(1)Mosquito collection and identification

Mosquitoes of *Culex pipiens* complex were collected in northern, central, southern, and eastern Taiwan by light or BG-sentinel traps in the routine surveillance program for *Anopheles minimus* Theobald or airport/harbors from 2012-2013. Additionally, more samples were also collected in metropolitan cities of Taiwan, northern Philippines and Japan from Japan NIID. All samples were identified under microscope following Lien (2004) and kept in a freezer until further experiments.

(2) Mosquito DNA extraction

First, pipet 20μL QUIAGEN protease into the bottom of a 1.5mL microcentrifuge, and added 200μL sample to the microcentrifuge tube. To add 200μL buffer AL to sample and mixed by pulse-vortexing for 15 seconds, than incubated at 65°C for 10 min. Briefly centrifuge the tube and removed drops from the inside of the lid. Added 200μL ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5mL microcentrifuge tube to remove drops from the inside of the lid. Carefully apply the mixture to the QIAamp Mini spin column without wetting the rim. Close the cap, and centrifuge at 8,000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 500μL Buffer AW1 without wetting the rim. To close the cap and centrifuge at 8000 rpm for 1 min and place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the collection tube containing the filtrate. Finally, place the QIAamp Mini spin column in a clean 1.5mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200μL Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 8,000 rpm for 1 min.

(3) PCR for mosquito species and DNA sequence analysis

After collection the mosquitoes DNA sample, we next performed polymerase chain reaction to differentiate the mosquitoes species or detect the avian malaria. To add 10 X buffer (Invitrogen) 2.5μL, 1μL forward primer (4 mM), 1μL reverse primer (4 mM), 2μL dNTP (2.5mM), 1μL MgCl2 (50 mM), 0.2μL Taq enzyme (Invitrogen), 1μL DNA (10-100ng/μL), and 16.3μL distilled water to a final volume 25μL. In *Culex pipiens* complex identification experiment, we use the specific primer (*Cx. quinquefasciatus* Say ACEquin: 5'-CCTTCTTGAATGGCTGTGGCA-3', 274 bp; *Cx. pipiens pallens* ACEpall:5'-ATGGTGGAGACGCATGACG-3',478 bp; *Cx. pipiens* ACEpip: 5'-GGAAACAACGACGTATGTACT-3', 610 bp; Forward primer B1246s;5'-TGGAGCCTCCTCTTCACGG-3') to amplify the target sequence that we want. The amplification program consisted of activation, 5min at 95°C (1 cycle), followed by denaturation , 30sec at 95°C, annealing, 30sec at 55°C, extension, 1min at 72°C (40 cycle), and final extension, 10min at 72°C (1 cycle). After confirming the mosquito species, we use the primer sequence F1457 (5'-GAGGAGATGTGGAATCCCAA-3'), B1246

(5'-TGGAGCCTCCTCTTCACGGC-3') to get the partial sequence of exon2 and exon3, and full intron II region sequence of ace-2 gene in mosquito nuclear DNA.

(5) TA cloning

After PCR, in order to get the correct sequence, we do the TA cloning. The TA cloning was performed by Zback Faster Ligation Kit (BIOTOOLS). To add 2X reaction buffer 5ul, DNA blunting enzyme 0.5ul, and PCR product 3ul, vortex briefly and centrifuge for 3-5 sec, then incubated the mixture at 70℃ for 5 min, then chill briefly on ice. Next, set up the ligation buffer, we added pZBack/blunt vector 1ul and T4 DNA ligase 0.5ul and incubation the mixture at room temperature for 5 min, finally use the ligation mixture directly for DH5α transformation. We added the ligation-reaction mixture to 50uL DH5α competent cell, after gently shock, placed the tube on ice for 30 min, then heat shock the cells for 90 sec at 42°C, and immediately return the tubes to ice for 3 min. To add 50uL cells to 500uL LB broth, and incubate for 1 hr at 37°C with shaking. Finally, we added 100ul the LB broth on LB agar plate with 0.002% ampicillin, incubated the plate at 37°C for 16hr and then selected the single colony for further sequencing.

(6) Phylogenetic analysis

The sequence alignment was performed by using MEGA version 5.2 Genetic distances and was calculated by using Kimura 2-parameter distance algorithm with 1,000 bootstrap replicates. We used neighbor-Joining method to generate the phylogenetic trees.

2. Avian malaria study

(1) Mosquito collection and identification

Mosquito collection was conducted for 2 successive nights in May and September 2013 at Yilan, Taiwan by using 10 suction traps enhanced with 1 kg of dry-ice. Adults were identified to species under microscope following Lien (2004) and kept in a freezer until the laboratory experiments.

(2) DNA extraction from mosquitoes using DNeasy Blood and Tissue kit

The details of protocol were described in Kim et al (2009b) and Kim and Tsuda (2010). In brief, head of mosquitoes were removed and pooled in a group of 1-10 mosquitoes per pool in a 1.5mL or 2.0mL tube. Add 180μL of Buffer ATL and homogenize mosquitoes in the tube. Add 20μL Proteinase K and vortex the tube. Incubate at 56°C for 1~3 hours (vortex the tube every 30 minutes). Add 200μL of Buffer AL and vortex well. Then, incubate at 56°C for 10 minutes and dd 200μL of Ethanol (90~100%). Pipet the mixture including any precipitate into the DNeasy Mini spincolumn placed in a collection tube. Centrifuge at 10,000rpm for 1 minute. Discard the collection tube, and place the spin column into a new collection tube. Add 500μL of Buffer AW1, and centrifuge at 10,000rpm for 1 minute. Discard the collection tube, and place the spin column into a new collection tube. Add 500μL of Buffer AW2, and

centrifuge at 14,000rpm for 3 minute. Empty the collection tube by discarding the flow-through. Reuse the same collection tube and centrifuge at 14,000rpm for 1 minute. Place the spincolumn in a clean 1.5 mL or 2 mL tube, and add 100μL of Buffer AE directly onto the DNeasy membrane. (Use a new pipet tip for each specimen to avoid contamination.). Incubate at room temperature for 1~3 minutes, then centrifuge at 10,000rpm for 1 minute. Measure the concentration of the extracted DNA.

(3) PCR for DNA sequence analysis

We performed the nested PCR followed the protocol provide by Dr. Yoshio Tsuda from Japan NIID. First, we performed the 1st PCR, we use the Fast-Run™ Taq Master Mix with Dye (ProTECH), and adjust the DNA concentration between 25-50ng/uL. We added 5X pre-mix enzyme 4uL, 0.6 μL forward primer (10mM),0.6μL reverse primer (10mM), 1uL DNA and 13.8uL ddH_2O to a final volume of 20uL. The PCR program consisted of 5min at 94°C (1 cycle), followed by denaturation, 30sec at 94°C, annealing, 30sec at 50°C, extension, 45sec at 72°C (25 cycle), and final extension, 10min at 72°C (1 cycle). Next, we use the 1st PCR product as the DNA template to perform the 2nd PCR. To add 5X pre-mix enzyme 10uL, 2 μL forward primer (10mM), 2μL reverse primer (10mM), 1.25uL DNA and 34.75uL ddH_2O to a final volume of 50uL, and the PCR condition were 94°C (1 cycle) , 30sec at 94°C, annealing, 30sec at 55°C, extension, 45sec at 72°C (35 cycle), and final extension, 10min at 72°C (1 cycle). The primer sequence used were HAEMNF (5'-CATATATTAAGAGAATTATGGAG-3'), HAEMNR(5'-AGAGGTGTAGCATATCTATCTAC-3'), HAEMF(5'-ATGGTGCTTTCGATATATGCATG-3'), and HAEMR(5'-GCATTATCTGGATGTGATAATGGT-3').

3. Japanese encephalitis virus study

(1)Mosquito collection and identification

Adult mosquitoes were collected at pig farms or wetland habitats for water-birds in northern, central, southern, and eastern parts of Taiwan during 2005 to 2012 by dry ice traps or sweep nets. Additionally, mosquitoes also collected in December 2012, April and November 2013 at northern Philippines by using 10 suction traps enhanced with 1 kg of dry-ice. Collected mosquitoes were transported either alive or on dry ice to the laboratory and were identified to species under microscope. Female mosquitoes were pooled in groups of 1-50 mosquitoes and were homogenized in a TissueLyzer (Qiagen GmbH, Hilden, Germany) for 90 sec at frequency 30 Hz for 2 times after addition of a 3 mm steel ball to each tube. The supernatants were sterilized by filtration and removed for RNA extraction and virus isolation.

(2) RNA extraction and real time RT-PCR

Viral RNA was extracted from mosquito suspensions by using the QIAamp viral RNA mini kit (Qiagen). Three sets of primers including flavivirus-specific (FL-F1:

5'-GCCATATGG TACATGTGGCTGGGAGC-3'; FL-R3:
5'-GTKATTCTTGTGTCCCAWCCGGCTGTGTCATC-3'; FL-R4:
5'-GTGATGCGRGTGTCCCAGCCRGCKGTGTCATC-3'), JEV-specific (JE3F1:
5'-CCCTCAGAACCGTCTCGGAA-3' and JE3R1:
5'-CTATTCCCAGGTGTCAATATGCTGT-3') and JEV GIII-specific (10F:
5'-CTGGGAATGGGCAATCGTG-3' and 5'-TGTCAATGCTTCCCTTCCC-3') primers were used for RT-PCR assay. Real-time RT-PCR was used to screen JEV in mosquito pools as previously described (Shu et al. 2003). DNA sequences of positive RT-PCR products were determined. Samples with positive RT-PCR were subjected to virus isolation.

(3)Virus isolation and genome sequencing

Cell culture technique using a mosquito C6/36 cell line or plaque assay using BHK cell line were used for virus isolation as described previously (Chang et al. 2010). Viral RNA was extracted from JEV-infected culture medium using the QIAamp viral RNA mini kit (Qiagen). Primers used for amplification and sequencing of complete open reading frame of JEV are listed in Table 1. The RT-PCR reaction was carried out using the Superscript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen). The cDNA synthesis step was performed at 55°C for 30 min; PCR at 94°C for 2 min; 40 cycles of 94°C for 15 sec, 50°C for 30 sec, and 68°C for 1 min; and a prolonged elongation at 68°C for 5 min. PCR products were purified using the Qiagen QIA quick Gel Extraction kit (QIAGEN). Nucleotide sequences were determined by the ABI Prism automated DNA sequencing kit and the ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's protocols. Overlapping nucleotide sequences were combined and edited with the Lasergene software package (DNASTAR Inc., Madison, WI). Nucleotide sequences of JEV strains were aligned, edited, and analyzed using ClustalW software. The phylogenetic analysis was performed using MEGA 5 (http://www.megasoftware.net/) (Tamura et al. 2011). Phylogenetic tree was generated using the maximum likelihood method based on the general time-reversible model. The reliability of the analysis was calculated using 1,000 bootstrap replications.

Result:

1. Mosquito study on *Culex pipiens* complex

In this study, we identified 71,366 *Culex pipiens* complex mosquitoes by morphology, which included 71,056 *Cx. quinquefasciatus* and 122 *Cx. pipiens* form *molestus*, and 18 *Cx. pipiens pallens* (Table 1). The PCR method to identify *Culex pipiens* complex were tested in our laboratory-breed *Cx. quinquefasciatus* (274 bp) and *Cx. pipiens* form *molestus* (610 bp) before used in the field samples (Fig. 1). 363 *Culex pipiens* complex mosquitoes were tested by PCR method, which included 260 *Cx. quinquefasciatus*, 93 *Cx. pipiens* form *molestus*, and 10 *Cx. pipiens pallens* (Table 2). The identification rate by the PCR method was 41.0% (149/363). The sample preservation for using PCR identification the mosquito species is very critical to obtain the good results. The hybrid species only found in *Cx. pipiens pallens* provided

by NIID, the CPP-01-JP, CPP-02-JP, and CPP-04-JP samples were the hybrid species of *Cx. pipiens pallens* and *Cx. quinquefasciatus*, CPP-09-JP was the hybrid species of *Cx. pipiens pallens* and *Cx. pipiens* form *molestus*.

After confirming the mosquito species, we operated the PCR experiment with F1457 and B1246 primer to get longer sequence for further phylogenetic analysis. We successfully sequenced 146 samples, including 64 *Cx. quinquefasciatus*, 39 *Cx. pipiens* form *molestus*, 4 *Cx. pipiens pallens* and some sequences downloaded from NCBI. We use partial *Cx. quinquefasciatus*, *Cx. pipiens* form *molestus*, and *Cx. pipiens pallens* sequence to do the phylogenetic analysis. In this analysis, we use the Neighbor joining method, and bootstrap analysis (1000 replicates) was applied to get the topological robustness, and the clades with at least 75% bootstrap value support were considered "supported". The result showed the *Cx. quinquefasciatus*, *Cx. pipiens* form *molestus*, and *Cx. pipiens pallens* can divided into three independent groups with good bootstrap value supported, indicated the all the molecular biology methods and the sequence can well differentiate these *Culex pipiens* complex species and one sibling species, *Cx. Australia* (Fig. 2).

In order to understand the origin of the invading *Cx. pipiens* form *molestus* in Taiwan, we analyzed the gene sequences of this species directly. First of all, we download the sequences from other countries in NCBI. After sequence alignment, and selected Kimura 2 Parameter (K2P) model, we performed phylogenetic analysis by neighbor joining methods and did 1000 replicates bootstrap value analysis. The result indicated these sequences can divide into two groups (bootstrap value: 71) (Fig. 3). One group included the gene sequences only from samples of various parts of Taiwan. The gene sequences in the second group were samples from several countries, including America, Japan, Iran, and Taiwan. However, all the sequences in this two groups can not divide into sub-group according to where these sequences from. Interestedly, TycA-02B sequence from Taoyuan International Airport grouped with two sequences from Japan, but the bootstrap value was too low (bootstrap value: 53).

We also analysis the *Cx. quinquefasciatus* mosquitoes sequence, directly (Fig. 4). The phylogenetic analysis performed by K2P model with neighbor joining method, the result was similar to the phylogenetic analysis result of *Cx. pipiens* form *molestus*. Almost all the sequence can not group well by their origin, only two sequences from Bangladesh (Bangladesh 3、Bangladesh 4), and two from Philippines (PHP08、PHP15B) can group together, respectively. Additionally, we also reanalyzed 6 samples (5 *Cx. pipiens* form *molestus* and 1 *Cx. quinquefasciatus*) by18S ribosomal DNA(1,120 bp) (Fig. 5). When blast with NCBI, the similarity of Taiwan samples with *Culex pipiens* clone PMA6J11 and Cx. *quinquefasciatus* from USA was 97% (Fig. 4).

2. Avian malaria study

Adult mosquitoes were collected by dry-ice traps at coastal area of Yilan, Taiwan in 20-22 May and 7-9 October 2013. A total of 5,362 females of 15 mosquito species were collected (Table 3). Avian *Plasmodium* parasite detection by nested PCR was

conducted and four genetically distinct lineages were identified from 3 mosquito species in May collection samples and two different avian *Plasmodium* parasites from eight mosquito species in October collection mosquitoes, respectively (Table 4). The six genetic lineages found in this study were identical to previously published sequences; *P. gallinaceum, P. elongatum*, *P. rouxi* from China, *P. lutzi* from Columbia, *P. juxtanucleare*, and *Plasmodium* species Tacy7 from Japan. The minimum infection rates per 1,000 mosquitoes for *Coquillettidia crassipes* (Van der Wulp), *Cx. sasai* Kano, Nitahara and Awaya, and *Cx. murrelli* Lien in May were between 51.3, 58.8, and 6.3, respectively. In October, the minimum infection rates per 1,000 mosquitoes for *Cx. sasai*, *Cx. murrelli*, *Cx. bitaeniorhynchus* Giles, *Armigeres subalbatus* (Coquillett), *Cx. sitiens* (Wiedemann), *Cx. fuscanus* (Wiedemann), *Cx. tritaeniorhynchus* Giles, and *Cx. annulus* Theobald were 66.7, 37.0, 100.0, 45.5, 6.9, 333.3, 6.6, and 14.0, respectively.

3. Japanese encephalitis virus study

A total of 90,574 JEV vector mosquitoes were collected and analyzed (Table 5). The most predominate species was *Cx. tritaeniorhynchus* Giles (98.5%, n = 89,189), followed by *Cx. annulus* (1.1%, n=991) and *Cx. fuscocephala* Theobald (0.4%, n=394). A total of 2,340 mosquito pools were subjected to real-time RT-PCR for the detection of JEV, among them, 480 pools were positive for JEV. The most frequently identified JEV positive mosquitoes was *Cx. tritaeniorhynchus* (468 positive pools), followed by *Cx. annulus* (9 positive pools), *Cx. fuscocephala* (3 positive pools), and their minimum infection rates per 1,000 individuals were 5.2, 9.1, and 7.6, respectively.

The JEV strains isolated from mosquitoes in Taiwan during 2005-2012 fell into two genotypes (GI and GIII). Before 2008, all of the JEV found in Taiwan belonged to GIII. Genotype 1 of JEV isolates were first identified in northern Taiwan in 2008, and then, the proportion of GI isolates in Taiwan was increasing rapidly. During 2009 to 2010, GI had become the predominant genotype of JEV circulating in Taiwan. From 2011, almost all of the JEV isolates obtained in Taiwan belonged to GI, except 2 GIII of JEV strains that were found in Kuantu Nature Park, Taipei City in 2012. Fig. 4 shows phylogenetic tree of E gene sequences of GI of JEV. The GI JEV strains in Taiwan can be grouped into 2 clusters. Cluster 1 contains JEV strains isolated from mosquitoes collected all over the country during 2008-2012, including the first two GI isolates (TPC0806c and YL0806f) identified in Taiwan. Subsequently, Cluster 1 strains of GI were found in central and northern Taiwan. These strains are most closely related to the viruses from China and Japan. Cluster 2 of GI contains virus strains were first identified in northern and central Taiwan in 2009, in the following year, these strains were found all over Taiwan. These strains are most closely related to the viruses from China and Japan.

Adult mosquitoes were collected by dry-ice traps at northern Philippines in December 2012, May and November, 2013. A total of 4,048 females of 18 mosquito species were collected (Table 6). However, no positive pools of JEV were detected.

Discussion:

In Taiwan, the *Culex pipiens* complex included only *Cx. quinquefasciatus* and *Cx. pipiens* form *molestus*. The former species was the predominant mosquito species of *Culex pipiens* complex, and were collected almost in all collection sites. The invading *Cx. pipiens* form *molestus* mosquitoes were only collected in the harbors, airports, and the northern collection sites. From phylogenetic analysis on partial ACE-2 gene segment, some mosquitoes collected in Taiwan grouped with the mosquitoes of other countries (such as America, Japan etc.). These 2 countries have very busy schedule of travels with Taiwan. In addition, adult mosquitoes of *Cx. quinquefasciatus* were commonly found inside the airplanes from Southeast Asia in the routine mosquito surveillance. Therefore, it is highly likely that the *Culex pipiens* complex of other countries can invade Taiwan through the international airports or harbors.

This study also showed that no hybrids were found in *Culex pipiens* complex species of Taiwan. We only found the hybrid species in Japanese mosquito samples of *Cx. pipiens pallens*. This confirmed that the *Culex pipiens pallens* were the hybrid species not the sub-species as the previous study (Miller et al., 1996). Mosquito samples of *Cx. pipiens* from *molestus* grouped into three clusters by phylogenetic analysis on partial ACE-2 gene but no geological differences were detected. It implied that the gene flow among these mosquitoes were very common and frequent. These nocturnal mosquitoes may be attracted by light and import or export from country to country through airplanes or ships. More direct cross-strait flights between Taiwan and mainland China, Japan or USA, the *Culex pipiens* complex were common mosquito species in cities, which were also the vector of West Nile virus and *Wuchereria bancrofti*. Therefore, mosquito surveillance and control in airports or harbors is important to prevent these invading vector mosquito species or pathogens they transmitted.

In this year's study, mosquito species found positive for avian *Plasmodium* parasites were *Co. crassipes*, *Cx. sasai*, *Cx. murrelli*, *Cx. bitaeniorhynchus*, *Armigeres subalbatus*, *Cx. sitiens*, *Cx. fuscanus*, *Cx. tritaeniorhynchus*, and *Cx. annulus*. Mosquito species found positive for avian *Plasmodium* parasites in Japan from Dr. Tsuda' study were *Cx. pipiens pallens*, *Cx. pipiens* form *molestus*, *Cx. quinquefasciatus*, *Cx. iantomii, Cx. tritaeniorhynchus, Cx. bitaeniorhynchus, Cx. nigropunctatus, Lt. vorax*, *Lt. fuscanus*, *Armigeres subalbatus*, and *Ae. albopictus*. Comparing the results, we found the common vector species between Taiwan and Japan were *Cx. tritaeniorhynchus, Cx. bitaeniorhynchus, Ar. subalbatus*, and *Cx.* (*Lutzia*) *fuscanus*. *Culex sasai, Cx. murrelli, Cx. sitiens, and Cx. annulus* found positive for avian *Plasmodium* in this study for both months or high number of the positive pools might be important vectors of avian malaria in Yilan. In this study no

Cx. quinquefasciatus were positive for avian *Plasmodium*, which was quite different from the result of Japan. *Culex pipiens* complex including *Cx. quinquefasciatus* is one of famous vectors of avian *Plasmodium* in Japan (Kim and Tsuda 2010). The avian Plasmodium lineages found in this study included two widely distributing lineages (*P. elongatum* and *P. juxtanucleare*) and lineages distributing neighboring countries; in Japan (P. tacy7 and *P. gallinaceum*), in China (may be *P. rouxi*), and in Philippines (*P. gallinaceum*). However, the gene sequence of *Plasmodium lutzi* similar to the sample from Columbia (Mantilla et al. 2013) need further study. These results supported our idea of the close genetic relationship of mosquito-borne pathogens between Taiwan and Japan, and the importance of migrating birds for introduction of novel pathogens from oversea regions.

In our study, phylogenetic analysis of E gene sequences of JEV supports the multiple introductions and maintenance of transmission cycles of GI of JEV strains in Taiwan. Gao et al. (2013) recently reported that the southernmost region (Thailand, Vietnam, and Yunnan Province, China) might be the source of GI of JEV transmission from its origin to the Asian continent including Taiwan. In their study, Cluster 1 and Cluster 2 of GI JEV strains in Taiwan belonged to the lineages of the eastern coastal Asian endemic cycle and the central Asia endemic cycle, respectively, suggesting that GI of JEV strains were likely introduced constantly from China and Japan into Taiwan in the recent years. In our study, we found GI of JEV first appeared in northern Taiwan in 2008, in the following year, GI strains were found in northern and central Taiwan. Subsequently, these viruses were spread all over Taiwan after 2009. The direction of transmission of GI of JEV in Taiwan seems in accordance with the transmission mode of JEV (GI) proposed by Gao et al (2013).

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Table 1. The sample origin and numbers of *Culex pipiens* complex collected in 2012-2013.

Collection sites	*Cx. quinquefasciatus*	*Cx. pipiens form molestus*	*Cx. pipiens pallens*
Northern Taiwan	7429	67	0
Central Taiwan	2972	11	0
Southern Taiwan	59,231	27	0
Eastern Taiwan	778	0	0
Taiwan remote islands	556	0	0
Airplanes from southeastern countries	37	0	0
Philippines	34	0	0
Japan	19	17	18
Total	71,056	122	18

Table 2. Species identification of *Culex pipiens* complex by PCR methods.

Mosquito species*		PCR identification					
		CQ	CPM	CPP	Hybrid	Unkown	Total
Morphological identification	CQ	106	1	0	0	153	260
	CPM	0	37	0	0	56	93
	CPP	0	0	1	4	5	10
	Total	106	38	1	4	214	363

*CQ: *Culex quinquefasciatus*, CPM: *Culex pipiens* form *molestus*,
CPP: *Culex pipiens pallens*.

Table 3. Mosquito species and number of females collected by dry-ice traps at Suao, Yilan, Taiwan in May and October 2013.

Species	May		October		Total	
	Female	Male	Female	Male	Female	Male
Aedes albopictus	26	0	243	0	269	0
Armigeres subalbatus	149	0	22	0	171	0
Coqillettidia crassipes	39	0	105	1	144	1
Culex annulus	58	0	499	0	557	0
Cx. tritaeniorhynchus	191	0	2,564	0	2,755	0
Cx. sitiens	8	0	866	0	874	0
Cx. bitaeniorhynchus	83	0	12	0	95	0
Cx. sasai	34	0	15	0	49	0
Cx. nigropunctatus	1	0	7	0	8	0
Cx. malayi	9	0	14	0	23	0
Cx. rubithoracis	5	0	6	0	11	0
Cx. murrelli	319	0	27	0	346	0
Cx. fuscanus	0	0	3	0	3	0
Cx. quinquefasciatus	29	0	2	0	31	0
Mansonia uniformis	12	0	14	0	26	0
All Groups	963	0	4,399	1	5,362	1

Table 4. Detection of avian *Plasmodium* parasite DNA from mosquitoes collected at Suao, Yilan, Taiwan during May and September 2011.

Month	Species(females)	No. examined	No. pools	*Plasmodium* no. positive	Lineage	Distribution	MIR*
May	*Coquillettidia crassipes*	39	4	2	*Plasmodium gallinaceum*	Japan, Philippines	51.3
	Culex sasai	34	4	2	*Plasmodium elongatum*	World wide	58.8
	Culex murrelli	319	32	2	*Plasmodium rouxi* *Plasmodium lutzi*	China Colombia	6.3
	Culex bitaeniorhynchus	83	9	0			0.0
	Culex tritaeniorhynchus	191	20	0			0.0
	Armigeres subalbatus	149	15	0			0.0
	Aedes albopictus	26	3	0			0.0
	Culex rubithoracis	5	1	0			0.0
	Culex malayi	9	1	0			0.0
	Mansonia uniformis	12	2	0			0.0
	Culex nigropunctatus	1	1	0			0.0
	Culex quinquefasciatus	29	3	0			0.0
	Culex annulus	58	6	0			0.0
	Culex sitiens	8	1	0			0.0
October	*Coquillettidia crassipes*	104	11	0			0.0
	Culex sasai	15	2	1	*Plasmodium* sp. Tacy7	Japan	66.7
	Culex murrelli	27	3	1	*Plasmodium* sp. Tacy7	Japan	37.0
	Culex bitaeniorhynchus	10	1	1	*Plasmodium* sp. Tacy7	Japan	100.0
	Armigeres subalbatus	22	3	1	*Plasmodium* sp. Tacy7	Japan	45.5
	Aedes albopictus	243	25	0			0.0
	Culex rubithoracis	5	1	0			0.0
	Culex malayi	14	2	0			0.0
	Mansonia uniformis	14	2	0			0.0

Culex nigropunctatus	7	1	0			0.0
Culex quinquefasciatus	2	1	0			0.0
Culex sitiens	866	87	6	*Plasmodium* sp. Tacy7 *x4*	Japan	6.9
				Plasmodium juxtanucleare x2	World wide	
Culex fuscanus	3	1	1	*Plasmodium juxtanucleare*	World wide	333.3
Culex tritaeniorhynchus	2,564	257	17	*Plasmodium sp.* Tacy7 （AB601436 ） x8	Japan	6.6
				Plasmodium juxtanucleare x9	World wide	
Culex annulus	499	50	7	*Plasmodium sp.* Tacy7 （AB601436 ） x4	Japan	14.0
				Plasmodium juxtanucleare x3	World wide	

*Minimum infection rate for 1,000.

Table 5. Summary of JE Vector species collected from 2005 to 2012 and their minimum infection rates of JEV.

Species	No. Individuals	No. pools	No. Pos Pools	MIR*
Culex annulus	991	79	9	9.1
Culex fuscocephala	394	19	3	7.6
Culex tritaeniorhynchus	89,189	2,242	468	5.2
Total	90,574	2,340	480	5.3

*Minimum infection rate for 1,000.

Table 6. The number of mosquitoes collected in northern Philippines from 2012-2013 by 10 suction traps for 2 nights.

Mosquito species	2012		2013				Total	
	December		April		November			
	Female	Male	Female	Male	Female	Male	Female	Male
Aedes vexans	0	0	0	0	13	0	13	0
Anopheles annularis	0	0	0	0	4	0	4	0
Anopheles barbirostris	4	0	0	0	0	0	4	0
Anopheles barbumbrosus	0	0	17	0	23	0	40	0
Anopheles indifinitus	0	0	26	0	12	0	38	0
Anopheles ludlowae	0	0	1	0	0	0	1	0
Anopheles minimus	1	0	0	0	8	0	9	0
Anopheles sinensis	0	0	0	0	1	0	1	0
Anopheles tessellatus	0	0	2	0	1	0	3	0
Armigeres subalbatus	4	0	6	0	68	0	78	0
Coquillettidia crassipes	1	0	5	0	18	0	24	0
Culex fuscocephala	410	0	4	0	193	0	607	0
Culex gelidus	1	0	36	0	133	0	170	0
Culex quinquefasciatus	323	4	46	5	167	5	536	14
Culex sitiens	0	0	33	0	205	0	238	0
Culex tritaeniorhynchus	1	0	10	0	563	0	574	0
Culex vishnui	89	0	1,040	0	364	0	1,493	0
Mansonia uniformis	0	0	33	0	182	15	215	15
Total	834	4	1,259	5	1,955	20	4,048	29

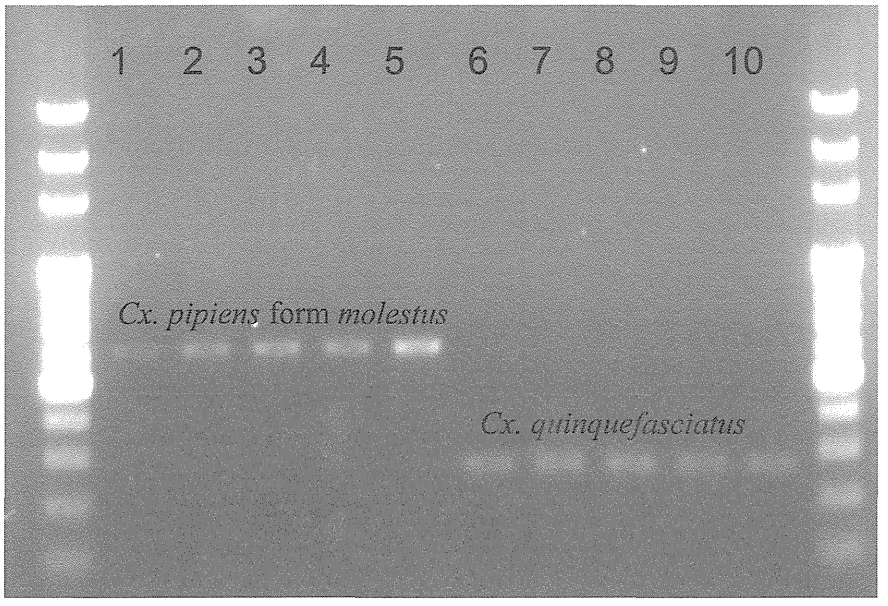

Fig. 1. Testing of PCR identification on Culex pipiens complex, Lane1-5: *Cx. pipiens* form *molestus* (610 bp). Lane6-10: *Cx. quinquefasciatus* (274 bp).

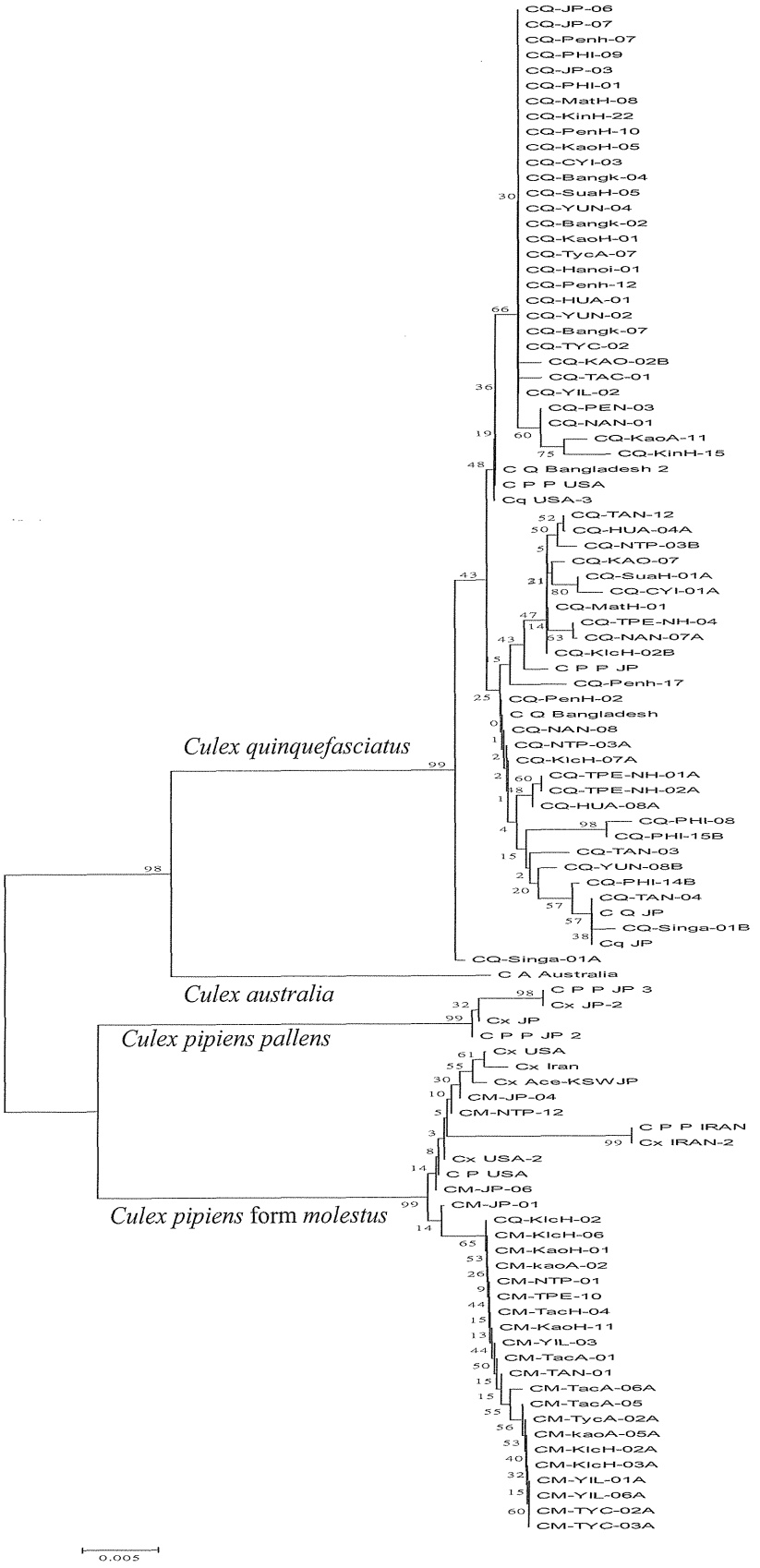

Fig. 2. The phylogenetic analysis of *Culex pipiens* complex on partial ACE-gene sequences. (JP:Japan, PHI: Philippens, Bangk: Thailand, Hanol: Vitman, Singa: Singapore)

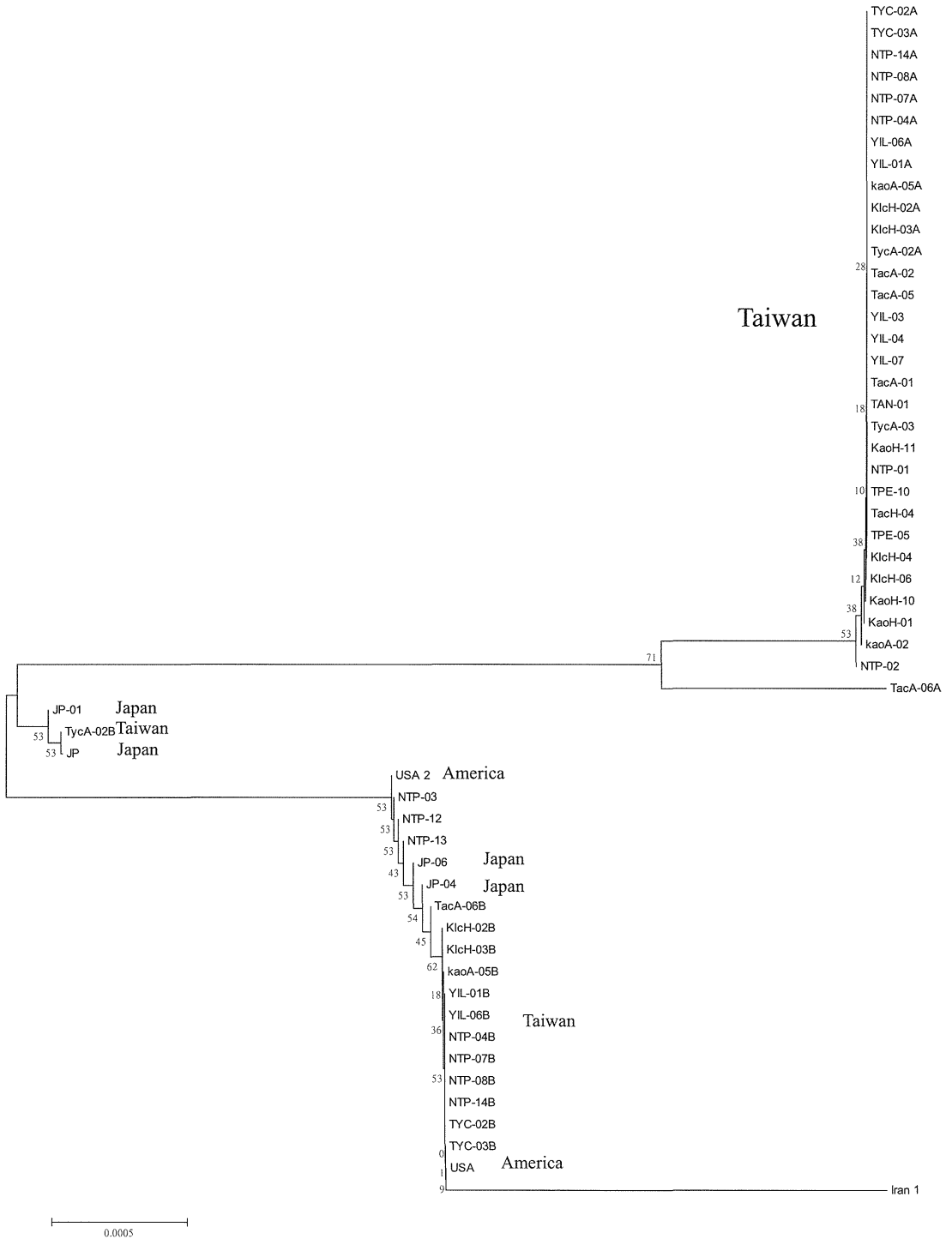

Fig. 3. The phylogenetic analysis of *Culex pipiens* form *molestus* on partial ACE-gene sequences.

Fig. 4. The phylogenetic analysis of *Culex quinquefasciatus* on partial ACE-gene sequences. (JP:Japan, PHI: Philippens, Bangk: Thailand, Hanol: Vitman, Singa: Singapore)