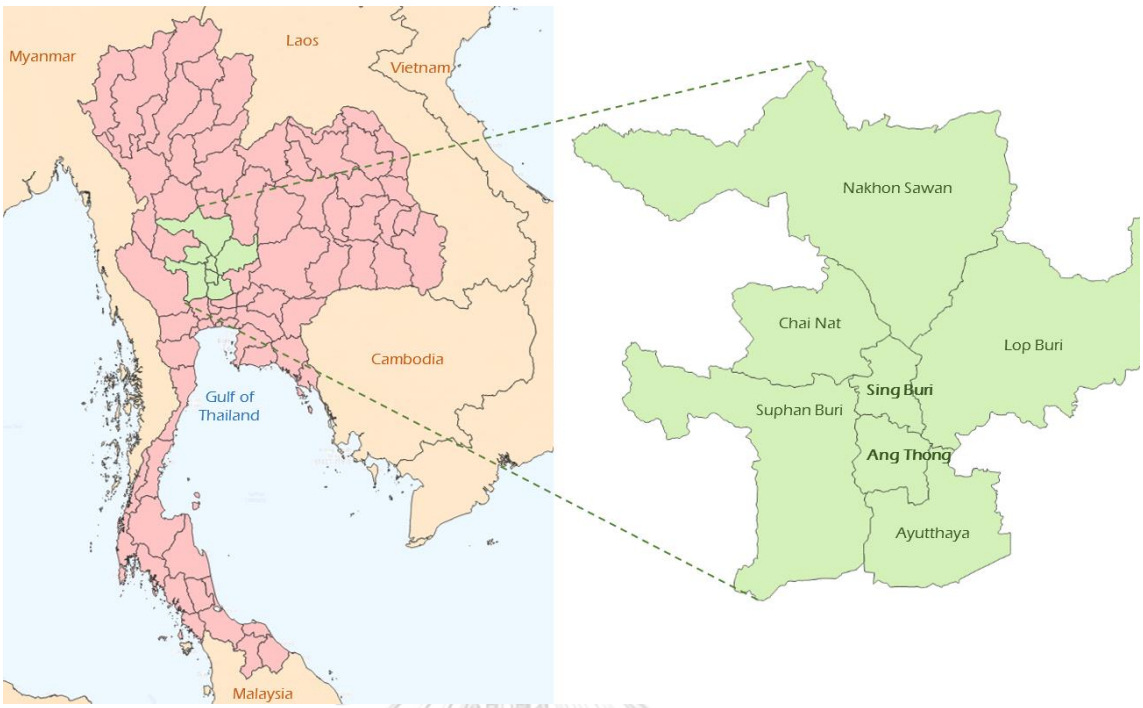


Figure 6: Map of Thailand indicating the location of Sing Buri and Ang Thong Provinces, and surrounding Provinces.

It was created using software QGIS 3.6.1 with data extracted from the GADM database.

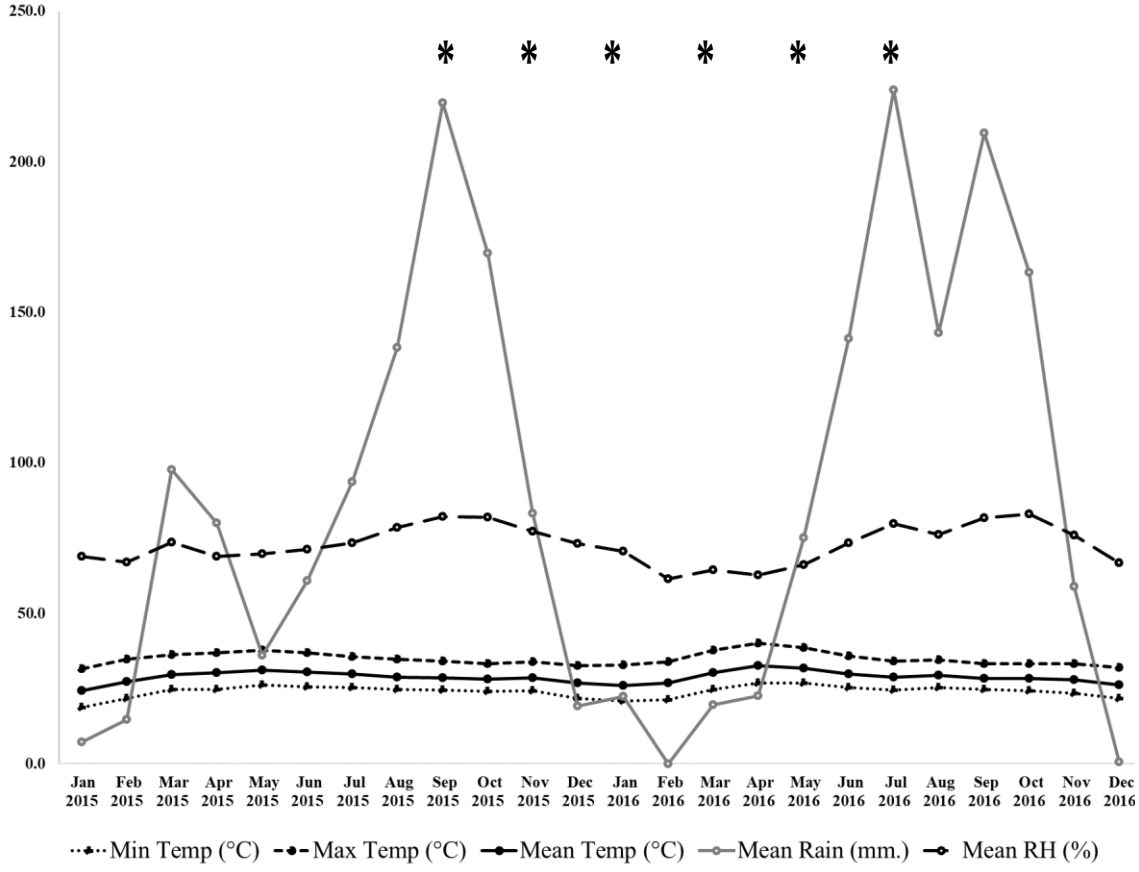


Figure 7: Average values of meteorological data collected from weather stations in four Thai provinces (Ayutthaya, Chinat, Lopburi, and Suphanburi Provinces) surrounding the study sites in Sing Buri and Ang Thong Provinces (* indicates the collection times of the mosquito samples).

2.3 Results

Mosquito distribution in duck farms in Thailand

Mosquitoes were collected from two duck farms in Sing Buri and Ang Thong Provinces, central Thailand between September 2015 and July 2016. They were collected on six occasions at two month-intervals in September 2015, November 2015, January 2016, March 2016, May 2016, and July 2016. The species and number of collected mosquitoes are shown in Table 1. A total of 30,841 mosquitoes was collected and identified to seven species (*Anopheles* (*An*.) *barbirostris*, *An. stephensi*, *Cx. gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus, Mansonia* (*Ma*.) *annulifera*, and *Ma. uniformis*). The most commonly collected species from each duck farm and each collection time was *Cx. tritaeniorhynchus*.

DTMUV detection in mosquitoes collected from duck farms in Thailand

A total of 273 mosquito pools were examined for DTMUV infection by RT-PCR using specific primers (Tables 1-2), and the PCR amplicon from each positive sample was sequenced and analyzed. Only one mosquito pool of *Cx. tritaeniorhynchus* collected from Farm 1 in Sing Buri Province was positive for DTMUV infection, representing 0.52% (1/192) of *Cx. tritaeniorhynchus* pools or 0.37% (1/273) for all tested mosquito pools in this study.

Table 1: Species and number of mosquitoes collected from the duck farms in Sing Buri and Ang Thong Provinces, Thailand

		Farm 1 Sing Buri	Farm 2 Sing Buri	Farm 3 Ang Thong	Farm 4 Ang Thong
September 2015	*Anopheles barbirostris*	18 (0/1)	46 (0/1)	5 (0/1)	40 (0/1)
	Anopleles stephensi	0	1 (0/1)	1 (0/1)	0
	Culex gelidus	93 (0/2)	157 (0/4)	57 (0/1)	560 (0/8)
	Culex tritaeniorhynchus	1,619 (0/16)	8,292 (0/20)	1,106 (0/15)	3,986 (0/20)
	Mansonia annulifera	0	1 (0/1)	8 (0/1)	38 (0/1)
	Mansonia uniformis	0	36 (0/1)	2 (0/1)	29 (0/1)
	Total	1,730 (0/19)	8,533 (0/28)	1,179 (0/20)	4,653 (0/31)
November 2015	*Anopheles barbirostris*	45 (0/1)	354 (0/3)	26 (0/1)	74 (0/1)
	Anopleles stephensi	1 (0/1)	0	1 (0/1)	1 (0/1)
	Culex gelidus	86 (0/2)	63 (0/2)	8 (0/1)	50 (0/1)
	Culex tritaeniorhynchus	1,281 **(1/16)**	4,494 (0/20)	787 (0/14)	713 (0/14)
	Mansonia annulifera	0	0	14 (0/1)	10 (0/1)
	Mansonia uniformis	40 (0/1)	15 (0/1)	55 (0/1)	78 (0/1)
	Total	1,453 **(1/21)**	4,926 (0/26)	891 (0/19)	926 (0/19)
January 2016	*Anopheles barbirostris*	38 (0/1)	7 (0/1)	3 (0/1)	7 (0/1)
	Culex gelidus	3 (0/1)	2 (0/1)	0	1 (0/1)
	Culex quinquefasciatus	0	0	0	1 (0/1)
	Culex tritaeniorhynchus	497 (0/9)	113 (0/2)	18 (0/1)	27 (0/1)
	Mansonia uniformis	8 (0/1)	20 (0/1)	2 (0/1)	0
	Total	546 (0/12)	142 (0/5)	23 (0/3)	36 (0/4)

Table 1: (Cont.) Species and number of mosquitoes collected from the duck farms in Sing Buri and Ang Thong Provinces, Thailand

		Farm 1 Sing Buri	Farm 2 Sing Buri	Farm 3 Ang Thong	Farm 4 Ang Thong
March 2016	*Culex gelidus*	1 (0/1)	0	8 (0/1)	0
	Culex tritaeniorhynchus	8 (0/1)	7 (0/1)	19 (0/1)	2 (0/1)
	Mansonia uniformis	0	0	1 (0/1)	0
	Total	9 (0/2)	7 (0/1)	28 (0/3)	2 (0/1)
May 2016	*Culex gelidus*	1 (0/1)	0	1 (0/1)	1 (0/1)
	Culex tritaeniorhynchus	21 (0/1)	11 (0/1)	12 (0/1)	0
	Mansonia annulifera	0	0	1 (0/1)	0
	Total	22 (0/2)	11 (0/1)	14 (0/3)	1 (0/1)
July 2016	*Anopheles barbirostris*	5 (0/1)	9 (0/1)	13 (0/1)	10 (0/1)
	Culex gelidus	48 (0/1)	136 (0/3)	6 (0/1)	8 (0/1)
	Culex tritaeniorhynchus	681 (0/10)	3,409 (0/15)	62 (0/2)	1,313 (0/10)
	Mansonia annulifera	1 (0/1)	2 (0/1)	0	1 (0/1)
	Mansonia uniformis	0	1 (0/1)	0	4 (0/1)
	Total	735 (0/13)	3,557 (0/21)	81 (0/4)	1,336 (0/14)

Table 2: Summary of DTMUV infected and tested mosquito pools collected from each duck farm in Sing Buri and Ang Thong Provinces, Thailand

	Farm 1 Sing Buri	Farm 2 Sing Buri	Farm 3 Ang Thong	Farm 4 Ang Thong	Total
Anopheles barbirostris	0/4	0/6	0/4	0/4	0/18
Anopheles stephensi	0/1	0/1	0/2	0/1	0/5
Culex gelidus	0/8	0/10	0/5	0/12	0/35
Culex quinquefasciatus	0	0	0	0/1	0/1
Culex tritaeniorhynchus	**1/53**	0/59	0/34	0/46	1/192
Mansonia annulifera	0/1	0/2	0/3	0/3	0/9
Mansonia uniformis	0/2	0/4	0/4	0/3	0/13
Total	**1/69**	0/82	0/52	0/70	1/273

Phylogenetic and genetic analyses of the mosquito-derived Thai DTMUV

To genetically characterize the Thai DTMUV isolated from *Culex* mosquito in 2015, the virus designated as *Culex*/TH/CU_2015 was subjected to whole genome sequencing. The whole genome length of *Culex*/TH/CU_2015 is 10,278 nucleotides, encoding 3,425 amino acids, which is similar to that of the previously reported DTMUV isolates (Ninvilai et al., 2018, Thontiravong et al., 2015a). Phylogenetic analysis of the polyprotein gene sequence demonstrated that *Culex*/TH/CU_2015 was grouped into subcluster 2.1 and most closely related to the 2013 Thai DTMUVs (98.9% nucleotide and 99.8% amino acid identities) (Figure 8a and Table 3). However, the polyprotein gene sequence of *Culex*/TH/CU_2015 shared only 88.2% and 97.1% nucleotide identities with the mosquito derived TMUV isolated from *Cx. tritaeniorhynchus* in Malaysia (MM1775) and the mosquito derived DTMUV isolated from *Cx. pipiens* in China (SDMS), respectively (Table 3). In addition, the phylogenetic analysis of the complete E gene of *Culex*/TH/CU_2015 showed similar finding with that of polyprotein gene (Figure 8b and Table 3). This virus had the highest identity with Thai DTMUVs isolated from ducks during 2015-2016 (99.1-99.5% nucleotide and 99.0-99.8% amino acid identities), while it shared only 96.9% and 89.6% nucleotide identities with Chinese DTMUVs and Malaysian DTMUVs, respectively (Table 3).

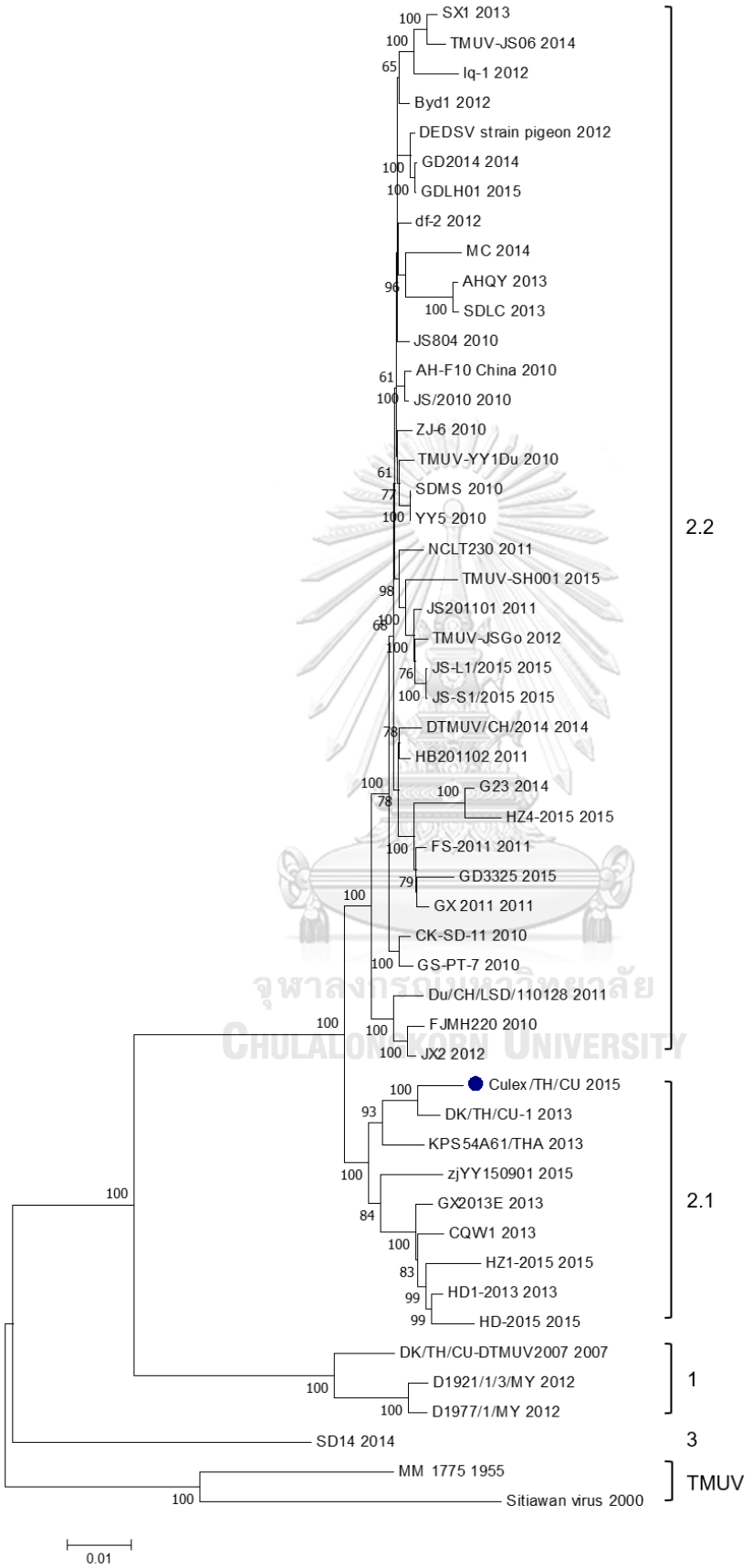
Table 3: Comparison of the nucleotide and amino acid identities of the polyprotein and E genes of a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) with reference DTMUVs and TMUVs

Reference virus	Accession No.	Host	Location	Year of collection	Polyprotein gene		E gene	
					%Nucleotide identity	%Amino acid identity	%Nucleotide identity	%Amino acid identity
MM1775	JX477685	*Culex tritaeniorhynchus*	Malaysia	1995	88.2	96.7	88.2	96.2
Sitiawan virus	JX477686	Chicken	Malaysia	2000	87.1	96.4	86.6	96.2
Cluster 1								
DK/TH/CU-DTMUV2007	MF621927	Duck	Thailand	2007	91.7	97.7	91.9	97.8
D1921/1/3/MY	KX097990	Duck	Malaysia	2012	91.2	97.5	89.6	96.4
D1977/1/MY	KX097989	Duck	Malaysia	2012	91.2	97.5	89.6	96.6
Cluster 2.1								
DK/TH/CU-1	KR061333	Duck	Thailand	2013	98.9	99.8	98.9	99.4
KPS54A61	KF573582	Duck	Thailand	2013	98.1	99.4	97.9	99.4
CQW1	KM233707	Duck	China	2013	97.7	99.6	97.5	99.4
HD-2015	KX686572	Duck	China	2015	97.3	99.3	96.9	98.8
HZ1-2015	KX686570	Duck	China	2015	97.2	99.2	96.9	98.8
zjYY150901	MF522174	Duck	China	2015	97.3	99.4	97.1	98.8
DK/TH/CU-10	MK276415	Duck	Thailand	2015	ND*	-	99.2	99.6
DK/TH/CU-9	MK276414	Duck	Thailand	2015	-	-	99.5	99.6
DK/TH/CU-13	MK276416	Duck	Thailand	2015	-	-	97.9	99.0
DK/TH/CU-48	MK276426	Duck	Thailand	2016	-	-	99.5	99.8
DK/TH/CU-85	MK276431	Duck	Thailand	2016	-	-	99.1	99.6
DK/TH/CU-71	MK276429	Duck	Thailand	2016	-	-	98.9	99.4
DK/TH/CU-164	MK276456	Duck	Thailand	2017	-	-	99.4	99.6
DK/TH/CU-170	MK276457	Duck	Thailand	2017	-	-	99.3	99.6
DK/TH/CU-172	MK276458	Duck	Thailand	2017	-	-	99.1	99.6
DK/TH/CU-175	MK276459	Duck	Thailand	2017	-	-	98.7	99.6

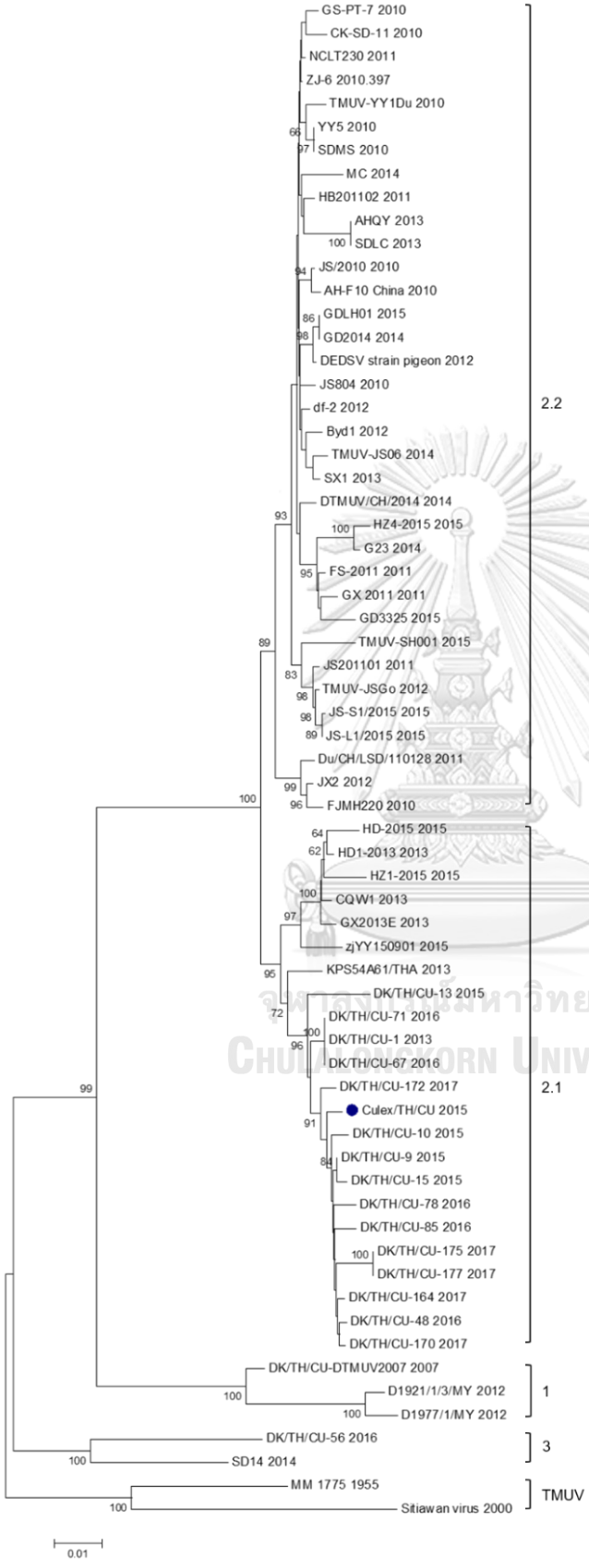
ND* = not determined since polyprotein gene sequence is not available.

Table 3: (Cont.) Comparison of the nucleotide and amino acid identities of the polyprotein and E genes of a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) with reference DTMUVs and TMUVs

Reference virus	Accession No.	Host	Location	Year of collection	Polyprotein gene		E gene	
					%Nucleotide identity	%Amino acid identity	%Nucleotide identity	%Amino acid identity
Cluster 2.2								
JS804	JF895923	Goose	China	2010	97.2	99.3	97.4	98.4
CK-SD-11	JQ627862	Chicken	China	2010	97.1	99.3	97	98.4
SDMS	KC333867	*Culex pipiens*	China	2010	97.1	96.4	97.2	98.8
AH-F10	KM102539	Duck	China	2010	97.1	99.4	97.4	99.0
Du/CH/LSD/110128	KC136210	Duck	China	2011	97.0	99.4	97.1	98.6
JS201101	KY623426	Duck	China	2011	97.0	99.4	97.0	99.0
HB201102	KY623427	Duck	China	2011	97.2	99.4	97.2	98.8
TMUV-JSGo	AB917090	Goose	China	2012	96.9	99.4	97.0	98.8
DEDSV strain pigeon	JQ920425	Pigeon	China	2012	97.1	99.5	97.3	98.8
df-2	KJ489355	Duck	China	2012	97.1	99.5	97.4	98.8
SX1	KM066945	Chicken	China	2013	96.7	98.7	97.2	98.2
AHQY	KJ740748	Duck	China	2013	96.5	99.2	96.5	98.8
SDLC	KJ740747	Duck	China	2013	96.5	99.2	96.5	98.8
G23	KT239021	Goose	China	2014	96.5	99.4	96.5	98.8
JS06	KR869106	Chicken	China	2014	-	-	97.0	98.0
DTMUV/CH/2014	KP096415	Duck	China	2014	97.0	99.4	97.1	98.8
GD2014	KU323595	Duck	China	2014	97.1	99.4	97.2	98.6
GDLH01	KT824876	Duck	China	2015	97.1	99.4	97.2	98.6
HZ4-2015	KX686571	Duck	China	2015	96.1	98.8	96.5	98.8
TMUV-SH001	KP742476	Duck	China	2015	96.6	99.2	96.3	98.4
Cluster 3								
DK/TH/CU-56	MK276427	Duck	Thailand	2016	-	-	88.9	95.0
SD14	MH748542	Duck	China	2014	89.6	96.4	89.8	94.8



a)



b)

Figure 8: Phylogenetic analysis of a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) and selected reference strains of DTMUV and TMUV.
The phylogenetic tree was constructed using neighbour-joining (NJ) algorithm based on the nucleotide sequences of the polyprotein (10,278 bp) (a) and complete E (1,503 bp) (b) genes of a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) and selected reference strains of DTMUVs and TMUVs identified from Thailand, Malaysia, and China. A black circle indicates a mosquito-derived Thai DTMUV identified in this study (*Culex*/TH/CU_2015). A similar result was observed when applying the maximum-likelihood (ML) algorithm.

2.4 Discussion

There are several types of duck raising in Thailand that range in scale from small backyard to industrial scale farms. This study, however, only evaluated the mosquito distribution and DTMUV detection in four duck farms and not in free-grazing duck raising areas, which might have a different mosquito distribution and DTMUV detection prevalence. Note that free-grazing ducks are commonly raised and widespread in several Asian countries including Thailand. Previously, a total of 1,000 blood samples were collected from free grazing ducks in Thailand during 2008–2015 and 9.10% of them showed DTMUV neutralizing antibodies, while DTMUV seropositive ducks have been detected in Thailand since 2008 (Tunterak et al., 2018). Another serological survey of DTMUV infection conducted across 20 Provinces located in the major free-grazing duck raising areas of Thailand in 2016 revealed 30.4% of 1,200 blood samples were positive for DTMUV neutralizing antibodies and 93.3% of flocks had at least one DTMUV seropositive duck. Additionally, DTMUV seropositive ducks were observed in all Provinces tested (Tunterak et al., 2018). Ninvilai et al. (2018) also indicated the presence of DTMUV in Thai ducks since 2007,

prior to the first report of DTMUV in China in 2010. However, phylogenetic analysis of the polyprotein gene sequence revealed that this virus belonged within DTMUV cluster 1, which is genetically different from the currently circulating Thai and Chinese DTMUV that belong to cluster 2.1. Thus, DTMUV may have a high genetic diversity and have been circulating in Asia long before 2010.

Mosquitoes are important vectors for the transmission of DTMUV but the involvement of mosquito vectors in the transmission of this virus in duck farms in Thailand is still unclear. This study indicated that at least seven mosquito species from three genera were found in proximity to duck farms in Thailand, which were *An. barbirostris*, *An. stephensi*, *Cx. gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus, Ma. annulifera* and *Ma. uniformis*. The two most commonly collected species were *Cx. tritaeniorhynchus* and *Cx. gelidus*, while only one *Cx. quinquefasciatus* sample was collected from Ang Thong Province in July 2016. The highest number of collected mosquitoes was in September 2015 and then gradually decreased in November 2015, January 2016, and March 2016, and then increased in May and July 2016. Thus, the number of collected mosquitoes was related to the amount of rainfall. The average rainfall ranged from 0–223.9 mm, relative humidity from 61.5–82.2% and temperature from 26.0–32.6 °C during the study period (Figure 6).

In China, *Cx. pipiens* mosquitoes were collected during the summers of 2010–2012 in the Shandong Province of China during the outbreak of TMUV in duck farms, where 58.8% of mosquito pools tested were positive for TMUV when examined by both RT-PCR and virus isolation assays. This was the first detection of TMUV in mosquitoes in China (Tang et al., 2015). For the relation between mosquito vectors and TMUV in Thailand, a laboratory study on the vector competence of mosquitoes (F1 progeny) that were originally captured in Kamphaeng Phet Province in 2005 revealed that *Cx. vishnui* developed high viral titers after feeding on TMUV-infected chicks and transmitted the virus to naive chickens but *Cx. fuscocephala* appeared

less susceptible to infection (O'Guinn et al., 2013a). A field study in Kanchanaburi Province in 2015 found *Cx. quinquefasciatus* were the most commonly collected mosquito species near a chicken farm and 1/70 mosquito pools was positive for TMUV by RT-PCR and virus isolation. This provided potential support for TMUV transmission by this *Culex* species in a natural habitat (Nitatpattana et al., 2017). However, these TMUV were not involved or responsible for the disease outbreak in ducks in Thailand. Currently, the relationship between *Cx. tritaeniorhynchus* and DMTUV in Thailand is still limited even though this species of mosquitoes can be found all over Thailand, especially in the farming areas.

Cx. tritaeniorhynchus is the most important mosquito vector for transmission of several diseases in humans and animals and is a primary vector for Japanese encephalitis virus in Asia (Gingrich et al., 1992, Leake et al., 1986). Several studies have examined the distribution and variation of mosquitoes in human communities and animal nested areas in Thailand. The mosquito distribution in suburban sites of Bangkok during 1986–1987 found *Cx. tritaeniorhynchus* and *Cx. gelidus* were the major populations. Their abundance was high in the monsoon season (May–October), moderate in the transition period (March–April and November–December), and low in the dry season (January–February) in 1987 (Gingrich et al., 1992).

Several studies on the abundance of mosquitoes in animal nested areas, particularly avian species, have been conducted in Thailand. At a nesting colony of ardeid birds in Phitsanulok Province from July 2009 to May 2010, a total of five genera and 14 species of mosquitoes were collected, with the five most abundant species being *Cx. tritaeniorhynchus* followed by *Cx. vishnui*, *Cx. gelidus*, *An. peditaeniatus*, and *Cx. quinquefasciatus*, with peak densities in July (Changbunjong et al., 2013). Another investigation in the Asian open billed stork nesting area in Pathum Thani province, central Thailand from March 2008 to January 2009 revealed seven genera and 18 species of mosquitoes with *Cx. tritaeniorhynchus* as the most

common species in each month, except for November when *Cx. gelidus* was the most common species (Tiawsirisup et al., 2012). Mosquito distribution were also studied in a bat cave and its surrounding area in Lopburi province, central Thailand from May 2009 to April 2010, where five genera and eight species of mosquitoes were collected from the bat cave and eight genera with 16 species of mosquitoes collected from the area close to the bat cave. The dominant species of the collected mosquitoes from these two areas were *Cx. quinquefasciatus, Cx. tritaeniorhynchus*, and *Armigeres subalbatus*.

The finding of this study is the first report that indicates the detection of DTMUV in *Cx. tritaeniorhynchus* collected from a duck farm in Sing Buri Province, Thailand. The polyprotein gene sequence revealed that it was closely related to the DTMUV isolated from ducks in Thailand, which might indicate the possible role of this mosquito species in the transmission cycle of DTMUV in duck farms in Thailand. However, more studies about vector competence of this mosquito for DTMUV in laboratory conditions are required to indicate the virus infection, dissemination, and horizontal or vertical transmission in mosquitoes. Further exploration of DTMUV epidemics should also be performed by collecting mosquitoes from different types of duck raising in Thailand, since the mosquito distribution and role of each mosquito species in each type of duck raising might be different.

CHAPTER III

Vector competence of *Culex tritaeniorhynchus* and *Culex quinquefasciatus* for duck Tembusu virus

Manuscript in preparation

Jitra Sanisuriwong, Aunyaratana Thontiravong, Sonthaya Tiawsirisup

3.1 Introduction

Flaviviruses are important arthropod-borne viruses found in both humans and animals. The transmission cycle of these viruses involves arthropod vectors and reservoir vertebrate hosts. Flaviviruses can be divided into two clades including the vector-borne virus and the others with no known vector (Chambers et al., 1990). The vector-borne virus clade can be subdivided into a mosquito-borne and a tick-borne clade. While the mosquito clade is divided into *Culex* which is associated with encephalitis diseases and *Aedes* which are associated with hemorrhagic diseases. Non-vectored flaviviruses are probably maintained in nature by animal to animal transmission via saliva and urinary shedding (Chen and Wilson, 2005).

Flaviviruses cause hemorrhagic disease, encephalitis, fever, paralysis, and jaundice in humans. Humans and animals can be infected after bitten by infected arthropod vectors (Chambers et al., 1990). Important flaviviruses include yellow fever virus, dengue virus, West Nile virus, St. Louis encephalitis virus, Japanese encephalitis virus, tick borne encephalitis virus, Kyasaner forest disease virus, Omsk hemorrhagic fever virus, Tembusu virus, and Zika virus. However, important flaviviruses in birds are West Nile, Israel turkey encephalitis, Bagaza, Usutu, and Tembusu virus which are transmitted through mosquito biting and caused a specified pathogenicity for birds.

These viruses cause severe symptoms (i.e., neurological disorder, egg production drop and/or mortalities) among avian hosts (Benzarti et al., 2019). Japanese encephalitis, West Nile, and Tembusu virus are associated with *Culex* mosquito vectors that are more likely to take blood meals from poultry and cause infections in the nervous system (Gould and Solomon, 2008).

Duck Tembusu virus (DTMUV) is an emerging infectious disease in ducks. Infected ducks showed consistently acute anorexia, diarrhea, egg production drop, ovary-oviduct disease, and severe neurologic disorders. It is a single stranded positive-sense RNA virus belonging to the genus *Flavivirus* of the family *Flaviviridae* (Su et al., 2011). DTMUV is a mosquito-borne flavivirus, which various species of mosquitoes are important vectors for the transmission cycle of this virus in nature (Sanisuriwong et al., 2020, Tang et al., 2015).

Transmission cycles of vector-borne viruses are the relationship between the virus, invertebrate hosts (vector), and vertebrate hosts (humans and/or animals). Vector competence is affected by both intrinsic and extrinsic factors and it is defined as the capacity of a mosquito to acquire the pathogen and support its transmission (Souza-Neto et al., 2019). Intrinsic factors include mosquito host preferences and the ability of mosquitoes to become infected with a virus after ingestion of an infective blood meal and to transmit virus by bite or through the egg. While, extrinsic factors include density and composition of both male and female mosquitoes, vertebrate populations, and environmental conditions.

In 1970s, Tembusu virus (TMUV) was first isolated from *Culex* (*Cx.*) *tritaeniorhynchus* and *Cx. gelidus* mosquitoes in Malaysia (Platt et al., 1975). During 1982-2002, TMUVs were detected from *Cx. tritaeniorhynchus*, *Cx. gelidus*, and *Cx. vishnui* mosquitoes collected from Kamphaeng Phet and Chiang Mai provinces, Thailand (Leake et al., 1986, Pandey et al., 1999) and from *Cx. quinquefasciatus* mosquitoes collected from Kanchanaburi province, Thailand (Nitatpattana et al.,

2017). Later in 2010, DTMUVs were reported in China and it was first reported in Thailand in 2013 (Cao et al., 2011, Chakritbudsabong et al., 2015, Su et al., 2011, Thontiravong et al., 2015a). During 2015-2017, DTMUV were detected from various duck farms in several provinces in Thailand (Ninvilai et al., 2019, Tunterak et al., 2018). Retrospective study; however; indicated that DTMUV was presented in Thailand since 2007 (Ninvilai et al., 2018). DTMUV was first detected in *Cx. tritaeniorhynchus* mosquitoes collected from duck farm in Sing Buri province, Thailand in 2015 (Sanisuriwong et al., 2020).

Cx. tritaeniorhynchus is the most important mosquito vector to transmit several diseases in humans and animals (e.g., Japanese encephalitis virus). *Cx. tritaeniorhynchus* and *Cx. gelidus* are the major population of the mosquitoes found in several areas include parks and duck farms in Thailand (Sanisuriwong et al., 2020, Tiawsirisup and Nuchprayoon, 2010, Tiawsirisup et al., 2008). On the other hands, *Cx. quinquefasciatus* are dominant mosquitoes found in urban and semi-urban areas in Thailand (Pipitgool et al., 1998). The role of mosquitoes in the transmission of DTMUV in Thailand remains unclear and the studies about mosquito vectors for DTMUV in Thailand are limited. Therefore, the vector competence of *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* for DTMUV transmission was examined in this study.

3.2 Materials and Methods

Mosquitoes

Field-collected *Culex (Cx.) tritaeniorhynchus* and laboratory-reared *Cx. quinquefasciatus* mosquitoes were used in this study. *Cx. tritaeniorhynchus* were collected from rice field in Bangkok area, Thailand using CDC light traps with dry ice.

The mosquitoes were morphologically identified (Rattanarithikul and Panthusiri, 1994) and *Cx. tritaeniorhynchus* were separated from other species of mosquitoes using aspirator. Approximately 10% of collected *Cx. tritaeniorhynchus* were randomly separated and tested for duck Tembusu virus (DTMUV) infection by using RT-PCR. If there was no DTMUV infection in these tested mosquitoes, the left of them will be used for the vector competence study in the laboratory. Field-collected *Cx. tritaeniorhynchus* were used in this study because there was no colony of these mosquitoes in our laboratory and we also would like to study in the mosquitoes which have similar biology and physiology with natural mosquitoes in Thailand.

Laboratory-reared *Cx. quinquefasciatus* were kindly provided by the Department of Medical Sciences, Ministry of Public Health, Thailand. They have been maintained at 25ºC and 80± 5% relative humidity with 12:12 hr light/dark cycle in the insectary, the Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand for more than 10 generations. This study was conducted in compliance with the Chulalongkorn University Animal Care and Use Committee, Thailand (IACUC Institute No. B 2559/00018.010: Animal Use Protocol No. 1731070).

Virus

The DK/TH/CU-1 strain of DTMUV was kindly provided by the Virology Unit, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. It was originally isolated from an infected duck in Nakhon Ratchasima province, Thailand. This virus was used as a positive control for reverse transcription polymerase chain reaction (RT-PCR).

Reverse transcription polymerase reaction (RT-PCR) for DTMUV detection

The DTMUV detection was performed by RT-PCR amplification of the viral NS5 gene. The specific primers for this reaction were forward 5'-GCAGGTTCAGGAAGTGAGAGG-3' and reverse 5'-GGATTGTCTTGGTCATAATGCC-3' to yield a PCR product of 617 bp (Thontiravong et al., 2015a). Viral nucleic was extracted from mosquitoes using the viral nucleic acid extraction kit II (Geneaid, Taiwan). Total RNA samples were amplified using the SuperScript® III One-Step RT-PCR with Platinum® Taq DNA Polymerase (Invitrogen, USA) in a 25 µL volume comprised of 1.5 µL of RNA, 12.5 µL of 2x reaction mix (0.4 mM dNTP, 3.3 mM $MgSO_4$) (Invitrogen, USA), 1 µL of forward and reverse primer (10 µM), 1 µL of SuperScript III RT/Platinum Taq Mix (Invitrogen, USA), and 8 µL of distilled water (Invitrogen, USA). The thermal cycling was performed at 48°C for 45 min, 94°C for 3 min and then 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and then a final 72°C for 10 min using a Perkin Elmer Cetus 9600 thermocycler (Perkin Elmer, Waltham, USA). The reaction was then mixed with 6 µL of loading buffer (BlueJuice Gel Loading Buffer, Invitrogen, USA) and resolved by 2% (w/v) agarose gel (UltraPure, Invitrogen, USA) electrophoresis.

Experimental design

DTMUV infection, dissemination, and transmission were examined in field-collected *Cx. tritaeniorhynchus* and laboratory-reared *Cx. quinquefasciatus* mosquitoes. A group of 100 mosquitoes was starved from water and sucrose for 24 hr and allowed to feed on DMTUV infected blood meals using artificial membrane

feeder. The blood meal consists of DTMUV, 20% fetal bovine serum, 1% sucrose, and 70% (v/v) packed sheep erythrocytes. The feeder was placed on screened lid of the mosquito carton. Four groups of *Cx. tritaeniorhynchus* were allowed to feed on different titers of DTMUV which were 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL and two groups of *Cx. quinquefasciatus* were allowed to feed on two titers of DTMUV which were 10^4 and 10^5 $TCID_{50}$/mL for 45 min. The fully-fed mosquitoes were kept for 14 days and examined for the virus infection, dissemination, and transmission. DTMUV in body, legs and wings, and saliva of the mosquito were examined to indicate the virus infection, dissemination, and transmission, respectively. Body, legs and wings, and saliva samples from each mosquito were examined separately.

The mosquito was immobilized by keeping at 4°C for 10 min, wings and legs were removed and placed in a centrifuge tube containing 300 µL of 10% FBS in Minimal Essential Medium (MEM) for the detection of virus dissemination. The mosquito proboscis was then inserted into a 10-µL-capillary tube containing 5% sucrose and 0.5% fetal bovine serum (FBS) in phosphate buffer saline (PBS) about 20 min for saliva collection (Tiawsirisup et al., 2005). Each saliva sample was transferred into a separate centrifuge tube containing 100 µL of 10% FBS in MEM for the detection of virus transmission. After 20 min of salivation, the mosquito's body was removed from the capillary tube and placed in a separate centrifuge tube containing 300 µL of 10% FBS in MEM for the detection of virus infection. Total RNA was extracted from each sample by using viral nucleic acid extraction kit II (Geneaid, Taiwan). All samples were tested for the RNA of DTMUV by using RT-PCR technique.

3.3 Results

Four groups of *Culex (Cx.) tritaeniorhynchus* were allowed to feed on four levels of DTMUV which were 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL and two groups of *Cx.*

quinquefasciatus were allowed to feed on two levels of DTMUV which were 10^4 and 10^5 $TCID_{50}$/mL. DTMUV infection in *Cx. tritaeniorhynchus* were 1.6%, 10.2%, 35.8%, and 59.3% after feeding on 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively. DTMUV dissemination and transmission in *Cx. tritaeniorhynchus* were 20.3% and 16.9% after feeding on 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively. However, there was no virus dissemination and transmission in *Cx. tritaeniorhynchus* after feeding on 10^2, 10^3, and 10^4 $TCID_{50}$/mL of DTMUV in the blood meals. DTMUV infection in *Cx. quinquefasciatus* were only 2.5% and 2.3% after feeding on 10^4 and 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively; however, there was no virus dissemination and transmission found in all tested mosquitoes. Duck Tembusu virus (DTMUV) infection, dissemination, and transmission by *Culex tritaeniorhynchus* and *Culex quinquefasciatus* on day 14 after feeding on DTMUV infected blood meal showed in table 4 and 5.

Table 4: Duck Tembusu virus (DTMUV) infection, dissemination, and transmission by *Culex tritaeniorhynchus* on day 14 after feeding on DTMUV infected blood meal

DTMUV titer in blood meal ($TCID_{50}$/mL)	No. mosquitoes tested	Percent Infection* (95% CI)	Percent Dissemination** (95% CI)	Percent Transmission*** (95% CI)
10^2	60	1.6[a] (0, 5)	0[a]	0[a]
10^3	49	10.2[a] (1, 19)	0[a]	0[a]
10^4	53	35.8[a] (23, 49)	0[a]	0[a]
10^5	59	59.3[b] (46, 72)	20.3[b] (10, 31)	16.9[b] (7, 27)

*Percent infection indicated by the percentage of blood-fed mosquitoes with DTMUV present in their bodies

**Percent dissemination indicated by the percentage of blood-fed mosquitoes with DTMUV in legs and wings

***Percent transmission indicated by the percentage of blood-fed mosquitoes that imbibed and deposited DTMUV into feeding solution contained in capillary tubes

Values followed by the same letter within percent infection, percent dissemination, and percent transmission are not significantly different ($P \geq 0.05$).

The percent infection in *Cx. tritaeniorhynchus* mosquitoes was higher than percent dissemination and percent transmission on day 14 after feeding on 10^3, and 10^4 $TCID_{50}$/mL of DTMUV in the blood meals ($P<0.05$).

Table 5: Duck Tembusu virus (DTMUV) infection, dissemination, and transmission by *Culex quinquefasciatus* on day 14 after feeding on DTMUV infected blood meal

DTMUV titer in blood meal ($TCID_{50}$/mL)	No. mosquitoes tested	Percent Infection* (95% CI)	Percent Dissemination**	Percent Transmission***
10^4	40	2.5 (0, 8)	0	0
10^5	44	2.3 (0, 7)	0	0

*Percent infection indicated by the percentage of blood-fed mosquitoes with DTMUV present in their bodies

**Percent dissemination indicated by the percentage of blood-fed mosquitoes with DTMUV in legs and wings

***Percent transmission indicated by the percentage of blood-fed mosquitoes that imbibed and deposited DTMUV into feeding solution contained in capillary tubes

3.4 Discussion

Duck Tembusu virus (DTMUV) is evaluated as important contagious virus in duck. It is a mosquito-borne virus; however; horizontal transmissions through aerosol, direct contact, ingestion or inhalation of contaminated materials are also occurred and considered as important route of transmission (Li et al., 2015). DTMUV was also isolated from duck embryos which might indicate the vertical transmission of this virus from breeding ducks to ducklings (Zhang et al., 2015).

The potential of *Culex (Cx.) tritaeniorhynchus* and *Cx. quinquefasciatus* to transmit DTMUV was examined in this study. The finding from this study demonstrated the ability of DTMUV to infect, disseminate, and transmit in *Cx. tritaeniorhynchus*; however, there was no DTMUV dissemination and transmission found in *Cx. quinquefasciatus* mosquitoes. In nature, Tembusu virus (TMUV) is primarily associated with *Cx. tritaeniorhynchus* and *Cx. vishnui*, which the morphological characteristics are very similar, and both of them are considered important vectors of Japanese Encephalitis virus. *Cx. tritaeniorhynchus* is distributed across South-East Asia and adjacent tropical areas, Middle East, Africa, and has recently been reported in Europe (Longbottom et al., 2017). The distribution of *Cx. vishnui* occurs across the Central parts of the Asia-Pacific region: eastern and southern India, Bangladesh, Myanmar, Laos, Thailand, Vietnam, Malaysia, and Indonesia (Samy et al., 2018). A larval stage of *Cx. tritaeniorhynchus* and *Cx. vishnui* prefers to live in groundwater (e.g., puddles, rice paddies, ponds, and ditches) and an adult stage likes to feed on cows and pigs; however, they also feed on humans, wild birds, and poultry (i.e., domestic chickens, turkeys, and ducks) depends on the integrity of the host.

The distribution of *Cx. quinquefasciatus* occurs across southern North America, South America, sub-Saharan Africa, south Asia, and most of Australia and New Zealand (Samy et al., 2016). They lay eggs in both natural and polluted water in

various containers (e.g., bird baths and old tires) and the larval stage an also live in both clean and wastewater (Grech et al., 2013). An adult stage likes to feed at night. They are potential vectors of West Nile virus, St. Louis encephalitis virus, lymphatic filariasis, and canine dirofilariasis (Tiawsirisup and Nithiuthai, 2006).

The surveillance in 1982 in Thailand indicated the detection of TMUV in *Cx. tritaeniorynchus*, *Cx. vishnui,* and *Cx. gelidus* with the minimum infection rate of 0.04, 0.2, and 0.1, respectively (Leake et al., 1986). Another study in 2002 in Thailand also revealed the detection of TMUV in *Cx. vishnui* (O'Guinn et al., 2013a). The study in the laboratory condition found TMUV development in *Cx. vishnui* with high viral titers and ready to transmit the virus. However, *Cx. fuscocephala* appeared less susceptible to infection and there was no virus transmission by bite, indicating a salivary gland barrier in this mosquito (O'Guinn et al., 2013a). Moreover, Nitatpattana et al. (2017) reported the detection of TMUV in *Cx. quinquefasciatus* in Thailand. The relation in TMUV-mosquito-avian transmission cycle in nature remains unclear; however, vector competence study showed that the field mosquitoes can be efficient vectors for spreading the virus (O'Guinn et al., 2013a).

During the outbreaks of TMUV in duck farms in China in 2010-2012, TMUV was first isolated from *Culex* mosquitoes collected in Shandong province, China (Tang et al., 2015). DTMUV was also detected in *Cx. tritaeniorhynchus* mosquitoes collected from duck farm in Sing Buri province, Thailand in 2015 (Sanisuriwong et al., 2020). Another study on mosquito vector competence for the virus that caused duck egg-drop syndrome in China which this virus was called Baiyangdian virus (BYDV) by the researchers. They found that *Cx. tritaeniorhynchus, Cx. pipiens pallens, Cx. pipiens quinquefasciatus*, and *Aedes albopictus* can become infected with BYD-1 virus and the transmission study indicated the abilities of *Cx. pipiens quinquefasciatus* and *Cx. tritaeniorhynchus* to transmit the virus (Guo et al., 2020).

This study evaluated the vector competence of *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes to infect and transmit DTMUV in Thailand. The results show that *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* can become infected with DTMUV after feeding on different virus titers in infected blood meals using artificial membrane feeder. DTMUV infection in *Cx. tritaeniorhynchus* were 1.6%, 10.2%, 35.8%, and 59.3% after feeding on 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively while DTMUV infection in *Cx. quinquefasciatus* were only 2.5% and 2.3% after feeding on 10^4 and 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively. DTMUV dissemination and transmission in *Cx. tritaeniorhynchus* were 20.3% and 16.9% after feeding on 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively while there was no virus dissemination and transmission in *Cx. quinquefasciatus* found in this study. These findings indicated possible role of *Cx. tritaeniorhynchus* in DTMUV transmission cycle in duck farms in Thailand. However, more study on transovarial transmission of DTMUV in *Cx. tritaeniorhynchus* need to be investigated to indicate the maintenance of DMTUV in *Cx. tritaeniorhynchus* mosquito population in Thailand.

CHAPTER IV

Transovarial transmission of duck Tembusu virus in *Culex tritaeniorhynchus* mosquitoes

Manuscript in preparation

Jitra Sanisuriwong, Aunyaratana Thontiravong, Sonthaya Tiawsirisup

4.1 Introduction

Duck Tembusu virus (DTMUV) is an emerging infectious disease in ducks described in China, Malaysia and Thailand. It is a single-stranded positive-sense RNA virus belonging to the Ntaya virus group of the *Flavivirus* genus and *Flaviviridae* family. Important flaviviruses include yellow fever, dengue, West Nile, St. Louis encephalitis, Japanese encephalitis, tick-borne encephalitis, Kyasaner Forest disease, Omsk hemorrhagic fever, Tembusu, and Zika virus. Japanese encephalitis, Tembusu, and West Nile virus are associated with *Culex* mosquito vectors that are more likely to take blood meals from poultry and cause nervous system infection (Tiawsirisup et al., 2004). While yellow fever and dengue virus are associated with *Aedes* mosquito vectors that are more likely to feed on mammals and humans. Both yellow fever and dengue virus cause vascular system infection. Moreover, some flaviviruses were transmitted by ticks and cause hemorrhagic or neurological disease (Gould and Solomon, 2008). The majority of the *Flavivirus* genus is horizontally transmitted between vertebrate hosts and vectors (e.g., mosquitoes and ticks).

Mosquito-borne flaviviruses are transmitted in nature and can be maintained in the environment. The transmission between mosquito vectors and vertebrate hosts is called horizontal transmission and causes diseases in humans and animals.

Culex mosquitoes are the important vectors for Japanese Encephalitis virus, West Nile virus, Bagaza virus, Tembusu virus, and other arthropod-borne viruses (i.e., Zika virus). In addition, mosquitoes are important vectors for the spreading of various disease and the existing of the virus in nature.

Transovarial transmission is the transmission of an infectious agent from parent mosquitoes to eggs and infected eggs develop to infected adult mosquitoes. Therefore, the infected adult mosquitoes can infect other humans or animals. Transovarial transmission is an important transmission route within mosquito population in nature. Several *Flavivirus* genera used transovarial transmission as an important route to maintain themselves in nature. West Nile, Japanese encephalitis, Zika virus and Bagaza virus can transmit through transovarial transmission in *Culex (Cx.) vishnui*, *Cx. tritaeniorhynchus*, *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus,* respectively (Mishra and Mourya, 2001, Phumee et al., 2019, Rosen et al., 1980, Sudeep et al., 2013).

Tembusu virus (TMUV), the most closely related virus to DTMUV, was first isolated from *Cx. tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia in 1955, while DTMUV was first identified as the causative agent of an egg-drop syndrome, neurological signs or death in infected egg-laying and breeder ducks in China in 2010 (Cao et al., 2011, Su et al., 2011). For DTMUV infection in ducks in Thailand, a severe infectious disease in broiler and layer ducks has emerged since 2013. The outbreaks have been reported from duck farms in various major duck production areas in Thailand including Sing Buri and Ang Thong Provinces (Ninvilai et al., 2018, Ninvilai et al., 2019, Tunterak et al., 2018). DTMUV is mosquito-borne flavivirus, where various species of mosquitoes are important vectors in the transmission cycle of this virus in nature.

DTMUV was first detected in *Cx. pipiens* mosquitoes collected from Shandong province, China in 2010–2012 (Tang et al., 2015) and was first detected in *Cx.*

tritaeniorynchus mosquitoes collected from a duck farm in Sing Buri province, Thailand in 2015 (Sanisuriwong et al., 2020). However, the development of this virus in *Cx. tritaeniorynchus* and the exact role of *Cx. tritaeniorynchus* in the ecology of DTMUV in Thailand remain unclear. Therefore, this study was conducted to examine the transovarial transmission of DTMUV in *Cx. tritaeniorhynchus* in the laboratory condition. The findings from this study will explain the involvement of this mosquito in the transmission cycle and ecology of this virus and lead to the planning for disease prevention and control in duck farms in Thailand.

4.2 Materials and Methods

Mosquitoes

Transovarial transmission of duck Tembusu virus (DTMUV) in *Culex (Cx.) tritaeniorhynchus* mosquitoes was investigated in this study. Field-collected *Cx. tritaeniorhynchus* were used in this study. The mosquitos were collected from rice field in Bangkok, Thailand using CDC light traps with dry ice. The mosquitoes were morphologically identified (Rattanarithikul and Panthusiri, 1994) and alive *Cx. tritaeniorhynchus* were sampled from other species of mosquitoes using mouth aspirator.

Approximately 10% of collected *Cx. tritaeniorhynchus* were randomly separated and tested for DTMUV infection by using RT-PCR. If there was no DTMUV infection in these tested mosquitoes, the remaining of them were used for the vector competence study in the laboratory. Field-collected *Cx. tritaeniorhynchus* were used in this study because we would like to study in the mosquitoes which have similar biology and physiology with mosquitoes in nature in Thailand. They were maintained at 25ºC and 80± 5% relative humidity with 12:12 hr light/dark cycle in the insectary,

the Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. This study was conducted in compliance with the Chulalongkorn University Animal Care and Use Committee, Thailand (IACUC Institute No. B 2559/00018.010: Animal Use Protocol No. 1731070).

Virus

The DK/TH/CU-1 strain of DTMUV was kindly provided by the Virology Unit, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. It was originally isolated from an infected duck in Nakhon Ratchasima Province, Thailand. This virus was used as a positive control for reverse transcription polymerase chain reaction (RT-PCR).

Reverse transcription polymerase reaction (RT-PCR) for DTMUV detection

The DTMUV detections in mosquito samples were performed by RT-PCR amplification of the viral NS5 gene. Viral nucleic was extracted from mosquitoes using the viral nucleic acid extraction kit II (Geneaid, Taiwan). Total RNA samples were amplified using the SuperScript® III One-Step RT-PCR with Platinum® Taq DNA Polymerase (Invitrogen, USA) in a 25 µL volume comprised of 1.5 µL of RNA, 12.5 µL of 2x reaction mix (0.4 mM dNTP, 3.3 mM $MgSO_4$) (Invitrogen, USA), 1 µL of forward and reverse primer (10 µM), 1 µL of SuperScript III RT/Platinum Taq Mix (Invitrogen, USA), and 8 µL of distilled water (Invitrogen, USA). The specific primers for this reaction were forward 5'-GCAGGTTCAGGAAGTGAGAGG-3' and reverse 5'-GGATTGTCTTGGTCATAATGCC-3' to yield a PCR product of 617 bp (Thontiravong et al., 2015a). The thermal cycling was performed at 48°C for 45 min, 94°C for 3 min and

then 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and then a final 72°C for 10 min using a Perkin Elmer Cetus 9600 thermocycler (Perkin Elmer, Waltham, USA). The reaction was then mixed with 6 µL of loading buffer (BlueJuice Gel Loading Buffer, Invitrogen, USA) and determined by 2% (w/v) agarose gel (UltraPure, Invitrogen, USA) electrophoresis.

Experimental design

Field-collected *Cx. tritaeniorhynchus* mosquitoes were used in this study. Approximately 10% of collected mosquitoes were randomly sampled and tested for DTMUV infection by using RT-PCR. If there was no DTMUV infection in these tested mosquitoes, the remaining of them were used in this study. The mosquitoes were starved from water and sucrose for 24 hr before were allowed to feed on DMTUV infected blood meals using artificial membrane feeder. The blood meal consisted of DTMUV, 20% fetal bovine serum, 1% sucrose, and 70% (v/v) packed sheep erythrocytes. The feeder was placed on screened lid of the mosquito carton. A group of 50-120 mosquitoes was allowed to feed on 10^5 $TCID_{50}$/ mL of DTMUV for 45 min. Only fully blood-fed mosquitoes were included in this study. Each blood-fed mosquito was individually kept in a plastic cup with water to allow the mosquito to lay eggs. After egg-laying, the mosquitoes were tested for DTMUV infection by using RT-PCR. Each mosquito egg raft was kept separately, and the larvae were allowed to hatch and develop into a pupal and adult stage. Total RNA was extracted from each pool of male or female adult mosquitoes that hatched from each egg raft by using viral nucleic acid extraction kit II (Geneaid, Taiwan) and tested for DTMUV by using RT-PCR.

4.3 Results

There were 9 experiments included in this study which each group consisted of 50-120 mosquitoes that were allowed to feed on 10^5 $TCID_{50}$/mL of DTMUV. A total of 750 mosquitoes were allowed to feed on DTMUV infected blood meal and there were 335 fully blood-fed mosquitoes. A total of 43 DTMUV infected and 37 non-infected female mosquitoes with eggs were included in this study. A total of 182 (75 male and 107 female) F1 mosquitoes from DTMUV infected mosquitoes and 145 (51 male and 94 female) F1 mosquitoes from non-infected mosquitoes were tested for DTMUV infection; however, all of them were negative for DTMUV (Table 6-9).

Table 6: Examination of transovarial transmission of duck Tembusu virus (DTMUV) in infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of DTMUV. Individual pool of F1 male and female mosquitoes from the same F0 female mosquito was tested for DTMUV by using RT-PCR.

Study	Blood-fed/total mosquitoes	#Infected mosquitoes with eggs	Infected/tested F1 mosquitoes	
			Male	Female
1	20/50	1	0/36	0/31
2	28/50	0	-	-
3	40/50	0	-	-
4	42/60	12	0/11	0/14
5	25/120	0	-	-
6	37/80	3	0/2	0/17
7	31/100	0	-	-
8	32/120	6	0/3	0/14
9	80/120	21	0/23	0/31
Total	335/750	43	0/75	0/107

Table 7: Number of F1 mosquitoes from each infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of duck Tembusu virus (DTMUV).

Study	#Infected mosquitoes with eggs	Egg No.	Infected/tested F1 mosquitoes	
			Male	Female
1	1	#1	0/36	0/31
		Total (1)	0/36	0/31
4	12	#1	0/2	0/1
		#2	-	0/3
		#3	0/1	0/1
		#4	0/1	0/1
		#5	0/1	0/1
		#6	-	0/1
		#7	0/2	0/1
		#8	0/1	0/1
		#9	0/3	-
		#10	-	0/1
		#11	-	0/2
		#12	-	0/1
		Total (12)	0/11	0/14
6	3	#1	0/2	0/6
		#2	-	0/9
		#3	-	0/2
		Total (3)	0/2	0/17

Table 7: (Cont.) Number of F1 mosquitoes from each infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of duck Tembusu virus (DTMUV).

Study	#Infected mosquitoes with eggs	Egg No.	Infected/tested F1 mosquitoes	
			Male	Female
8	6	#1	-	0/3
		#2	-	0/3
		#3	0/1	0/2
		#4	-	0/1
		#5	0/1	0/1
		#6	0/1	0/4
		Total (6)	0/3	0/14
9	21	#1	0/6	0/1
		#2	0/1	0/1
		#3	0/1	0/7
		#4	-	0/2
		#5	-	0/1
		#6	0/1	0/3
		#7	0/4	0/2
		#8	0/1	-
		#9	-	0/1
		#10	0/1	0/1

Table 7: (Cont.) Number of F1 mosquitoes from each infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of duck Tembusu virus (DTMUV).

Study	#Infected mosquitoes with eggs	Egg No.	Infected/tested F1 mosquitoes	
			Male	Female
		#11	0/3	-
		#12	0/1	0/1
		#13	-	0/3
		#14	0/2	0/1
		#15	0/1	0/1
		#16	-	0/1
		#17	-	0/1
		#18	0/1	-
		#19	-	0/2
		#20	-	0/1
		#21	-	0/1
		Total (21)	0/23	0/31

Table 8: Examination of transovarial transmission of duck Tembusu virus (DTMUV) in non-infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of DTMUV. Individual pool of F1 male and female mosquitoes from the same F0 female mosquito was tested for DTMUV by using RT-PCR.

Study	Blood-fed/total mosquitoes	#Non-infected mosquitoes with eggs	Infected/tested F1 mosquitoes	
			Male	Female
1	20/50	0	-	-
2	28/50	3	0/5	0/10
3	40/50	2	0/12	0/18
4	42/60	5	0/13	0/9
5	25/120	4	0/5	0/6
6	37/80	12	0/3	0/21
7	31/100	2	0/12	0/17
8	32/120	4	-	0/6
9	80/120	5	0/1	0/7
Total	335/750	37	0/51	0/94

Table 9: Number of F1 mosquitoes from each non-infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of duck Tembusu virus (DTMUV).

Study	#Non-infected mosquitoes with eggs	Egg No.	Infected/tested F1 mosquitoes	
			Male	Female
2	3	#1	0/2	0/3
		#2	-	0/5
		#3	0/3	0/2
		Total (3)	0/5	0/10
3	2	#1	0/8	0/7
		#2	0/4	0/11
		Total (2)	0/12	0/18
4	5	#1	0/3	0/2
		#2	0/3	0/1
		#3	0/2	0/3
		#4	-	0/2
		#5	0/5	0/1
		Total (5)	0/13	0/9
5	4	#1	0/1	0/1
		#2	-	0/2
		#3	0/3	0/1
		#4	0/1	0/2
		Total (4)	0/5	0/6
6	12	#1	-	0/2
		#2	-	0/1

Table 9: (Cont.) Number of F1 mosquitoes from each non-infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of duck Tembusu virus (DTMUV).

Study	#Non-infected mosquitoes with eggs	Egg No.	Infected/tested F1 mosquitoes	
			Male	Female
		#3	-	0/4
		#4	0/1	0/3
		#5	0/0	0/1
		#6	0/1	0/1
		#7	0/1	0/3
		#8	-	0/2
		#9	-	0/1
		#10	-	0/1
		#11	-	0/1
		#12	-	0/1
		Total (12)	0/3	0/21
7	2	#1	0/5	0/9
		#2	0/7	0/8
		Total (2)	0/12	0/17
8	4	#1	-	0/3
		#2	-	0/1
		#3	-	0/1
		#4	-	0/1
		Total (4)	-	0/6

Table 9: (Cont.) Number of F1 mosquitoes from each non-infected *Culex tritaeniorhynchus* after feeding on infected blood meal with 10^5 $TCID_{50}$/mL of duck Tembusu virus (DTMUV).

Study	#Non-infected mosquitoes with eggs	Egg No.	Infected/tested F1 mosquitoes	
			Male	Female
9	5	#1	-	0/1
		#2	-	0/1
		#3	-	0/1
		#4	0/1	0/2
		#5	-	0/2
		Total (5)	0/1	0/7

4.4 Discussion

Duck Tembusu virus (DTMUV) is a mosquito-borne flavivirus which was first identified in China in 2010 (Su et al., 2011). During 2013, DTMUV was detected in Sing Buri and Ang Thong Provinces, Thailand (Ninvilai et al., 2018, Tunterak et al., 2018). Vertical or transovarial transmissions of some flaviviruses have been reported in *Culex* mosquitoes (Mishra and Mourya, 2001, Rosen et al., 1980, Saiyasombat et al., 2011, Sudeep et al., 2013). *Culex (Cx.) tritaeniorhynchus* are predominant mosquitoes that can be found in different habitats in Thailand (Changbunjong et al., 2013, Sanisuriwong et al., 2020, Tiawsirisup and Nuchprayoon, 2010, Tiawsirisup et al., 2008). Therefore, *Cx. tritaeniorhynchus* mosquitoes have important roles in the spreading of several flaviviruses (Changbunjong et al., 2013, O'Guinn et al., 2013a, Sanisuriwong et al., 2020).

The maintenance and transmission of mosquito-borne pathogens are depended on the availability of competent mosquito vectors in nature. An ability of

DTMUV to replicate in mosquitoes and its potential to cause infection and disease in host make it is an important emerging disease in ducks. Transovarial transmission is an efficient mechanism by which the mosquito-borne pathogens are maintained in mosquitoes in nature. Transovarial transmission and tissue tropisms of *Culex* flavivirus have been showed in *Cx. pipiens* which all positive females produced infected offspring and the virus also infected the ovaries as early as 4 days post-inoculation after needle inoculation. Additionally, viral RNA was detected in various tissues (e.g., salivary glands, ovaries, testes, head, fat bodies, and midguts of *Cx. pipiens* (Saiyasombat et al., 2011).

Our previous study found a relatively high infection, dissemination, and transmission rate of DTMUV in *Cx. tritaeniorhynchus* mosquitoes. In this study, *Cx. tritaeniorhynchus* were allowed to feed on10^5 $TCID_{50}$/mL of DTMUV via artificial membrane feeder and DTMUV infection in offspring or transovarial transmission was examined. DTMUV can pass through salivary gland but cannot be able to pass into the ovary which the ovary might has a barrier that prevent the virus to disseminate and propagate in the ovary. This finding indicated that there was no transovarial transmission of DTMUV in *Cx. tritaeniorhynchus* and transovarial transmission may be not the mechanism by which DTMUV is maintained in *Cx. tritaeniorhynchus* mosquitoes in nature in Thailand.

CHAPTER V

General discussion, conclusion and further recommendations

In 2010, severe outbreaks of duck infectious diseases were occurred around the major duck producing farms in China (Su et al., 2011). Infected ducks showed egg production drop, ovary-oviduct disease, and severe neurologic disorders including an incapability to stand, ataxia, and paralysis. In Thailand, there was reported in layer and broiler duck farms in 2013 (Thontiravong et al., 2015a). Duck Tembusu virus (DTMUV) can cause disease in ducks, but also it can cause in other species (i.e., house sparrows, chickens, and geese) (Liu et al., 2012a, Huang et al., 2011). DTMUV is a mosquito borne flavivirus which the mosquitoes are important vectors for the transmission cycle of this virus. Moreover, the transmission of the DTMUV are several routes (i.e., direct contact, horizontal transmission, fecal-oral route, and aerosol transmission) (Yan et al., 2011c, Cao et al., 2011, Tang et al., 2012a).

The mosquito diversity and distribution in duck farms in Thailand, DTMUV infection in field collected mosquitoes in DTMUV endemic areas, the vector competence of *Culex (Cx.) tritaeniorhynchus* for DTMUV, and the transovarial transmission of DTMUV in *Cx. tritaeniorhynchus* were investigated in this study. The first study, a total of 30,841 mosquitoes were collected and identified to seven species (*Anopheles* (*An.*) *barbirostris*, *An. stephensi*, *Culex* (*Cx.*) *gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus, Mansonia* (*Ma.*) *annulifera* and *Ma. uniformis*). While, the most common collected species from each duck farm and each collection time was *Cx. tritaeniorhynchus*. A total of 273 mosquito pools were examined, with only one pool of *Cx. tritaeniorhynchus* collected from Sing Buri Province in November 2015 testing positive for DTMUV. The obtained nucleotide

sequencing of DTMUV from *Cx. tritaeniorhynchus* in this study was aligned and compared with available DNA sequences in the GenBank database and it is closely related with the DTMUV isolated from duck in Thailand.

The second study was conducted to examine vector competence of *Cx. tritaeniorhynchus* and *Cx. quinquafasciatus* for DTMUV. *Cx. tritaeniorhynchus* were allowed to feed on four levels of DTMUV which were 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL and *Cx. quinquefasciatus* were allowed to feed on two levels of DTMUV which were 10^4 and 10^5 $TCID_{50}$/mL. DTMUV infection in *Cx. tritaeniorhynchus* were 1.6%, 10.2%, 35.8%, and 59.3% after feeding on 10^2, 10^3, 10^4, and 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively. DTMUV dissemination and transmission in *Cx. tritaeniorhynchus* were 20.3% and 16.9% after feeding on 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively. However, there was no virus dissemination and transmission in *Cx. tritaeniorhynchus* after feeding on 10^2, 10^3, and 10^4 $TCID_{50}$/mL of DTMUV in the blood meals. While, DTMUV infection in *Cx. quinquefasciatus* were only 2.5% and 2.3% after feeding on 10^4 and 10^5 $TCID_{50}$/mL of DTMUV in the blood meals, respectively; however, there was no virus dissemination and transmission found in all tested *Cx. quinquefasciatus* mosquitoes.

The third study was performed to examine the transovarial transmission of DTMUV in *Cx. tritaeniorhynchus*. The mosquitoes were allowed to feed on infected blood meal with 10^5 $TCID_{50}$/mL of DTMUV and to lay eggs. After egg-laying, the mosquitoes were tested for DTMUV infection by using RT-PCR. F1 mosquitoes from DTMUV infected mosquitoes were tested for DTMUV infection; however, all of them were negative for DTMUV. This finding indicated that there was no transovarial transmission of DTMUV in *Cx. tritaeniorhynchus*.

Each mosquito species has different ability to be infected with DTMUV or even the same mosquito species can be infected differently depending on many factors including amount of the virus in blood meals, environmental factors (i.e.,

humidity and temperature), and anatomical and physiological barriers in the mosquitoes (i.e., midgut, salivary gland and ovarian barrier). However, mosquitoes are important vectors for the spreading of DTMUV in nature. In addition, the methods for further studies of mosquito infection capabilities may need to be examined. Other routes of mosquito infection should be performed (i.e., taking blood meals from infected ducks and intrathoracic injection). Direct detection of the virus from the mosquito's ovary is another method that can be used for the study of transovarial transmission in mosquitoes. Finally, the findings from this study will be useful for the controlling and preventing of DTMUV in Thailand.

Appendix

Appendix A

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RAPID COMMUNICATION

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Duck Tembusu virus detection and characterization from mosquitoes in duck farms, Thailand

Jitra Sanisuriwong[1] | Nichapat Yurayart[1] | Aunyaratana Thontiravong[1,2] | Sonthaya Tiawsirisup[1,3]

[1]Animal Vector-Borne Disease Research Unit, Veterinary Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

[2]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

[3]Veterinary Parasitology Research Group, Veterinary Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

Correspondence
Sonthaya Tiawsirisup, Animal Vector-Borne Disease Research Unit, Veterinary Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.
Emails: sonthaya.t@chula.ac.th; sonthayatiaw@hotmail.com

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Abstract

Duck Tembusu virus (DTMUV), an emerging infectious disease in ducks, belongs to the *Flavivirus* genus and Flaviviridae family. The transmission of DTUMV involves mosquito vectors; however, the exact role of mosquitoes in the ecology of DTMUV in Thailand remains unclear. This study was conducted to examine DTMUV detection and characterization from mosquitoes in duck farms in central Thailand. Mosquitoes were collected from two duck farms in Sing Buri Province and two duck farms in Ang Thong Province from September 2015 to July 2016 using four CDC-light traps. A total of 30,841 mosquitoes were collected and identified to seven species (*Anopheles* (*An.*) *barbirostris*, *An. stephensi*, *Culex* (*Cx.*) *gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, *Mansonia* (*Ma.*) *annulifera* and *Ma. uniformis*). The most common collected species from each duck farm and each collection time was *Cx. tritaeniorhynchus*. Mosquitoes were pooled according to species, location, and collection time and then examined for DTMUV by RT-PCR. A total of 273 mosquito pools were examined, with only one pool of *Cx. tritaeniorhynchus* collected from Sing Buri Province in November 2015 testing positive for DTMUV. Phylogenetic analysis of the polyprotein genes demonstrated that a mosquito-derived Thai DTMUV was grouped into subcluster 2.1 and most closely related to the 2013 Thai DTMUVs. Thus, this study indicated that *Cx. tritaeniorhynchus* may play a role as a vector in the transmission of DTMUV in Thailand. However, additional studies concerning the vector competence of this mosquito for DTMUV are needed.

KEYWORDS
detection, duck farms, duck Tembusu virus, mosquito, Thailand, vector

1 | INTRODUCTION

Duck Tembusu virus (DTMUV) is an emerging infectious disease in ducks described in China, Malaysia and Thailand. It is a single-stranded positive-sense RNA virus belonging to the Ntaya virus group of the genus *Flavivirus* and family Flaviviridae. This virus affects various hosts, such as ducks, geese, chickens, sparrows, pigeons, mice and mosquitoes (Tang et al., 2015; Yu, Lin, Tang, & Diao, 2018). Tembusu virus (TMUV), the most closely related virus to DTMUV, was first isolated from *Culex* (*Cx.*) *tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia in 1955, while DTMUV was first identified as the causative agent of egg-drop syndrome, neurological signs or death in infected egg-laying and breeder ducks in China in 2010 (Cao et al., 2011; Su et al., 2011).

For DTMUV infection in ducks in Thailand, a severe infectious disease in broiler and layer ducks has emerged since 2013. The outbreaks have been reported from free-grazing ducks and duck farms in various major duck production areas in Thailand including Sing Buri and Ang Thong Provinces (Ninvilai et al., 2018; Ninvilai, Tunterak, Oraveerakul, Amonsin, & Thontiravong, 2019; Tunterak

et al., 2018). Phylogenetic analysis of the polyprotein gene sequence of the Thai DTMUV isolates indicated that it is closely related to Chinese DTMUV (Thontiravong et al., 2015). However, an unknown contagious disease associated with severe neurological signs and egg production losses in ducks, resembling DTMUV infection, has been observed in Thailand since 2007 (Ninvilai et al., 2018).

As known, DTMUV is mosquito-borne flavivirus. It was first detected in *Cx. pipiens* mosquitoes collected from Shandong Province, China in 2010–2012 (Tang et al., 2015). However, subsequent in vivo studies indicated horizontal transmission of the virus among ducks by direct contact and aerosol transmission (Li et al., 2015), and by vertical transmission from breeding ducks to ducklings (Zhang et al., 2015).

In Thailand, TMUV was detected from pools of *Cx. gelidus*, *Cx. tritaeniorynchus* and *Cx. vishnui* mosquitoes collected from Kamphaengphet Province in 1982 (Leake et al., 1986), *Cx. tritaeniorhynchus* mosquitoes collected from Chiang Mai Province in 1992 (Pandey et al., 1999), and *Cx. quinquefasciatus* mosquitoes collected from Kanchanaburi Province in 2015 (Nitatpattana et al., 2017). Phylogenetic analysis of TMUV isolated from Kanchanaburi Province indicated that it is different from the DTMUV isolated from ducks in Thailand (Chakritbudsabong et al., 2015; Thontiravong et al., 2015). However, the exact role of mosquitoes in the ecology of DTMUV in Thailand remains unclear. Therefore, this study was conducted to examine DTMUV detection and characterization from mosquitoes in major duck production areas in central Thailand.

2 | MATERIALS AND METHODS

2.1 | Field mosquito collection

The mosquitoes were randomly collected from two duck farms in Sing Buri Province and two duck farms in Ang Thong Province between September 2015 and July 2016. These duck farms in these two provinces were selected as study sites based on the high-density duck raising areas of Thailand, DTMUV infection history and the farmers' cooperation (Niamsang, 2015; Ninvilai et al., 2019; Tunterak et al., 2018). Of 5 DTMUV affected duck farms from 1,292 duck farms in these two provinces (Niamsang, 2015; Ninvilai et al., 2019), these farms were selected as study sites in this study. Neurological signs of the infected ducks also be found during the mosquito sample collection. The mosquitoes were collected on six occasions at two -month intervals for one year to indicate the relation between mosquito species and season in Thailand. Four CDC-light traps were placed all over the duck housing area for each farm and the distance between each trap was at least 20 m. Traps were operated from dark until dawn because this period was preferred for *Culex* mosquito blood feeding which *Culex* was a majority mosquito genus found in duck farms. The mosquitoes were morphologically identified (Rattanarithikul & Panthusiri, 1994) and examined for DTMUV. This study was conducted in compliance with the Chulalongkorn University Animal Care and Use Committee, Thailand (IACUC Institute No. B 2559/00018.010: Animal Use Protocol No. 1631045).

2.2 | Detection of DTMUV

2.2.1 | Virus

The DK/TH/CU-1 strain of DTMUV was kindly provided by the Virology Unit, Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. It was originally isolated from an infected duck in Nakhon Ratchasima Province, Thailand. This virus was used as a positive control for reverse transcription-polymerase chain reaction (RT-PCR).

2.2.2 | Viral nucleic acid extraction

The collected mosquitoes were split into pools of 1–50 mosquitoes according to species, location and collection time. The maximum number of mosquitoes per pool was 50 mosquitoes; however, if the numbers of collected mosquitoes did not reach this number, then as many as possible were selected. For each mosquito species of the same location and collection time, 20 mosquito pools were the quota selected as representative for DTMUV detection. The DTMUV detection status of the collected mosquitoes was examined using RT-PCR. Viral nucleic was extracted from pools of mosquitoes using the viral nucleic acid extraction kit II (Geneaid, Taiwan). A total of 273 mosquito pools (18 pools of *Anopheles barbirostris*, 5 pools of *Anopheles stephensi*, 35 pools of *Culex gelidus*, 1 pool of *Culex quinquefasciatus*, 192 pools of *Culex tritaeniorhynchus*, 9 pools of *Mansonia annulifera* and 13 pools of *Mansonia uniformis*) were tested in this study.

2.2.3 | RT-PCR of the NS5 gene fragment of DTMUV

The DTMUV detection was performed by RT-PCR amplification of the viral NS5 gene (Thontiravong et al., 2015). Total RNA samples were amplified using the SuperScript® III One-Step RT-PCR with Platinum® Taq DNA Polymerase (Invitrogen). After agarose gel electrophoresis, the DNA band of the expected product size was purified using the GenepHlowTM Gel/PCR Cleanup Kit (Geneaid, Taiwan). The purified amplicon was submitted to a commercial service for DNA sequencing (First Base Laboratories, Kuala Lumpur, Malaysia). The obtained nucleotide sequencing results were aligned and trimmed using the ClustalW multiple alignment in BioEdit (Thompson, Higgins, & Gibson, 1994), and compared with available DNA sequences in the GenBank database to identify nucleotide identities among isolates using the Basic Local Alignment Search Tool (Altschul, Gish, Miller, Myers, & Lipman, 1990).

2.3 | Whole-genome sequencing and phylogenetic analysis

Viral RNA of Thai DTMUV from *Culex* mosquito in 2015 (*Culex*/TH/CU_2015) was subjected to whole-genome sequencing using the primer set for amplification of the polyprotein gene sequence of DTMUV as described previously (Thontiravong et al., 2015). The nucleotide sequences were assembled using SeqMan software v.5.03 (DNASTAR Inc.). A polyprotein gene sequence of *Culex*/TH/CU_2015 was submitted to GenBank database under the accession number MH460536. Phylogenetic analysis was performed by comparing the polyprotein gene sequence of the *Culex*/TH/CU_2015 with that of the previously reported Chinese, Malaysian, and Thai DTMUVs and TMUV strains available in the GenBank database. The phylogenetic trees were constructed by neighbour-joining (NJ) and maximum-likelihood (ML) algorithms with 1,000 bootstraps using MEGA v.6.0 program (Tamura et al., 2011). The nucleotide and amino acid sequences of the *Culex*/TH/CU_2015 and the selected reference DTMUV and TMUV strains were aligned and compared using MegAlign software v.5.03 (DNASTAR Inc.).

TABLE 1 Species and number of mosquitoes collected from the duck farms in Sing Buri and Ang Thong Provinces, Thailand

		Number of mosquitoes (positive/tested pools)			
Collected time	Mosquito species	Farm 1 Sing Buri	Farm 2 Sing Buri	Farm 3 Ang Thong	Farm 4 Ang Thong
September 2015	*Anopheles barbirostris*	18 (0/1)	46 (0/1)	5 (0/1)	40 (0/1)
	Anopleles stephensi	0	1 (0/1)	1 (0/1)	0
	Culex gelidus	93 (0/2)	157 (0/4)	57 (0/1)	560 (0/8)
	Culex tritaeniorhynchus	1,619 (0/16)	8,292 (0/20)	1,106 (0/15)	3,986 (0/20)
	Mansonia annulifera	0	1 (0/1)	8 (0/1)	38 (0/1)
	Mansonia uniformis	0	36 (0/1)	2 (0/1)	29 (0/1)
	Total	1,730 (0/19)	8,533 (0/28)	1,179 (0/20)	4,653 (0/31)
November 2015	*Anopheles barbirostris*	45 (0/1)	354 (0/3)	26 (0/1)	74 (0/1)
	Anopleles stephensi	1 (0/1)	0	1 (0/1)	1 (0/1)
	Culex gelidus	86 (0/2)	63 (0/2)	8 (0/1)	50 (0/1)
	Culex tritaeniorhynchus	1,281 **(1/16)**	4,494 (0/20)	787 (0/14)	713 (0/14)
	Mansonia annulifera	0	0	14 (0/1)	10 (0/1)
	Mansonia uniformis	40 (0/1)	15 (0/1)	55 (0/1)	78 (0/1)
	Total	1,453 **(1/21)**	4,926 (0/26)	891 (0/19)	926 (0/19)
January 2016	*Anopheles barbirostris*	38 (0/1)	7 (0/1)	3 (0/1)	7 (0/1)
	Culex gelidus	3 (0/1)	2 (0/1)	0	1 (0/1)
	Culex quinquefasciatus	0	0	0	1 (0/1)
	Culex tritaeniorhynchus	497 (0/9)	113 (0/2)	18 (0/1)	27 (0/1)
	Mansonia uniformis	8 (0/1)	20 (0/1)	2 (0/1)	0
	Total	546 (0/12)	142 (0/5)	23 (0/3)	36 (0/4)
March 2016	*Culex gelidus*	1 (0/1)	0	8 (0/1)	0
	Culex tritaeniorhynchus	8 (0/1)	7 (0/1)	19 (0/1)	2 (0/1)
	Mansonia uniformis	0	0	1 (0/1)	0
	Total	9 (0/2)	7 (0/1)	28 (0/3)	2 (0/1)
May 2016	*Culex gelidus*	1 (0/1)	0	1 (0/1)	1 (0/1)
	Culex tritaeniorhynchus	21 (0/1)	11 (0/1)	12 (0/1)	0
	Mansonia annulifera	0	0	1 (0/1)	0
	Total	22 (0/2)	11 (0/1)	14 (0/3)	1 (0/1)
July 2016	*Anopheles barbirostris*	5 (0/1)	9 (0/1)	13 (0/1)	10 (0/1)
	Culex gelidus	48 (0/1)	136 (0/3)	6 (0/1)	8 (0/1)
	Culex tritaeniorhynchus	681 (0/10)	3,409 (0/15)	62 (0/2)	1,313 (0/10)
	Mansonia annulifera	1 (0/1)	2 (0/1)	0	1 (0/1)
	Mansonia uniformis	0	1 (0/1)	0	4 (0/1)
	Total	735 (0/13)	3,557 (0/21)	81 (0/4)	1,336 (0/14)

The bold values indicate the Duck Tembusu virus infected mosquito pool.

3 | RESULTS AND DISCUSSION

The mosquitoes were collected on six occasions at two-month intervals between September 2015 and July 2016. The species and number of collected mosquitoes are shown in Table 1. A total of 30,841 mosquitoes were collected and identified to seven species (*Anopheles* (*An.*) *barbirostris*, *An. stephensi*, *Cx. gelidus*, *Cx. quinquefasciatus*, *Cx. tritaeniorhynchus*, *Mansonia* (*Ma.*) *annulifera* and *Ma. uniformis*). The most commonly collected species from each duck farm and each collection time was *Cx. tritaeniorhynchus*. A total of 273 mosquito pools were examined for DTMUV by RT-PCR. Only one mosquito pool of *Cx. tritaeniorhynchus* collected from Farm 1 in Sing Buri Province was positive for DTMUV, representing 0.52% (1/192) of *Cx. tritaeniorhynchus* pools or 0.37% (1/273) for all tested mosquito pools in this study (Tables 1 and 2).

The sensitivity of RT-PCR was determined in this study and it can detect DTMUV as low as 1 EID_{50}/ml. No cross-reaction was observed with other viruses (i.e. Newcastle disease, avian influenza, duck plaque and other flaviviruses) and mosquitoes (i.e. *Aedes aegypti*, *Ae. albopictus* and *Culex quinquefasciatus*). However, only one mosquito pool of *Cx. tritaeniorhynchus* was positive for DTMUV infection in this study. It indicated the low DTMUV circulation in mosquito population in duck farms in Thailand. More sensitive assay may be needed for DTMUV detection in mosquito population, for example, a nanoparticle-assisted polymerase chain reaction (nano-PCR) assay. This assay was developed for DTMUV detection, and it was 10-fold more sensitive than a conventional PCR assay which the lower detection limit of this assay was 1.8×10^2 copies per µl (Wanzhe et al., 2016).

To genetically characterize the Thai DTMUV from *Culex* mosquito in 2015, the virus designated as *Culex*/TH/CU_2015 was subjected to whole-genome sequencing. The whole-genome length of *Culex*/TH/CU_2015 is 10,278 nucleotides, encoding 3,425 amino acids, which is similar to that of the previously reported DTMUV isolates (Ninvilai et al., 2018; Thontiravong et al., 2015). Phylogenetic analysis of the polyprotein gene sequence demonstrated that *Culex*/TH/CU_2015 was grouped into subcluster 2.1 and most closely related to the 2013 Thai DTMUVs (98.9% nucleotide and 99.8% amino acid identities) (Figure 1). However, the polyprotein gene sequence of *Culex*/TH/CU_2015 shared only 88.2% and 97.1% nucleotide identities with the mosquito-derived TMUV isolated from *Cx. tritaeniorhynchus* in Malaysia (MM1775) and the mosquito-derived DTMUV isolated from *Cx. pipiens* in China (SDMS), respectively. In addition, the phylogenetic analysis of the complete E gene of *Culex*/TH/CU_2015 showed similar finding with that of polyprotein gene (Figure 1). This virus had the highest identity with Thai DTMUVs isolated from ducks during 2015–2016 (99.1%–99.5% nucleotide and 99.0%–99.8% amino acid identities), while it shared only 96.9% and 89.6% nucleotide identities with Chinese DTMUVs and Malaysian DTMUVs, respectively.

Previously, a total of 1,000 blood samples were collected from free-grazing ducks in Thailand during 2008–2015 and 9.10% of them showed DTMUV neutralizing antibodies, while DTMUV seropositive ducks have been detected in Thailand since 2008 (Tunterak et al., 2018). Another serological survey of DTMUV infection conducted across 20 Provinces located in the major free-grazing duck raising areas of Thailand in 2016 revealed 30.4% of 1,200 blood samples were positive for DTMUV neutralizing antibodies and 93.3% of flocks had at least one DTMUV seropositive duck. Additionally, DTMUV seropositive ducks were observed in all Provinces tested (Tunterak et al., 2018). Ninvilai et al. (2018) also indicated the presence of DTMUV in Thai ducks since 2007, prior to the first report of DTMUV in China in 2010. However, phylogenetic analysis of the polyprotein gene sequence revealed that this virus belonged within DTMUV cluster 1, which is genetically different from the currently circulating Thai and Chinese DTMUV that belong to cluster 2.1. Thus, DTMUV may have a high genetic diversity and have been circulating in Asia long before 2010.

In China, *Cx. pipiens* mosquitoes were collected during the summers of 2010–2012 in the Shandong Province of China during the outbreak of TMUV in duck farms, where 58.8% of mosquito pools tested were positive for TMUV when examined by both RT-PCR and virus isolation assays (Tang et al., 2015). However, only 0.52% of *Cx. tritaeniorhynchus* mosquito pools were positive for DTMUV in this study. The difference of DTMUV infection in mosquitoes between China and Thailand might occur because of high virus titre in ducks in China and higher susceptibility of *Cx. pipiens* to DMTUV infection than *Cx. tritaeniorhynchus* which are the vectors found in

TABLE 2 Summary of DTMUV infected and tested mosquito pools collected from each duck farm in Sing Buri and Ang Thong Provinces, Thailand

Mosquito species	Positive/tested pool				
	Farm 1 Sing Buri	Farm 2 Sing Buri	Farm 3 Ang Thong	Farm 4 Ang Thong	Total
Anopheles barbirostris	0/4	0/6	0/4	0/4	0/18
Anopheles stephensi	0/1	0/1	0/2	0/1	0/5
Culex gelidus	0/8	0/10	0/5	0/12	0/35
Culex quinquefasciatus	0	0	0	0/1	0/1
Culex tritaeniorhynchus	**1/53**	0/59	0/34	0/46	1/192
Mansonia annulifera	0/1	0/2	0/3	0/3	0/9
Mansonia uniformis	0/2	0/4	0/4	0/3	0/13
Total	**1/69**	0/82	0/52	0/70	1/273

The bold values indicate the Duck Tembusu virus infected mosquito pool.

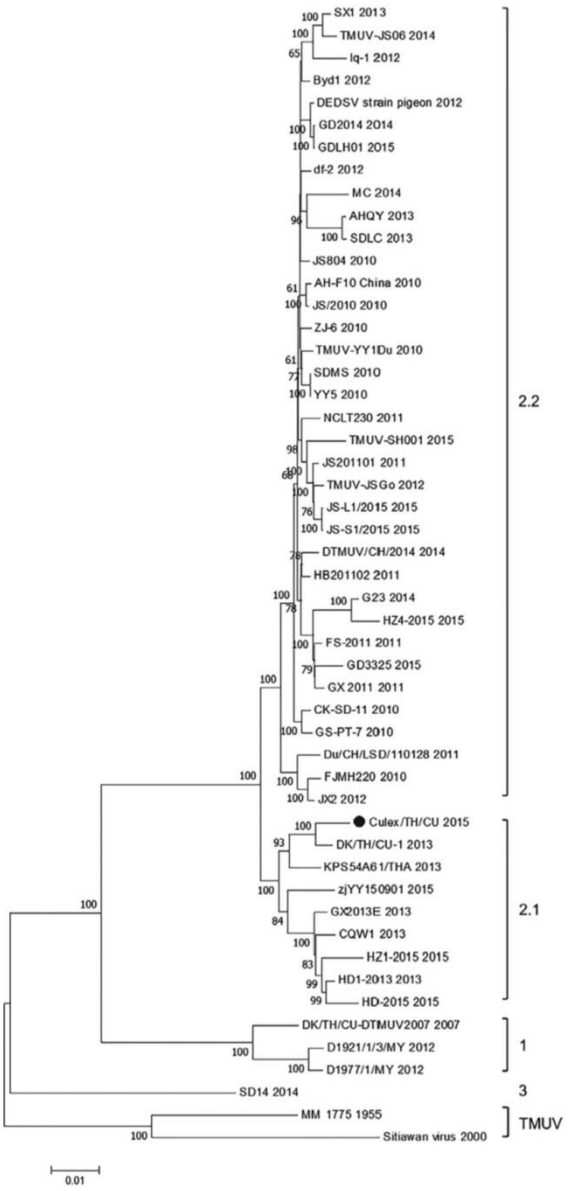

FIGURE 1 Phylogenetic analysis of a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) and selected reference strains of DTMUV and TMUV. The phylogenetic tree was constructed using neighbour-joining (NJ) algorithm based on the nucleotide sequences of the polyprotein (10,278 bp) genes of a mosquito-derived Thai DTMUV (*Culex*/TH/CU_2015) and selected reference strains of DTMUVs and TMUVs identified from Thailand, Malaysia and China. A black circle indicates a mosquito-derived Thai DTMUV identified in this study (*Culex*/TH/CU_2015). A similar result was observed when applying the maximum-likelihood (ML) algorithm

Thailand. For the relation between mosquito vectors and TMUV in Thailand, a laboratory study on the vector competence of mosquitoes (F1 progeny) that were originally captured in Kamphaeng Phet Province in 2005 revealed that *Cx. vishnui* developed high viral titres after feeding on TMUV-infected chicks and transmitted the virus to naive chickens but *Cx. fuscocephala* appeared less susceptible to infection (O'Guinn et al., 2013). A field study in Kanchanaburi Province in 2015 found *Cx. quinquefasciatus* was the most commonly collected mosquito species near a chicken farm, and 1/70 mosquito pools were positive for TMUV by RT-PCR and virus isolation. This provided potential support for TMUV transmission by this *Culex* species in a natural habitat (Nitatpattana et al., 2017). However, this TMUV was not involved or responsible for the disease outbreak in ducks in Thailand. Currently, the relationship between *Cx. tritaeniorhynchus* and DMTUV in Thailand is still limited even though this species of mosquitoes can be found all over Thailand, especially in the farming areas.

This study indicates the detection of DTMUV in *Cx. tritaeniorhynchus* collected from a duck farm in Sing Buri Province, Thailand. The polyprotein gene sequence revealed that it was closely related to the DTMUV isolated from ducks in Thailand, which might indicate the possible role of this mosquito species in the transmission cycle of DTMUV in duck farms in Thailand. However, more studies about vector competence of this mosquito for DTMUV in laboratory conditions are required to indicate the virus infection, dissemination and horizontal or vertical transmission in mosquitoes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

ORCID

Sonthaya Tiawsirisup https://orcid.org/0000-0002-6783-2050

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vertical transmission of Tembusu virus in ducks. *Veterinary microbiology,* 179, 149-154.

VITA

NAME	Jitra Sanisuriwong
DATE OF BIRTH	13 october 1979
PLACE OF BIRTH	Bangkok Province
INSTITUTIONS ATTENDED	Bachelor degree of Veterinary Medicine from Mahanakorn University of Technology, Bangkok, Thailand in 2004
HOME ADDRESS	34/1 moo.5 Sangkasantisuk Road, Kratumrai, Nong Chok, Bangkok, 10530
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