ตารางที่ 8 ผลการทดสอบการติดเชื้อของแบคทีเรีย *Wolbachia* และ bacteriophage WO ด้วยเทคนิค PCR โดยใช้ยีน *wsp* ของแบคทีเรีย *Wolbachia* และยีน *orf7* ของ bacteriophage WO จากแบคทีเรีย *Wolbachia* ที่พบในยุง ชนิดต่าง ๆ ในธรรมชาติ

Genus (subgenus) species	No. test	*Wolbachia* infection status*	No. test	Bacteriophage WO infection status*	Supergroup of *Wolbachia*	*Wolbachia* strain (s)	Accession no. of phage WO
Anopheles (Cellia)							
An. dirus C	1	-	1	-	-	-	-
An. dirus D	1	-	1	-	-	-	-
An. jamesi (Theobald)	1	-	1	-	-	-	-
An. minimus s.l. (Theobald)	1	-	1	-	-	-	-
An. nivipes (Theobald)	1	-	1	-	-	-	-
An. subpictus Grassi	1	-	1	-	-	-	-
An. vagus Doenitz	1	-	1	-	-	-	-
Armigeres (*Armigeres*)							
Ar. kesseli Ramalingam	4	++	4	++	B	wKes	AY515378, AY515569-71
Ar. subalbatus (Coquillett)	2	+	2	+	A	wSub	-
Armigeres (*Leicesteria*)							
Ar. flavus (Leicester)	1	+	1	-	-	-	-
Coquillettidia (*Coquillettidia*)							
Cq. crassipes (Van der Wulp)	1	++	1	++	B	wCras	AY515572
Culex (*Culex*)							
Cx. fuscocephala Theobald	1	++	1	++	B	wFusc	AY515576
Cx. gelidus Theobald	4	++	4	++	B	wGel	AY515577-80
Cx. quinquefasciatus Say	2	++	2	++	B	wPip	AY515581-82
Cx. sitiens Wiedemann	1	+	1	+	B	wSit	-
Cx. tritaeniorhynchus Giles	1	-	1	-	-	-	-
Cx. vishnui Theobald	1	+	1	+	-	-	-
Cx. whitmorei (Giles)	1	-	1	-	-	-	-

ตารางที่ 6 ผลการทดสอบการติดเชื้อของแบคทีเรีย Wolbachia และ bacteriophage WO ด้วยเทคนิค PCR โดยใช้ยีน wsp ของแบคทีเรีย Wolbachia และยีน orf7 ของ bacteriophage WO จากแบคทีเรีย Wolbachia ที่พบในยุง ชนิดต่างๆในธรรมชาติ

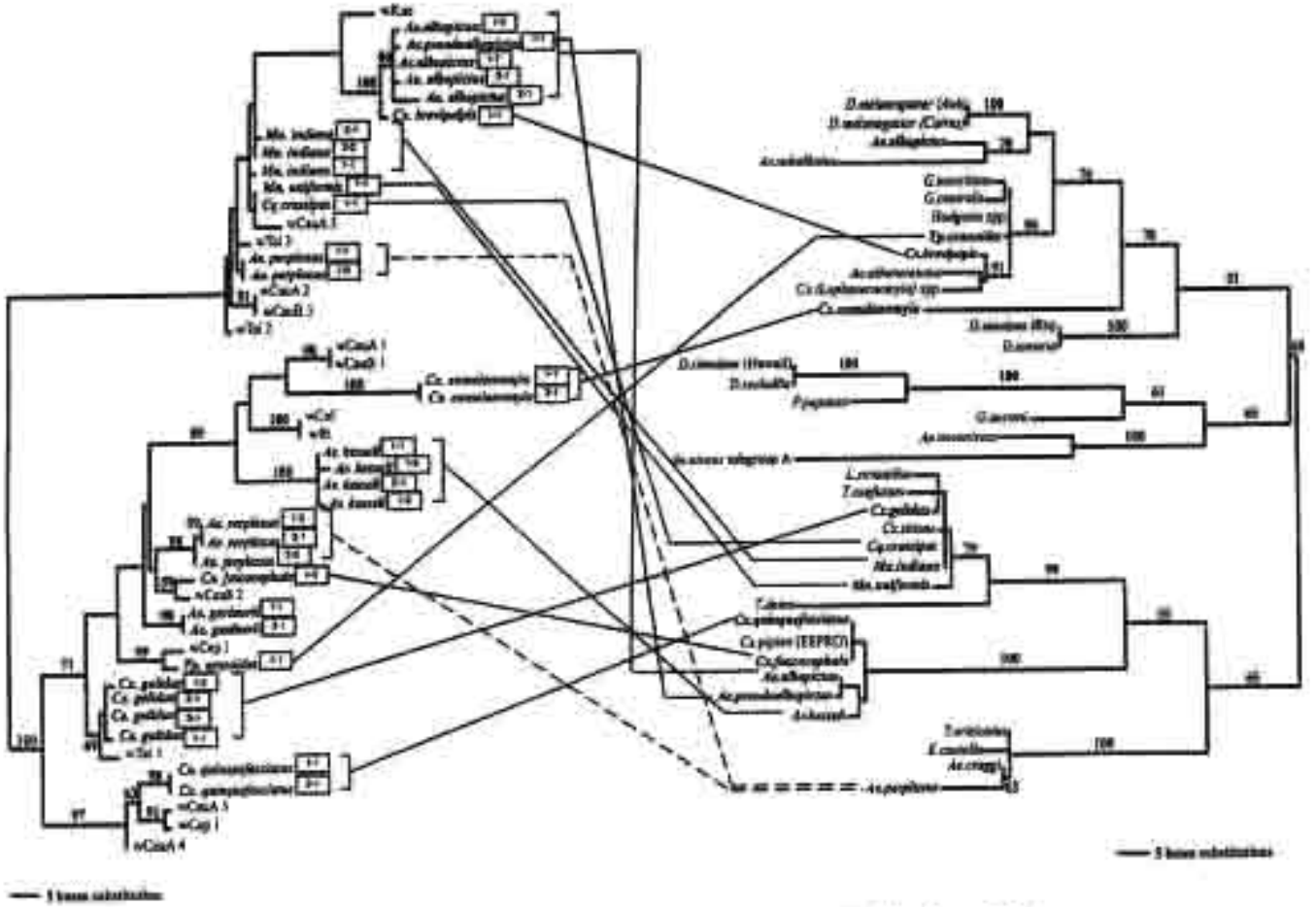
Genus (subgenus) species	No. test	*Wolbachia* infection status*	No. test	Bacteriophage WO infection status*	Supergroup of *Wolbachia*	*Wolbachia* strain (s)	Accession no. of phage WO
Culex (*Eumelanomyia*)							
Cx. brevipalpis (Giles)	1	++	1	++	A	wBre	AY515575
Cx. sumalaiensis	2	++	2	++	A	wEum	AY515573-74
Culex (*Lophoceromyia*)							
Cx. lophoceromyia	2	+	2	+	A	wLop	-
Culex (*Lutzia*)							
Cx. fuscanus Wiedemann	1	-	2	-	-	-	-
Hodgesia							
Hodgesia spp.	2	+	2	-	A	wHod	
Mansonia (*Mansonioides*)							
Mn. indiana Edwards	3	++	3	++	B	wInd	AY515583-85
Mn. uniformis (Theobald)	1	++	1	++	B	wUnif	AY515588
Orthopodomyia							
Or. andamanensis Barraud	1	-	1	-	-	-	-
Or. anopheloides (Giles)	2	-	1	-	-	-	-
Tripteroides (*Rachionotomyia*)							
Tp. aranoides (Theobald)	1	++	1	++	A	wAra	AY515587
Uranotaenia (*Pseudoficalbia*)							-
Ur. patriciae Peyton	1	+	1	+	-	-	
Other insects							
Corcyra cephalonica	-	++	-	++	-	wCep	AB036657, AB036658
Drosophila simulans (Coffs Harbour S-20)	-	++	-	++	-	wCof	AB036659

ตารางที่ 8 ผลการทดสอบการติดเชื้อของแบคทีเรีย Wolbachia และ bacteriophage WO ด้วยเทคนิค PCR โดยใช้ยีน wsp ของแบคทีเรีย Wolbachia และยีน orf7 ของ bacteriophage WO จากแบคทีเรีย Wolbachia ที่พบในยุง ชนิดต่างๆในธรรมชาติ

Genus (subgenus) Species	No. test	Wolbachia infection status*	No. test	Bacteriophage WO infection status*	Supergroup of Wolbachia	Wolbachia strain (s)	Accession no. of phage WO
Other insects							
Drosophila simulans (Riverside)	-	++	-	++	-	wRi	AB036661
Ephestia kuehniella (Tsuchiura)	-	++	-	++	B	wCauB	AB036654, AB036655, AB036656
Ephestia cautella (Tsuchiura)	-	++	-	++	A, B	wCauA, CauB	AB036654, AB036655, AB036656, AB036648, AB036650, AB036651, AB036652 AB036653
Ephestia kuehniella (Tsuchiura)	-	++	-	++	A	wCauA	AB036648, AB036650, AB036651, AB036652 AB036653
Ephestia kuehniella (Yokohama)	-	++	-	++	-	wKue	AB03660
Teleogryllus taiwanemma	-	++	-	++	-	wTai	AB036662 , AB036663, AB 036664

* – ไม่พบผลผลิต PCR, + พบผลผลิต PCR ในปริมาณน้อย, ++ พบผลผลิต PCR ในปริมาณที่มากพอจะนำมาหาลำดับเบสได้

จากนั้นผู้วิจัยทำการศึกษาวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการของ bacteriophage WO จากแบคทีเรีย *Wolbachia* ในยุงชนิดต่างๆ เปรียบเทียบกับสิ่งมีชีวิตอื่นที่มีใน GenBank โดยการทำ multiple sequence alignment ของยีน *orf7* ของ bacteriophage WO จากแบคทีเรีย *Wolbachia* ในยุงเปรียบเทียบกับลำดับดีเอ็นเอที่มีรายงานใน GenBank โดยใช้โปรแกรม Clustal X (1.83) และทำการวิเคราะห์โดยสร้าง phylogenetic tree ของ ยีน *orf7* (bacteriophage origin) ด้วยวิธี Parsimony โดยใช้โปรแกรม Clustal X (1.83) และ PAUP (4.0) จากนั้นสร้างต้นไม้วิวัฒนาการ ด้วยโปรแกรม TreeView เพื่อวิเคราะห์สายสัมพันธ์ทางวิวัฒนาการของ bacteriophage WO จากแบคทีเรีย *Wolbachia* ที่พบในยุงชนิดต่างๆ เปรียบเทียบกับสิ่งมีชีวิตอื่นๆที่มีรายงานใน GenBank จากนั้นทำการนำต้นไม้วิวัฒนาการของ bacteriophage WO มาเปรียบเทียบกับต้นไม้วิวัฒนาการของแบคทีเรีย *Wolb achia* ซึ่งพบในยุงแต่ละชนิด ซึ่งสามารถดูผลการทดลองได้ในภาพที่ 9



orf7 (bacteriophage WO origin)

wsp (*Wolbachia* origin)

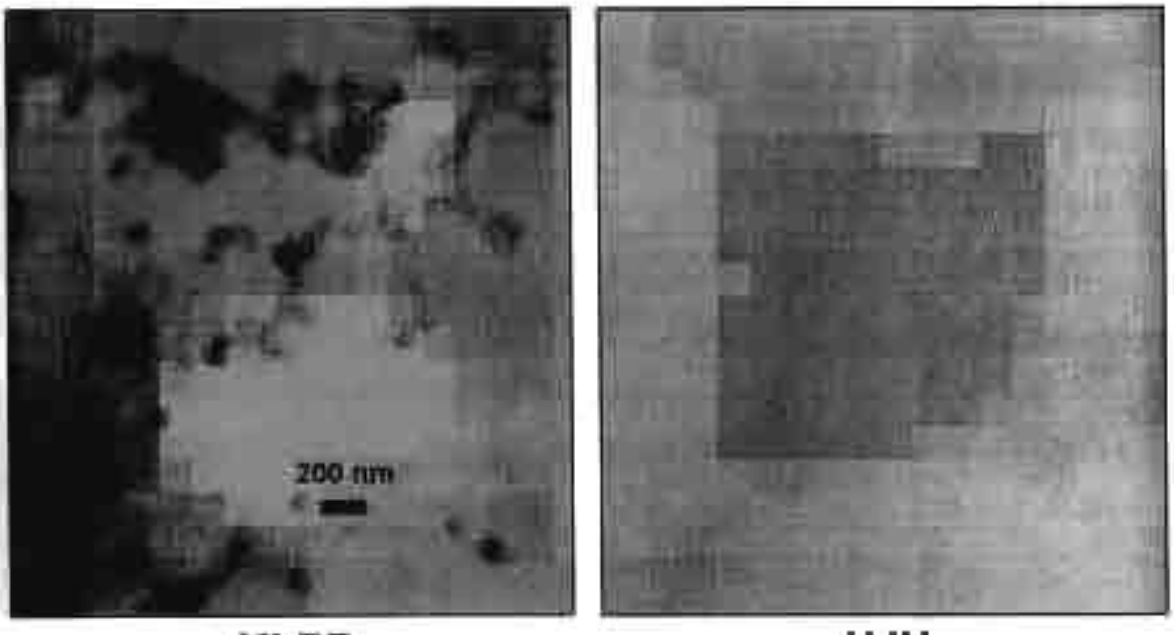
ภาพที่ 9 Phylogenetic tree ของยีน *orf7* (bacteriophage WO origin) และ Phylogenetic tree ของยีน *wsp* (*Wolbachia* origin) ซึ่งสร้างด้วยวิธี Parsimony โดยใช้โปรแกรม Clustal X (1.83) และ PAUP (4.0) จากนั้นสร้างต้นไม้วิวัฒนาการด้วยโปรแกรม TreeView เพื่อวิเคราะห์สายสัมพันธ์ทางวิวัฒนาการของ bacteriophage WO จากแบคทีเรีย *Wolbachia* ที่พบในยุงชนิดต่าง ๆเปรียบเทียบกันเองและเปรียบเทียบกับสิ่งมีชีวิตอื่นที่มีรายงานใน GenBank โดยตัวเลขที่อยู่ตาม Node ต่างๆ แสดงถึง bootstrapping probabilities (nrep=1000). ตัวเลขที่อยู่ในกรอบตัวเลขแรกแสดงถึงเลขที่ของตัวอย่างยุง ส่วนตัวเลขที่สองแสดงถึงเลขที่ของโคลนของผลิตภัณฑ์ PCR เฉพาะส่วนของยีน *orf7*

4.4 การศึกษาลักษณะทางกายภาพของ bacteriophage WO ที่พบในยุง superinfected *Aedes albopictus* (*w*AlbA + *w*AlbB) (KLPP) เพื่อยืนยันผลการทดสอบการติดเชื้อของ bacteriophage WO

เพื่อเป็นการยืนยันผลการทดสอบการติดเชื้อของ bacteriophage WO ของเชื้อแบคทีเรีย *Wolbachia* ในยุง ผู้วิจัยทำการถ่ายภาพจากกล้องจุลทรรศน์อิเล็คตรอน (electron microscoopes) ของอนุภาค bacteriophage WO ที่ผ่านการสกัดแยกจากแบคทีเรีย *Wolbachia* ที่อยู่ในรังไข่ของ *Aedes albopictus* (*w*AlbA+*w*AlbB) (KLPP) (ภาพที่ 10) จากภาพถ่ายนี้พบอนุภาคที่มีลักษณะคล้าย bacteriophage WO จำนวนมาก

4.5 ศึกษาคุณสมบัติการกระจายและความหนาแน่นของ *Wolbachia* และ bacteriophage WO ที่อาศัยอยู่ในยุงตามธรรมชาติชนิดต่างๆ

ผู้วิจัยทำการตรวจหาความหนาแน่นของ bacteriophage WO และ แบคทีเรีย *Wolbachia* ในยุงชนิดต่างๆในธรรมชาติ ด้วยเพื่อดูแนวโน้มการกระจายตัวของ bacteriophage WO และ แบคทีเรีย *Wolbachia* ในยุงชนิดต่างๆที่จับได้จากธรรมชาติ โดยวิธี PCR เชิงปริมาณ (quantitative PCR) ซึ่งสามารถดูผลความหนาแน่นของ bacteriophage WO ในยุงชนิดต่างๆในธรรมชาติได้ดังแสดงในตารางที่ 7 และ ผลความหนาแน่นของ bacteriophage WO เทียบกับความหนาแน่นของแบคทีเรีย *Wolbachia*ในยุงชนิดต่างๆในธรรมชาติได้ดังแสดงในตารางที่ 8



ภาพที่ 10 ภาพถ่ายจากกล้องจุลทรรศน์อิเล็คตรอน (electron microscope) ของ อนุภาค bacteriophage WO ที่ผ่านการสกัดแยกแล้วจากแบคทีเรีย *Wolbachia* ที่พบในยุงลาย superinfected *Aedes albopictus* (wAlbA+wAlbB) (KLPP) และ negative control *Aedes albopictus* (UJU) ที่ไม่ได้ติดเชื้อ *Wolbachia*

ตารางที่ 7 ความหนาแน่นของ bacteriophage WO ในแบคทีเรีย *Wolbachia* ที่พบในยุงให้อาศัยชนิดต่างๆในธรรมชาติ

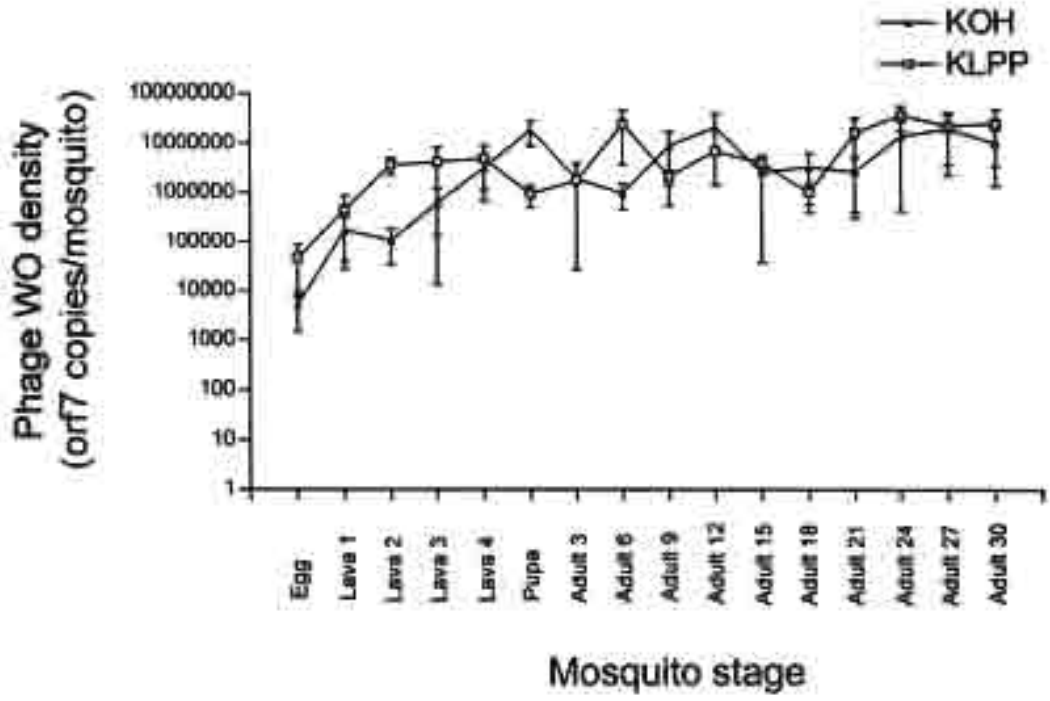
Mosquito strains	Wolbachia strains	No. of Testing	bacteriophageWO density orf7 copies ($\times 10^4$) / mosquito (mean ± SE)	Ratio
Ae.albopictus (Chachoengsao)	wAlbA, wAlbB	3	8.86 ± 2.4	0.12
Ae.albopictus (Kanchanaburi) (KLPP)	wAlbA, wAlbB	18	15.08 ± 3.1	0.20
Ae.albopictus (Samui) (KOH)	wAlbA	14	4.99 ± 2.63	0.07
Ae.gardnerii (Kanchanaburi)	wGar	4	14.39 ± 2.70	0.19
Ae.perplexus (Kanchanaburi)	wPerp	4	12.07 ± 2.63	0.16
Ae.pseudoalbopictus (Kanchanaburi)	wPseu	3	1.59 ± 0.21	0.02
Ar.kesseli (Chiangrai)	wKes	5	57.52 ± 9.74	0.77
Ar.kesseli (Kanchanaburi)	wKes	2	0.58 ± 0.11	0.01
Cq.crassipes (Kanchanaburi)	wCras	2	1.88 ± 0.08	0.03
Cx.fuscocephala (Chachoengsao)	wFusc	7	2.27 ± 1.03	0.03
Cx.gelidus (Chiangmai)	wGel	4	11.96 ± 4.10	0.16
Cx.gelidus (Prachuap Khiri Khan)	wGel	4	1.94 ± 0.48	0.03
Cx.quinquefasciatus (Chonburi)	wPip	2	74.23 ± 16.67	1
Cx.quinquefasciatus (Prachuap Khiri Khan)	wPip	4	8.99 ± 2.27	0.12
Cx.quinquefasciatus (Sakhon Nakhon)	wPip	6	1.81± 0.55	0.02
Cx.brevipalpis (Kanchanaburi)	wBre	4	1.79 ± 0.41	0.02
Cx.aumelanonyia (Kanchanaburi)	wEum	2	6.41 ± 0.16	0.09
Mn.indiana (Prathumthani)	wInd	2	0.73 ± 0.13	0.01
Mn.indiana (Ubon Ratchathani)	wInd	4	0.44 ± 0.12	0.01
Mn.uniformis (Ubon Ratchathani)	wUnif	3	3.39 ± 0.63	0.05
Tp.aranoides (Kanchanaburi)	wAra	2	4.40 ± 0.45	0.06

ตารางที่ 8 ความหนาแน่นของ bacteriophage WO เปรียบเทียบกับแบคทีเรีย *Wolbachia* ที่พบในยุงให้อาศัยชนิดต่างๆในธรรมชาติ

Mosquito strains	*Wolbachia* strains	*Wolbachia* supergroups	*Wolbachia* density *wsp* copies / mosquito (mean ± SE)	bacteriophageWO density *orf7* copies / mosquito (mean ± SE)
Ae.albopictus (Kanchanaburi)	wAlbA	A	$8.36 \times 10^{9} \pm 2.82 \times 10^{9}$	$15.08 \times 10^{5} \pm 3.10 \times 10^{5}$
Cx.brevipalpis (Kanchanaburi)	wBre	A	$1.12 \times 10^{9} \pm 4.15 \times 10^{8}$	$1.79 \times 10^{5} \pm 4.1 \times 10^{4}$
Cx.eumelanomyia (Kanchanaburi)	wEum	A	$9.79 \times 10^{8} \pm 4.31 \times 10^{7}$	$6.41 \times 10^{5} \pm 1.6 \times 10^{5}$
Tp.aranoides (Kanchanaburi)	wAra	A	$6.58 \times 10^{9} \pm 4.80 \times 10^{9}$	$4.40 \times 10^{5} \pm 4.5 \times 10^{5}$
Ae.albopictus (Kanchanaburi)	wAlbB	B	$9.14 \times 10^{10} \pm 6.18 \times 10^{10}$	$15.08 \times 10^{5} \pm 3.10 \times 10^{5}$
Ae.perplexus (Kanchanaburi)	wPerp	B	$1.48 \times 10^{11} \pm 7.63 \times 10^{10}$	$12.07 \times 10^{5} \pm 2.63 \times 10^{5}$
Ae.pseudoalbopictus (Kanchanaburi)	wPseu	B	$1.20 \times 10^{10} \pm 9.68 \times 10^{9}$	$1.59 \times 10^{5} \pm 2.1 \times 10^{5}$
Ar.kesseli (Kanchanaburi)	wKes	B	$6.62 \times 10^{11} \pm 4.64 \times 10^{11}$	$0.58 \times 10^{5} \pm 1.1 \times 10^{5}$
Cq.crassipes (Kanchanaburi)	wCras	B	$1.68 \times 10^{10} \pm 1.34 \times 10^{10}$	$1.88 \times 10^{5} \pm 8 \times 10^{4}$
Cx.fuscocephala (Chachoengsao)	wFusc	B	$1.36 \times 10^{10} \pm 1.03 \times 10^{10}$	$2.27 \times 10^{5} \pm 1.03 \times 10^{5}$
Cx.gelidus (Chiangmai)	wGel	B	$1.53 \times 10^{10} \pm 1.26 \times 10^{10}$	$11.96 \times 10^{5} \pm 4.10 \times 10^{5}$
Mn.indiana (Pathumthani)	wInd	B	$3.28 \times 10^{11} \pm 3.18 \times 10^{11}$	$0.73 \times 10^{5} \pm 1.3 \times 10^{5}$
Mn.uniformis (Ubon Ratchathani)	wUnif	B	$2.07 \times 10^{10} \pm 4.25 \times 10^{9}$	$3.39 \times 10^{5} \pm 6.3 \times 10^{4}$

4.6 ศึกษาเปรียบเทียบ Growth kinetics ของความหนาแน่นของไวรัส bacteriophage WO ที่พบในแบคทีเรีย *Wolbachia* ในยุงลาย *Aedes albopictus* ตามธรรมชาติ

ปริมาณ bacteriophage WO ทั้งหมดในยุง KLPP, KOH มีปริมาณเริ่มต้นในไข่จนถึงระยะลูกน้ำแตกต่างกันเล็กน้อยโดยในยุง KLPP จะสูงกว่า KOH แต่เมื่อเข้าระยะดักแด้จนถึงระยะต้นของตัวเต็มวัยปริมาณของ bacteriophage WO จะใกล้เคียงกันและปริมาณของ bacteriophage WO ในยุง KLPP จะสูงกว่า KOH อีกเมื่อเข้าระยะของตัวเต็มวัยได้ 21 วัน ดังภาพที่ 11

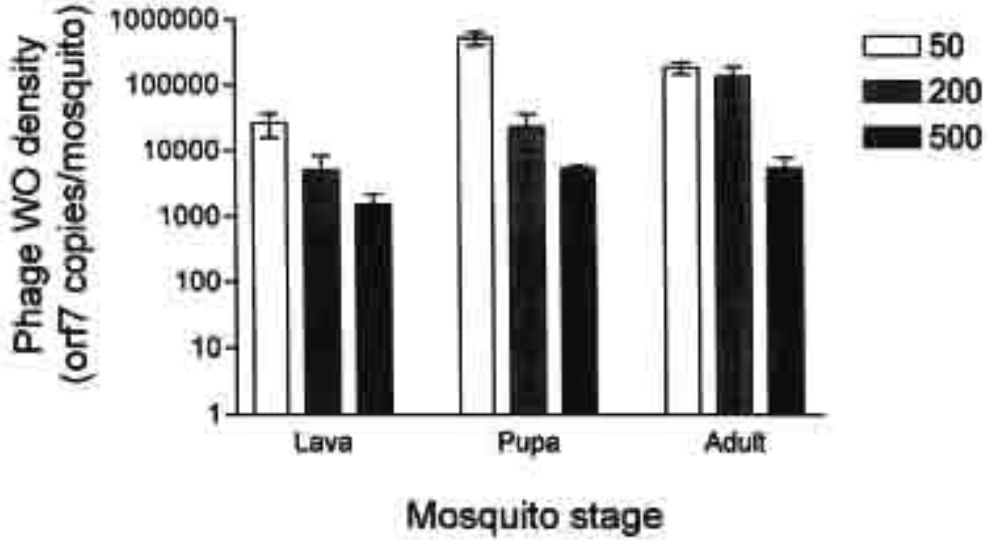


ภาพที่ 11 กราฟแสดงความสัมพันธ์ระหว่างปริมาณ bacteriophage WO ทั้งหมดในยุงให้อาศัย KLPP และ KOH ระยะต่างๆ

โดย KLPP เป็นยุง *Ae. albopictus* ที่มีเชื้อแบคทีเรีย *Wolbachia* สองสายพันธุ์ (A&B), KOH เป็นยุง *Ae. albopictus* ที่มีเชื้อแบคทีเรีย *Wolbachia* หนึ่งสายพันธุ์ (A)

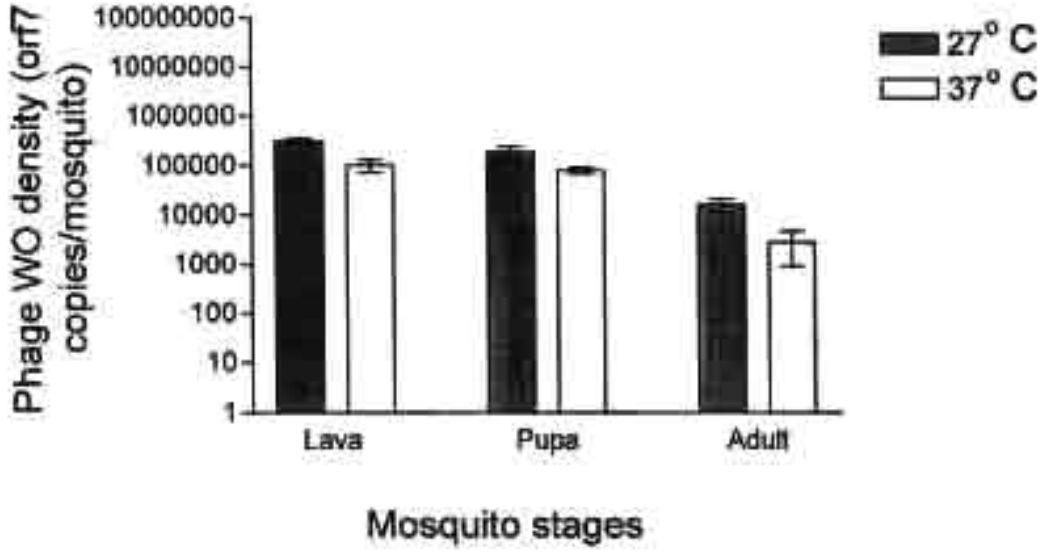
4.7 ศึกษาปัจจัยในการดำรงชีวิตของยุงลายที่อาจมีผลกระทบต่อความหนาแน่นของไวรัส (bacteriophage WO) ที่พบในแบคทีเรีย *Wolbachia*ในยุงลาย

ปริมาณ bacteriophage WO ทั้งหมดในยุง KLPP ระยะต่างๆ ซึ่งแบ่งการเลี้ยงเป็น 3 แบบ คือ ที่ความหนาแน่นของจำนวนประชากรยุงปกติ (50 ตัว) ที่ความหนาแน่นของจำนวนประชากรยุงปานกลาง (200 ตัว) ที่ความหนาแน่นของจำนวนประชากรยุงสูง (500 ตัว) ส่วนสภาวะที่เกี่ยวข้องอื่นๆ เช่น อุณหภูมิ ภาชนะที่ใช้เลี้ยง อาหาร จะควบคุมให้เท่ากันนั้น พบว่าที่ ความหนาแน่นของจำนวนประชากรยุงปกติจะมีปริมาณ bacteriophage WO สูงสุด รองลงมาคือที่ความหนาแน่นของจำนวนประชากรยุงปานกลาง และปริมาณ bacteriophage WO ต่ำสุดที่ที่ความหนาแน่นของจำนวนประชากรยุงสูง ดังภาพที่ 12



ภาพที่ 12 กราฟแสดงความสัมพันธ์ระหว่างปริมาณ bacteriophage WO ทั้งหมดในยุง KLPP ระยะต่างๆ ซึ่งแบ่งการเลี้ยงที่ความหนาแน่นของจำนวนประชากรยุงแตกต่างกันเป็น 3 แบบ

ปริมาณ bacteriophage WO ทั้งหมดในยุง KLPP ระยะต่างๆ ซึ่งแบ่งการเลี้ยงที่ 2 อุณหภูมิ คือในภาวะอุณหภูมิปกติ (27°C) และภาวะอุณหภูมิสูงกว่าปกติ (37°C) พบว่าที่อุณหภูมิปกติ (27°C) จะมีปริมาณ bacteriophage WO สูงกว่า ใน ภาวะอุณหภูมิสูงกว่าปกติ (37°C) ดังภาพที่ 13



ภาพที่ 13 กราฟแสดงความสัมพันธ์ระหว่างปริมาณ bacteriophage WO ทั้งหมดในยุงให้อาศัย (KLPP) ระยะต่างๆ ซึ่งแบ่งการเลี้ยงที่ 2 อุณหภูมิ คือในภาวะอุณหภูมิปกติ (27°C) และภาวะสูงกว่าปกติ (37°C)

5. Output ที่ได้จากโครงการ

ข้าพเจ้าได้ทำการเขียนผลงานเพื่อตีพิมพ์ในวารสารวิชาการระดับนานาชาติสำหรับในปีแรกเรียบร้อยแล้วในขอบเขตที่วางไว้ในปีแรกตามที่ได้เสนอไปในการรายงานความก้าวหน้ารอบ 1 ปี แต่ต่อมาได้มีผลงานวิจัยที่คล้าย ๆ กัน แต่ทำในสิ่งมีชีวิตที่แตกต่างกันตีพิมพ์ออกมา อาจารย์ที่ปรึกษาจึงแนะนำให้ทำงานวิจัยเพิ่มเติม ทำให้การตีพิมพ์ผลงานไม่ได้เป็นไปตามที่กำหนดไว้ ดังนั้นข้าพเจ้าจึงได้ทำงานวิจัยเพิ่มขึ้นอีกนอกเหนือจากที่ได้วางแผนไว้ในตอนต้นตามที่อาจารย์ที่ปรึกษาแนะนำ และบัดนี้ข้าพเจ้าได้เขียนผลงานวิจัยใหม่อีกครั้งเพื่อตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

โดยชื่อเรื่องที่คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาตินั้นคือ Molecular evidence of co-transmission with differences in density between *Wolbachia* and bacteriophage WO in their mosquito hosts และ ชื่อวารสารคือ Insect Molecularе Biology (impact factor = 2.89) ซึ่ง manuscript สามารถดูได้ในภาคผนวกและสำหรับปีที่ 2 ซึ่งครบกำหนดสัญญานี้ข้าพเจ้าได้ทำงานวิจัยเสร็จเรียบร้อยแล้วตามแผนที่ได้วางไว้และ manuscript ฉบับที่ 2 นี้กำลังอยู่ในระหว่างการตรวจแก้ไข

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ภาคผนวก

บทความ Manuscript ที่ได้จากงานวิจัยในโครงการ

Molecular evidence for co-transmission with differences in density between *Wolbachia* and bacteriophage WO in their mosquito hosts

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Abstract

Bacteriophages of the *Wolbachia* bacteria were proposed as a transformation tool in an attempt to genetically modify mosquito vectors. In this paper, we report the presence of the bacteriophage WO among natural populations of several mosquito hosts. Our survey revealed that 22 out of 25 *Wolbachia*-infected species of mosquitoes contained phage WO. Phylogenetic relationship based on *orf7* sequences showed that a single strain of phage WO was found in most singly or doubly *Wolbachia*-infected mosquito species. Only one species, i.e., *Aedes perplexus*, was found to harbor at least two different phage types. In addition, our results suggested that phage WO in mosquitoes preferred to co-transmit with their *Wolbachia* hosts and were likely to be specific in each mosquito species. This finding was different from those previously reported in *Drosophila*, moths and parasitoid wasps. Real-time quantitative PCR assay for the density of phage WO revealed that the average density was $7.76 \times 10^5 \pm 1.61 \times 10^5$ *orf7* copies per an individual mosquito. The phage WO of the Asian tiger mosquito, *Aedes albopictus* from recent colonies (KLPP) was confirmed by TEM. The phage WO density of double infected *Ae. albopictus* was determined to be three times higher than that of single infected one. However, the density of phage WO did not correlate with that of their *Wolbachia* hosts which were varied in different mosquito species. The viral-like particles were detected after purification and filtration of *Ae. albopictus* ovaries suggesting the presence of active phages in this mosquito vector. Our findings that

the phage WO from mosquito were host-specific and were active encourage further investigation to utilize these bacteriophages as transformation vectors.

Keywords: ***Wolbachia*****, bacteriophage, endosymbiont, mosquito**

Introduction

Mosquitoes are the important disease vectors causing morbidity and mortality especially in tropical and subtropical regions. The maintenance and transmission of pathogens, i.e., numerous viral infections and protozoa infections causing lymphatic filariasis, dengue fever, yellow fever, Japanese encephalitis, and malaria, are absolutely dependent on competent mosquito vectors (Beerntsen *et al.*, 2000). At present, the application of pesticides as a primary strategy for controlling mosquito-borne diseases has led to environmental problems and concerns. As a consequence, recent tools in molecular biology and genomics are being applied to develop genetically engineered mosquitoes that could resist pathogen development as an alternative vector control strategy.

The maternally inherited endosymbiotic bacteria in the Genus *Wolbachia* which infect numerous arthropod species have been proposed as a potential candidate to deliver pathogen-blocking genes into natural populations of medically important insects. These endosymbionts induce various reproductive disorders on their hosts, such as cytoplasmic incompatibility in mosquitoes (Yen & Barr, 1971), parthenogenesis in parasitoid wasps (Stouthamer *et al.*, 1993) male-killing in Mediterranean flour moths (Fujii *et al.*, 2001), and feminization in isopod crustaceans (Bouchon *et al.*, 1998). However, molecular mechanisms and *Wolbachia* genes those are responsible for these phenomena are still unknown. There are many hypotheses regarding this issue. One of them indicates that genes responsible for the reproductive effects on hosts do not locate on the bacterial chromosome but on extra-chromosomal factors such as plasmids or bacteriophages, and thus effects of *Wolbachia* depend on their infection status.

Recently, a novel report has identified the bacteriophage-like genetic element of *Wolbachia*, namely bacteriophage WO, in *Drosophila* and hymenopteran parasitoid. In addition, they showed that this phage can be either lysogenic and integrated into the *Wolbachia* chromosome or lytic and free in the cytoplasm (Masui *et al.*, 2000). There are many evidences that virulent determinants of pathenogenic bacteria are encoded in these bacteriophages. They code for a variety of toxin genes expressed by pathogenic bacteria. Therefore, an evolutionary advantage for phages might be due to enhance replication of bacteria carrying these virulence determinants (Cheetham & Katz, 1995).

In this paper, we examined the infection status of bacteriophage WO in several field-collected mosquito species. We also determined phylogenetic relationships and specificity of bacteriophage WO and *Wolbachia* in these mosquitoes. Lastly, we investigated the relative density of *Wolbachia* and their associated bacteriophages in each mosquito host.

Results

Phage WO infection among Wolbachia strains from mosquito hosts

The classical PCR analysis based on nucleotide sequences of putative minor capsid protein (*orf7*) was performed to initially examine the infection status of phage WO from mosquito species collected in different regions of Thailand. As shown in Table 1, phage WO was detected in 22 out of 25 mosquito species of mosquitoes that were originally found infected with *Wolbachia* bacteria. Most medically important genera of mosquitoes, i.e., *Aedes*, *Armigeres*, *Culex* and *Mansonia*, contained phage WO. *Anopheles* was only an exception which did not have any record of *Wolbachia* infection. Three species of *Wolbachia*-infected mosquitoes that were negative with *orf7* were *Ae. (Stegomyia)* sp., *Ar. flavus* and *Hodgesia sp.* These results imply that three mosquito species have no phage WO or the copy number of phage WO was extremely low and could not detect by a normal PCR technique, or the limitation of phage specific primers might occur because of the variability of phage WO sequence.

Phage-like particles (phage WO) were successfully isolated from a colony of *Wolbachia*-infected *Ae. albopictus* (KLPP) mosquitoes using 0.22 µm filters. This evidence indicated that the bacteriophage of *Wolbachia* bacteria infecting *Ae. albopictus* (KLPP) was active, and the appearance of packaged phage particles were confirmed by transmission electron microscopy (TEM) as shown in Figure 1. In contrast, no particle-like structure was detected in the preparation from mosquito samples obtained from *Wolbachia*-uninfected *Ae. albopictus* (UJU) colony.

Phylogenetic analysis of bacteriophage WO

The total numbers of 32 *orf7* sequences were obtained from 14 species of mosquitoes. Phylogenetic analysis using partial phage WO gene (*orf7*) was performed from 48 sequences; 32 sequences from 14 mosquito species (Table 1) and 16 sequences from GenBank (Masui *et al.*, 2000). In some mosquito species, i.e., *Ae. niveus* supergroup A, *Ae. novoniveus*, *Ar. subalbatus*, *Cx. sitiens*, *Cx. vishnui*, *Cx.* (*Lophoceraomyia*) sp. and *Ur. patriciae*, classical PCR detection of phage WO using *orf7* provided faint bands indicating the presence of phages. However, direct sequencing reactions from PCR products of these samples were not successful so they were not included in our phylogenetic study.

Phage WO which was found in mosquitoes had a pattern of the consensus parsimonious tree as shown in Figure 2. Results from phylogenetic analysis of phage WO using *orf7* mostly correlated with those of their *Wolbachia* hosts using *wsp* gene sequences (Ruang-areerate *et al.*, 2002). Phage WO of *Cq. crassipes*, *Mn. indiana*, *Mn. uniformis*, *Ae. pseudoalbopictus* and *Ae. albopictus* was clustered in the same group and correlated with supergroup B of *Wolbachia* infecting the same mosquito hosts. Phage WO of *Cx. brevipalpis* did not correlate with that of *Wolbachia* but this phage strain was closely related to the phage WO found in *Ae. pseudoalbopictus*, *Ae. albopictus* and phage WO wKue of *Ephestia kuehniella* (GenBank accession number: AB03660). In addition, phage WO from *Cx. gelidus* and wTal1 of *Teleogryllus taiwanemma* (GenBank accession number: AB036662) were in the same group but were not closely related to *Cq. crassipes*, *Mn. indiana*, and *Mn. uniformis* as shown in the *Wolbachia* phylogenetic tree. The *orf7* sequences of wCauA1 and wCauB1 (GenBank accession numbers: AB036654

and AB036649) were identical while those of *Tp. aranoides* and *w*Cep2 (GenBank accession number: AB036658) and those of *Cx. fuscocephala*, *Ae. gardneri* and *w*CauB2 (GenBank accession number: AB036655) were closely related. The *w*Cof (GenBank accession number: AB036659) from *Drosophila simulans* and *w*Ri (GenBank accession number: AB036661) from *Drosophila simulans* (Riverside) were in the same group. Phage WO of *Cx. quinquefasciatus*, *w*Cep1 (GenBank accession number: AB036657) from *Corcyra cepharonica*, *w*CauA4 (GenBank accession number: AB036652), *w*CauA5 (GenBank accession number: AB036653) from *Ephestia kuehniella* were related while those of *Ar. kesseli* and *Cx.* (*eumelannomyia*) were isolated and could not be classified in the same group. All results revealed that phage WO co-transmitted with their *Wolbachia* and they were quite specific in each mosquito species. However, the phylogenetic tree of the phage WO could not be clearly placed into either supergroups A or B as the phylogenetic tree of *Wolbachia*. The C and D clades were not included in the analysis because *Wolbachia* strains in C and D supergroups have been documented only in filarial nematode hosts (Taylor *et al.*, 2000) and none of *Wolbachia* strains in mosquitoes assemble out of A and B clades (Zhou *et al.*, 1998; Van Meer *et al.*, 1999).

Multiple *orf7* sequences from the same individual mosquitoes were obtained in order to check whether different strains of phage WO were present in one mosquito species or not. Therefore, in the following species: *Ae. albopictus*, *Ae. garderii*, *Ae. perplexus*, *Ar. kesseli*, *Cx. gelidus*, *Cx.* (*Eumelanomyia*) sp., *Cx. quinquefasiatus*, *Mn. indiana*, more than one clones of PCR products of *orf7* were cloned and sequenced. Our results indicated a single strain of phage WO was found in each mosquito species including *Ae. albopictus* which was generally infected with two strains of *Wolbachia*. In

contrast, the different situation was observed in *Ae. perplexus* which was infected with only one strain of *Wolbachia*. This species had at least two strains of phage WO, each of which were classified in different clades.

Density of bacteriophage WO and Wolbachia host in different mosquito species

The present study was designed to assess quantity of bacteriophage WO and the *Wolbachia* bacteria in natural mosquito species collected from geographically different locations using the real time quantitative-PCR (RTQ-PCR) technique. We constructed plasmid standard which had *orf7* gene of bacteriophege WO and another plasmid standard which had *wsp* gene of *Wolbachia* bacteria for using as RTQ-PCR standard. The target gene can be quantified in unknown samples of each mosquito host by including a serial dilution of such a standard in each PCR run with known amounts of input copy number. The results of bacteriophage WO and *Wolbachia* density in each mosquito species were shown in Table 2.

This is the first report for checking the precise density of bacteriophage WO in natural mosquitoes. The result demonstrated that *Ae. albopictus* (KLPP) from Kanchanaburi (West) showed highest density $15.08 \times 10^5 \pm 3.10 \times 10^5$ *orf7* copies / mosquito whereas the lowest density of bacteriophage WO showed in *Mn. indiana* from Ubon Ratchathani (North East) $0.44 \times 10^5 \pm 0.12 \times 10^5$ *orf7* copies / mosquito. The average density was $7.76 \times 10^5 \pm 1.61 \times 10^5$ *orf7* copies / mosquito.

Interestingly, individual samples of the same mosquito species collected from different geographic areas of Thailand were different in density of phage WO. The phage density of *Cx. gelidus* collected in Chiangmai Province (North) was different from that obtained from Prachuapkhirikhan Province (South). In addition, the phage density of *Cx.*

quinquefasciatus collected from Prachuapkhirikhan Province (South) was different from that in Sakhonnakhon Province (Northeast) and Kanchanaburi Province (West). However, the phage WO density of *Mn. indiana* from Ubonratchathani Province (Northeast) and Pathumthani Province (Central) were not distinctly different. The result of total *Wolbachia* bacterial density in each natural mosquito populations include both *Wolbachia* supergroup A and supergroup B demonstrated the range from $9.79 \times 10^{4} \pm 4.31 \times 10^{4}$ to $9.14 \times 10^{18} \pm 6.18 \times 10^{18}$ *wsp* copies / mosquito. The mean of *Wolbachia* density in mosquito was $7.04 \times 10^{17} \pm 5.57 \times 10^{17}$ *wsp* copies / mosquito. It is apparent that the density of supergroup A strain were lower than density of supergroup B. The mean of supergroup A *Wolbachia* density in mosquito was $6.77 \times 10^{5} \pm 1.80 \times 10^{5}$ *wsp* copies / mosquito (the range of supergroup A *Wolbachia* density in mosquito was $9.79 \times 10^{4} \pm 4.31 \times 10^{4}$ to $1.12 \times 10^{6} \pm 4.15 \times 10^{5}$ *wsp* copies / mosquito), but the mean of supergroup B *Wolbachia* density in mosquito was $1.02 \times 10^{18} \pm 8.02 \times 10^{17}$ *wsp* copies / mosquito (the range of supergroup B *Wolbachia* density in mosquito was $1.20 \times 10^{10} \pm 9.68 \times 10^{9}$ to $9.14 \times 10^{18} \pm 6.18 \times 10^{18}$ *wsp* copies / mosquito).

In the case of double and single infections of *Wolbachia* in *Ae. albopictus*, phage density of double-infected colony (KLPP) originally from Kanchanaburi Province ($15.08 \times 10^{5} \pm 3.10 \times 10^{5}$ *orf7* copies / mosquito) showed three times higher than that of single-infected colony (KOH) collected from Samui island ($4.99 \times 10^{5} \pm 2.63 \times 10^{5}$ *orf7* copies / mosquito). The environmental factors where collected the mosquitoes and also the status of *Wolbachia* infection may have an effect on the level of phage density. In addition, the chronological age and physiological status of the mosquito hosts should not be excluded.

Discussion

Our study is the first report of the bacteriophage-like genetic element in *Wolbachia* from natural mosquito hosts. The presence of bacteriophage WO infection among *Wolbachia* symbionts in different mosquito species was studied based on PCR detection of the putative minor capsid protein (ORF7). The results from all natural populations of 46 mosquito species demonstrate that 25 mosquito species have *Wolbachia* bacteria but phage WO were detected only 22 mosquito species because in *Aedes (Stegomyia)*, *Ar. flavus*, *Hodgesia spp.* may have *Wolbachia* but we could not detected phage WO. There are three possible events due to these results. First these three species have no phage WO but they have *Wolbachia*. Second, there were small amount of phage WO, thus absence of PCR bands were occurred. Third, the limitation of phage specific primers might occur because of the variability of phage WO sequence.

Moreover, the results of the electron microscope for detecting the phage particle in *Wolbachia* double infection *Ae. albopictus* (KLPP) also confirmed the present of phage WO. In addition, the filtering system and picture from TEM also confirmed that at least this phage was active.

Two earlier reports about phage WO infection in moth, *Drosophila*, and parasitoid wasp gave substantial evidence for horizontal transmission of this phage. (Masui *et al.*, 2000; Masui *et al.*, 2001; Gavotte *et al.*, 2004). In contrast, our results showed that horizontal transmission occurred at a very low rate and phage WO preferred to co-transmit with *Wolbachia* as indicated in the phylogenetic tree of phage WO and *Wolbachia* groups of mosquitoes. They mostly correlated with the phylogenetic tree of their *Wolbachia* within the same mosquito. These could be explained that mosquitoes

were likely unique in each species. Different species have distinct behaviors. The opportunity of one species meets with each other is very rare. Because each species has different times for forage and each species has a specific habitat. These supported the specificity of phage infection which has resisted capture of their new mosquito hosts and which prevents phage exchange between *Wolbachia* that co-occur within the same host cells. This is the advantage of phage WO which can be a good choice for using as a vector in the *Wolbachia* transformation to drive desirable genotypes into mosquito populations, for example, genes that prevent mosquito vectors from transmitting pathogens to humans, livestock, and plants (Sinkins *et al.*, 1997; Masui *et al.*, 2000).

In the phylogenetic analysis, the inconsistencies occurred between PCR typing of strains and full sequence analysis. These included PCR typing that indicated hosts contained multiple infections of *Wolbachia*. These might be many strains of phage WO in the same mosquito species, and these affected a direct sequence from the PCR but this problem was solved by cloning before sequencing of phage WO gene fragments. However, cloning resulted in only single infection. These suggested us to select the PCR clone more to compare the sequences in the same mosquito strains and in order to check whether different strains of phage WO were obtained in one mosquito species. Surprisingly, the results indicated that most mosquito species were found single strain of phage WO even *Ae. albopictus* which was found in Kanchanaburi province. It has two supergroup A and B of *Wolbachia* strains but, we could detect only one strain of phages in this mosquito species. These could be suggest that first, there was only one strain of phage WO infected in most mosquitoes or second, the strain of phage WO that we found

was a major population and other strains were very low density until we could not detect them.

In contrast, the result of phage WO based on *orf7* sequence in *Ae. perplexus* which had only one *Wolbachia* strain demonstrated multiple infection of phage WO at least two strains were discovered and they were classified in different clades. This evidence showed the capacity of one *Wolbachia* bacteria can carry more than one strain of phage WO.

In addition, some of the mosquito samples gave no PCR products for cloning due to the absent of phage WO in *Wolbachia* or the loss of DNA template for phage WO in the extracted samples (Table 1). In some cases they showed only a faint band of PCR product and we could not continue the process. As a result, we were not able to clone the following mosquito species: *Ae. niveus* supergroup A, *Ae. novoniveus*, *Ar. subalbatus*, *Cx. sitiens*, *Cx. vishnui*, *Cx. lophoceraomyia* and *Ur. patriciae*. The mismatch of primers may be one of related causes.

Comparison among the density of the phage WO in many mosquitoes from different places using quantitative PCR based on *orf7* sequence revealed variation in density of phage WO. Our results demonstrated a quantitative range of real-time PCR from $0.44 \times 10^5 \pm 0.12 \times 10^5$ *orf7* copies / mosquito to $15.08 \times 10^5 \pm 3.10 \times 10^5$ *orf7* copies / mosquito. It was the actual limits to compare our results to other insects since this is the first report demonstrated quantitative detection of phage WO using real-time PCR. However, the results from real-time PCR of natural mosquito species implied that the environmental habitats of the mosquitoes were important to judge the phage WO density because although the same mosquito species but they had different living habitats

resulting in the different phage WO density. In addition, the principal point was all mosquitoes that we used in phage WO density experiment were natural populations. Their ages, living climate, seasons of catching them, the amount or quantity of food for mosquito hosts were different. All of these factors may be involved in the bacteriophage WO density. Moreover, we also found that the case of *Wolbachia* double and single infections in *Ae. albopictus*, phage density of double infected *Ae. albopictus* (KLPP) showed three times higher than in single infected colony (KOH). These results implied that the level of phage WO density also involved in the status of *Wolbachia* infection.

Thus, *Wolbachia* bacteria density in each natural mosquito populations was performed. The results of the total *Wolbachia* density included both *Wolbachia* supergroup A and supergroup B demonstrated the range from $9.79 \times 10^{4} \pm 4.31 \times 10^{4}$ to $9.14 \times 10^{18} \pm 6.18 \times 10^{18}$ *wsp* copies / mosquito. The mean of *Wolbachia* density in mosquito was $7.04 \times 10^{17} \pm 5.57 \times 10^{17}$ *wsp* copies / mosquito. However, the results from the bacteriophage WO density did not correlate *Wolbachia* bacteria density.

The result of specific density of each *Wolbachia* strain showed that the supergroup A density was very different from supergroup B. The mean of supergroup A *Wolbachia* density in mosquito was $6.77 \times 10^{6} \pm 1.80 \times 10^{5}$ *wsp* copies / mosquito, but the mean of supergroup B *Wolbachia* density in mosquito was $1.02 \times 10^{18} \pm 8.02 \times 10^{17}$ *wsp* copies / mosquito. These results correspond to the previous result of in *Ae. albopictus* (Dutton & Sinkins, 2004) that the wAlbB strain was consistently found to be at higher density than *w*AlbA, which can explain a slightly lower rate of maternal transmission reported for *w*AlbA.

Nevertheless, further investigations need to be carried out such as the density of phage WO and *Wolbachia* in different development and the comparison of density dynamic between *Wolbachia* and phage WO which could be involve in cytoplasmic incompatibility effect of mosquitoes hosts.

Experimental procedures

Mosquito specimens

Wolbachia-infected mosquito specimens which used for phylogenetic analysis of phage-like particles were established as a previously report (Kittayapong *et al.*, 2000). These mosquitoes were collected using light traps, animal-baited nets, and mosquito-landing catches and subsequently stored at -70°C. The morphological keys of Buei and Rattanarithikul and Panthusiri (Rattanarithikul *et al.*, 1994) were used to identify species. The mosquito species used in this study were listed in Table 1.

Electron microscopy

Ten microlitres of fresh phage solution passed through a 0.22 µm filter were adsorbed on a copper-coated grid for 5 min, dried and stained with 2 % phosphotungstic acid for 1 min washed with water 3A grade 3 times. Grids were dried overnight and observed on a Hitachi H-300 operating at 75 kV.

DNA extraction and polymerase chain reaction (PCR) amplification

DNA was extracted using the STE boiling method from whole adult mosquitoes. PCR reaction was done to test for the presence of phage-like sequence in *Wolbachia*, using the following primer set: WOorf7F [5'-GAA ATG CTT GTT CAG CTA ATA GC-3']

and WOorf7R [5'-ATA AAT TCT CCT ATT TTT TCT GGC A-3'] in a 100 µl reaction volume. Each reaction consisted of 10 µl of 10X reaction buffer (Promega), 10 µl of 25 mM $MgCl_2$, 2.5 µl of 20 µM forward and reverse primers, 2.5 µl of dNTPs (10 mM each), 1 µl of extracted DNA template and 1 unit of *Taq* DNA polymerase (Promega). The primers that were used to amplify DNA for sequencing were designed from phage-like sequences (Masui *et al.*, 2000). Amplification was done under the following thermal profile: 95°C 3 min for 1 cycle, followed by 95°C 30 sec, 52°C 30 sec, and 72°C 1 min per cycle for 35 cycles and 72°C 5 min per cycle for 1 cycles. Ten microliters of each PCR product was run on a 1% agarose gel with a 1 Kb ladder (Promega) to determine the presence and size of amplified DNA. PCR products of the expected size (250-350 bp) were used for direct sequencing or cloning. Positive and negative controls in the PCR reaction were DNA from wCauB (B group *Wolbachia* infecting *Cadra cautella*) and distilled water as DNA template respectively.

Cloning and sequencing

Sequences that could not be obtained directly from PCR products were obtained by cloning the products in a pGEM-T vector (TA cloning kit, Promega, Madison, WI). The PCR products were analyzed using gel electrophoresis and DNA fragment was purified from agarose gel slices using QIAquick Gel Extraction kits (QIAGEN, USA) then ligated into a pGEM-T vector. Three clones were produced for each mosquito species. Clones were sequenced using an ABI automated sequencer. A consensus sequence was generated for each species from at least two clones. Sequences have been deposited in

GenBank under the following accession numbers AY515366-AY515378 and AY515569-AY515587.

Phylogenetic analysis of phage WO sequences

Our data set for phylogenetic analysis consisted of a total of 48 phage WO sequences; 32 phage WO sequences of 14 mosquito species and 16 phage WO sequences in GenBank reports (Masui *et al.*, 2000). The sequences were analyzed using Clustal X (Thompson *et al.*, 1997), PAUP 4.0b2 software (Swofford, 1998), and Modeltest 2.0 (Posada & Crandall, 1998). Owing to lack of suitable outgroup, the resulting tree was midpoint rooted.

Real time quantitative-PCR (RTQ-PCR)

To estimate phage WO and *Wolbachia* densities, the copy number of the partial *orf7* gene and *wsp* gene were measured by RTQ-PCR in ABI PRISM® 7000 Sequence Detection System (Applied Biosystems), respectively. The amplification reaction was monitored using a SYBR green. The PCR product were amplified by the primer WOorf7F [5′-GAA ATG CTT GTT CAG CTA ATA GC-3′] and WOorf7R [5′-ATA AAT TCT CCT ATT TTT TCT GGC A-3′] for phage WO and the primer 293GF [5′-GGT TTT GCT GGT CAA GTA A-3′] and 449AR [5′-GCA TCT TTG GTA ACT ACT TTT-3′] for *Wolbachia* supergroup A and the primer 293GF [5′-GGT TTT GCT GGT CAA GTA A-3′] and 444BR [5′-GCT GTA AAG AAC GTT GAT C-3′] *Wolbachia* supergroup B . The 25 μl reaction mixture consisted of 12.5 μl of 2X SYBR®Green PCR Master Mix (Applied Biosystems), 500 nM of each primers, 2 μl of template DNA, The RTQ-PCR cycling conditions were 3 min of 95°C followed by 45 cycles of 30 sec. at 95°C, 30 sec. at 52°C, and 30 sec. at

72°C for *orf7* and The conditions for *wsp* were 3 min of 95°C followed by 45 cycles of 1 min. at 95°C, 1 min. at 50°C, and 1 min. at 72°C. Standard solutions were prepared from the PCR products amplified by using the each specific primer. The products were electrophoresed on 1.5% agarose gel, and the DNA was quantified based on the optic absorbance at 260 nm and the copy number of the stock standards was calculated. Tenfold dilution series from 10^7 copy numbers to 10 copy numbers were used as the standard solution in RTQ-PCR.

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Figure legends

Figure 1. Electron micrograph of phage-like particles observed on supernatant of *Aedes albopictus* filtrated at 0.22 µm. Scale bar = 200 nm.

Figure 2. A phylogenetic trees based on genes of two origins from *wsp* representing *Wolbachia* and from *orf7* representing phage WO in Southeast Asian mosquitoes. These maximum parsimony trees were calculated using Clustal X, PAUP 4.0b2 and Modeltest 2.0. Numbers next to nodes indicate bootstrapping probabilities of 1000. Lines connecting genes of two origins show linkages among phage WO and *Wolbachia* hosts. Multiple sequences of phage WO detected in a single *Wolbachia* strain are numbered arbitrarily. The first number means the sample number whereas the second number after hyphen means the clone number.

Table 1. List of *Wolbachia* strains used in this study including group nomenclature, host species and GenBank accession numbers.

Table 2. The bacteriophage WO density in *Wolbachia* bacteria symbiont of mosquitoes.

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