Genetic relationship of vector mosquitoes and the vector-borne pathogens between Taiwan and Japan

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Summary:

This project of this year is to study the genetic relationship of vector mosquitoes and vector-borne pathogens between Taiwan and Japan to clarify the taxonomic status of *Culex annulus* and gene flow of *Cx. vishnui* subgroup. The results showed that *Cx. annulus* and *Cx. vishnui* is the same species which share the common gene pool between Taiwan and Japan, even extend to mainland China. The same situation was also applied to the population of *Cx. tritaeniorhynchus* between Taiwan and Japan. Additionally, we also presented our surveillance results of dengue cases in Taiwan in 2012. A total of 207 confirmed imported dengue cases were identified. The travelers were arriving from 11 countries in Southeast Asia, Western Pacific, and the Indian subcontinent. From the acute phase serum samples of imported dengue cases, 44 DENV-1, 35 DENV-2, 17 DENV-3, and 22 DENV-4 strains were identified by PCR. For local dengue outbreaks in Taiwan, a total of 1,271 dengue cases were laboratory confirmed with 36 cases of dengue hemorrhagic fever. Epidemiological study showed that 7 different DENV strains were circulated in Taiwan, including 3 DENV-1 strains circulating in Tainan City, Kaohsiung City, Taoyuan County, New Taipei City, 2 DENV-2 strains circulating in Kaohsiung City, Tainan City, Pingtung County, 1 DENV-3 strain circulating in Kaohsiung City; and 1 DENV-4 strain circulating in Kaohsiung City. Phylogenetic analyses of E gene sequences of DENVs isolated from indigenous and imported dengue cases showed that most epidemic DENVs circulated in Taiwan in 2012 were introduced from Southeast Asia. In addition, a DENV-1 strain introduced from the Central America caused overwinter outbreak in Tainan City during 2011-2012.

Purpose:

To study the genetic relationship of vector mosquitoes and vector-borne pathogens between Taiwan and Japan can provide basic information on the risk of vector-borne infectious diseases. Understanding the epidemiological situations of the diseases and the phenotypic and genotypic characteristics of viruses and vectors contributes to the development of new strategies for control and prevention. This year was focus on Japanese vectors, including *Culex tritaeniorhynchus* Giles, *Cx. annulus* Theobald, and *Cx. fuscocephala* Theobald, which belonged to *Cx. vishnui* subgroup. This subgroup also include *Cx. Pseudovishnui, Cx. perplexus, Cx. Alienus,* and *Cx. Incognitus*. These mosquitoes showed

similar phenotype and breeding habits, result in confusing us about mosquito classification. As a result, we applied the molecular biology methods to further classify the mosquito species. Additionally, dengue virus was also studied.

Method:

1. Mosquito study

(1)Mosquito collection and identification

We collect the at least 10 larva or adult mosquitoes (*Cx tritaeniorhynchus* Giles, *Cx. annulus* Theobald, *Cx. psudovishnu*i) in northern, southern, middle, eastern Taiwan and Philippine. We also apply the *Cx tritaeniorhynchus* Giles, *Cx. annulus* Theobald, *Cx. psudovishnu*i DNA sequence or mosquitoes from National Institute of Infectious Diseases for further study. The phenotype identification was performed by an experience-rich expert by stereo dissecting microscope follow Lien's method.

(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℃ for 10 min. Briefly centrifuge the tube and removed drops from the inside of the lid. Added 200 μl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml 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 8000 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 500 μl 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 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 μl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min.

(3)PCR

After collection the mosquitoes DNA sample, we next performed polymerase chain reaction. 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 distilledwater to a final volume 25μl. We use the specific primer to amplify the target sequence that we want. 18SF (GTAAGCTTCCTTTGTACACACCGCCCG), 28SR1 (GGGGTAGTCACACATTATTTG), 18SF4 (GGCTGGTCAGTCTATATCGC), 58SR1 (TTGCGGATGACCAGTCG). Two sets of primers including Flavivirus-specific primers (FL-F1: 5'-GCCATATGGTACATGTGGCTGGGAGC-3'; FL-R3: 5'-GTKATTCTT GTGTCCCAWCCGGCTGTGTCATC-3; FL-R4: GTGATGCGRGTGTCCCAGCCR GCKGTGTCATC-3') and JE virus-specific primers (10F : 5'-CTGGGA ATGGGCAAT CGTG-3', 325R:5'-TGTCAATGCTTCCCTTCCC-3') were used for real-time RT-PCR. Following the PCR program to amplify sequence: Activation, 3min at 95°C(1 cycle), Denaturation, 30sec at 95°C(40 cycle), Annealing, 30sec at 52°C(40 cycle), Extension, 1min at 72°C(40 cycle), and final Extension, 4min at 72°C (1 cycle).

(4) DNA sequence analysis and phylogenetic analyses

We analysis DNA sequence base on 18S rRNA, partial sequence of internal transcribed spacers2(ITS2), 5.8S rRNA gene, and full internal transcribed spacers 1(ITS1) sequence in mosquito ribosomal DNA spacer. Multiple sequence alignment was performed by using MEGA version 5. Genetic distances were calculated by using Kimura 2-parameter distance algorithm with 1,000 bootstrap replicates. We used neighbor-Joining method to generate the phylogenetic trees.

2. Dengue viruses

(1)Clinical samples and laboratory diagnosis

Human serum samples from clinically suspected DENV and other arbovirus infections were submitted to Research and Diagnostic Center, Centers for Disease Control, Taiwan (Taiwan CDC), Department of Health, for laboratory diagnosis. A confirmed dengue case was defined as febrile illness associated with a positive real-time reverse transcription (RT)-PCR test, isolation of DENV, or a ≥4-fold increase in DENV-specific IgM and IgG antibodies. A multiplex one-step real-time RT-PCR was developed to simultaneously detect and differentiate various flaviviruses and alphaviruses in the acute-phase serum samples using group-specific and virus-specific primers. In addition, a flavivirus/alphavirus-specific capture ELISA was developed to detect and differentiate various flavivirus/alphavirus infections.

(2)Virus isolation and identification

DENVs were isolated from the acute phase serum samples of confirmed cases. The virus isolation was performed using mosquito cell line (clone C6/36 of Aedes albopictus cells). For each acute phase serum, 4 μl of serum sample was diluted in 200 μl cultured medium

（RPMI , Gibco/BRL, Life Technologies, containing 1% FCS）and added to a 96-well microtiter plate, 50μl/well in quadruplicate. Then, 105 cells/100μl/well of C6/36 cell line were added into the microtiter plate and incubated at 30℃ for 2-7 days. Cells were harvested and virus isolates were identified by the indirect fluorescent antibody test with virus group-specific and serotype-specific monoclonal antibodies.

(3)Primers used for RT-PCR and nucleotide sequencing of DENV

The diagnostic tests for flavivirus infection from fevered patients on the basis of the results of one-step SYBR Green I-based real-time RT-PCR and envelope/membrane-specific capture IgM and IgG ELISA had been described previously. To screen viremic fever patients with alphavirus infection, a multiplex one-step SYBR Green I-based real-time RT-PCR was developed. A cocktail consisted with three sets of primers were mixed and used for RT-PCR screening. The alphavirus-specific primer set （AL-2: 5'-AAG CTY CGC GTC CTT TAC CAA AG-3' and AL-3: 5'-GTG GTG TCA AAC CCT ATC CA-3'） targeted a consensus region of the nonstructural protein 1 (nsp1) genes to detect all alphaviruses. Positive results were then confirmed by gene sequence analysis, virus isolation, and serological test.

(4)Preparation of viral RNA, RT-PCR amplification and nucleotide sequencing

Viral RNAs were extracted from either acute phase serum samples or culture supernatant of C6/36 cell line infected with each of the isolated DENV or CHIKV strains using the QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleotide sequences of partial NS5 gene fragment of DENV were determined directly from acute phase serum samples using RT-PCR product of one-step SYBR Green I quantitative RT-PCR . Partial NS5 gene sequencing was routinely performed to detect and differentiate serotype and genotype of the newly identified DENVs. For full-length structure gene sequencing, extracted viral RNA from culture supernatant of C6/36 cell line infected with each of the isolated DENV strains was used as a template for cDNA synthesis, which subsequently was used for PCR amplification. Two overlapping PCR products spanning the full-length structure gene were purified from agarose gels and directly sequenced in both directions using ABI Prism automated DNA sequencing kit and ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. Overlapping nucleotide sequences were combined for analysis and edited with the Laser software package (DNASTAR Inc, Madison, WI).

(5)Phylogenetic analysis

Phylogenetic analyses were conducted using PHYLIP (version 3.6) or MEGA version 5. Genetic distances were calculated by using Kimura 2-parameter distance algorithm with

1,000 bootstrap replicates. Neighbor-Joining method was used to generate the phylogenetic trees.

Result:

1. Mosquito study

We collected the mosquito samples in Taiwan, all mosquitoes carried to laboratory for further analysis. In Taiwan, we also collected mosquitoes in different areas, especially; we go to Wu-Wei Gang waterfowl, the protected areas in Yilan county. We detected the flavivirus and Japanese encephalitis virus in these collected mosquitoes, a total of 497 C*x. tritaeniorhynchus* females adults were grouped by date into 11 pools, and were analyzed for both flavivirus and JE virus infections by the real-time PCR. We didn't detect any flavivirus or JEV signal by real time PCR in these mosquitoes (Table 1).

After collection of mosquito samples, we created the *Cx. annulus*/ *Cx. vishnui* gene map. We aligned the 18S rRNA and 5.8S rRNA partial gene sequence and full length of internal transcribed spacers 1, and then did phylogenetic analysis. We analyzed 83 *Cx. annulus* DNA sequences, and one sequence from China, eight sequences from Japan. The multiple sequences alignment showed there was 96-100% similarity in these sequences. The phylogenetic analysis indicated that the Japan *Cx. vishnui* can't form a specific group, but grouped to other mosquito sequences collected from Taiwan. Four *Cx.. vishnui* sequences (CV-JP244-1, CV-JP205-1, CV-JP622 and CV-JP494-1) grouped to other 31 *Cx. annulus* sequences from Taipei city, Kaohsiung city, Yunlin county, Hualien county, and Tainan. Two sequences (CV-JP56R and CV-JP54) grouped to other 12 *Cx. annulus* sequences from different Taiwan areas, and other two *Cx. vishnui* sequences (CT11and CT-JP50-1) grouped to 17 *Cx. annulus* sequences from Taiwan, and one from China. (Fig.1)

We also analyzed the *Culex tritaeniorhynchus* gene map by 18S rRNA and internal transcribed spacers 2 (ITS2) partial sequence, internal transcribed spacers 1(ITS1) and full length of 5.8S rRNA. The mosquito samples also collected in Taiwan, and receive the related gene from Japan. After sequences alignment, there was 96-100% similarity between all the sequences. The phylogenetic tree showed the sequences from Japan can't grouped as a specific cluster, eight samples from Japan (CT-JP12-1, CT-22, CT-JP49-1, CT-JP50-2, CT-JP6-2, CT-JP9-1, CT-JP51-1 and CT-JP9-2) were grouped to twenty four sequences from Taiwan; other four samples from Japan (CT-JP15-2, CT-JP8-2, CT-JP8-1, and CV-JP15-1) also grouped to eleven sequences from Taiwan. (Fig.2)

2. The Epidemiology of Dengue

(1)Imported dengue cases in Taiwan, 2012

A total of 207 laboratory confirmed imported dengue cases were identified in Taiwan in 2012. Table 1 showed the summary of countries of origin and the DENV serotypes of

imported cases. The imported cases were arriving from 11 countries. Most of these imported cases were infected in the Philippines, Indonesia, Vietnam, and Thailand. DENV-1 and DENV-2 are the predominant serotypes of imported DENV strains. Among them, 42 DENV-1, 23 DENV-2, 12 DENV-3, and 19 DENV-4 strains were isolated. The nucleotide sequences of complete E gene sequences of DENV strains were determined and deposited in Taiwan Pathogenic Microorganism Genome Database for molecular epidemiological analysis.

(2) Multiple dengue epidemics in Taiwan, 2012

For local dengue outbreaks in Taiwan, a total of 1271 dengue patients were laboratory confirmed with 36 cases of DHF in 2012. The complete E gene sequences of DENV strains isolated from representative indigenous dengue cases were determined. Representative indigenous cases were selected based on the information of epidemiological investigation and preliminary results of partial NS5 gene sequences showing different infection time, infection place, and DENV serotypes and genotypes of these patients. Sequence analyses of DENV isolates obtained from acute phase serum samples of indigenous cases showed that 7 different DENV strains (3 DENV-1, 2 DENV-2, 1 DENV-3, and 1 DENV-4) were circulated in Tainan City (744 cases), Kaohsiung City (507 cases), Taoyuan County (7 cases), New Taipei Cities (5 cases), and Penghu County (2 cases).

(3) Nucleotide sequencing and phylogenetic analysis

Phylogenetic analyses of E gene sequences of DENVs isolated from indigenous and imported dengue cases showed that epidemic DENVs circulating in Taiwan in 2012 were likely introduced from the Americas, the Philippines and Cambodia (DENV-1); Indonesia and Thailand (DENV-2); Thailand (DENV-3) and the Philippines (DENV-4). Figure 3 shows the phylogenetic tree of complete E gene sequences of DENV-1 strains. Phylogenetic analysis showed that genotype I of DENV-1 contains virus strains isolated from imported cases from Indonesia, Malaysia, Myanmar, Cambodia and Vietnam. Genotype II of DENV-1 contains virus strains from the Philippines. Genotype III of DENV-1 contains virus strains from India and Malaysia. A DENV-1 strain, D1/Taiwan/700TN1109a/2011, is the major epidemic strain circulating in Tainan City during 2011-2012. Notably, this strain belonged to genotype III and is closely related to virus strains from Haidi in Central America. Figure 4 showed the phylogenetic tree derived from complete E gene sequences of DENV-2. Phylogenetic analysis showed that DENV-2 strains isolated from imported cases from Vietnam, Cambodia, and Thailand in 2012 belonged to the Asian genotype 1. DENV-2 strains isolated from imported cases from the Philippines, Indonesia, India and Bangladesh belonged to the Cosmopolitan genotype. A DENV-2 strain, D2/Taiwan/KH/2012, which belonged to the Cosmopolitan genotype, is the major epidemic strain circulating in Kaohsiung City in 2012. This strain is closely related to virus strains from Indonesia.

Discussion:

This study proof that the *Cx. vishnui* mosquito in Japan and *Cx. annulus* in Taiwan was the same species, from the phylogenetic analysis, we know that there was no geographic specific cluster. In Taiwan, previously study showed the phenotype of *Cx. vishnui* was different with *Cx. annulus*, so finally, this species mosquito was named the *Cx. annulus.* The molecular biology method clearly indicated *Cx. vishnui* and *Cx. annulus* was the same species and share common gene pool.

The increase in international trade and tourism facilitated the spread of disease vectors and pathogens. Our results showed the constant importation of various DENV strains into currently non-endemic areas, including Japan and Taiwan. Molecular epidemiologic studies analyzing DENV strains isolated from imported and indigenous cases showed that different serotypes, genotypes, and/or strains were responsible for outbreaks that occurred each year, and the epidemic strains disappeared with the ending of each local outbreak in Taiwan. Notably, our study showed that epidemic strains circulating in Taiwan were all introduced from Western Pacific and Southeast Asia, except an epidemic strain that circulating in Tainan City during 2011-2012 belonged to genotype III of DENV-1, which is closely related to virus from the Central America. The results demonstrated that local mosquitoes in Taiwan are competent vectors for transmission of a variety of DENV strains circulating in Southeast Asian, Western Pacific as well as Central America. More effective and efficient control program of dengue, including various surveillance systems, network of rapid diagnostic laboratories, and rapid response carried out by central and local health departments would be needed to control dengue and maintain Taiwan as a non-endemic country.

Virus	Total pools	Positive pools	No. of *Culex tritaeniorhynchus* tested	
detected	tested		May	September
JE virus	11	0	39	458
Flavivirus	11	0	39	458

Table 1. Virus infection in field-caught *Culex tritaeniorhynchs* adults collected in Wu-Wei Gang waterfowl protected areas in Yilan.

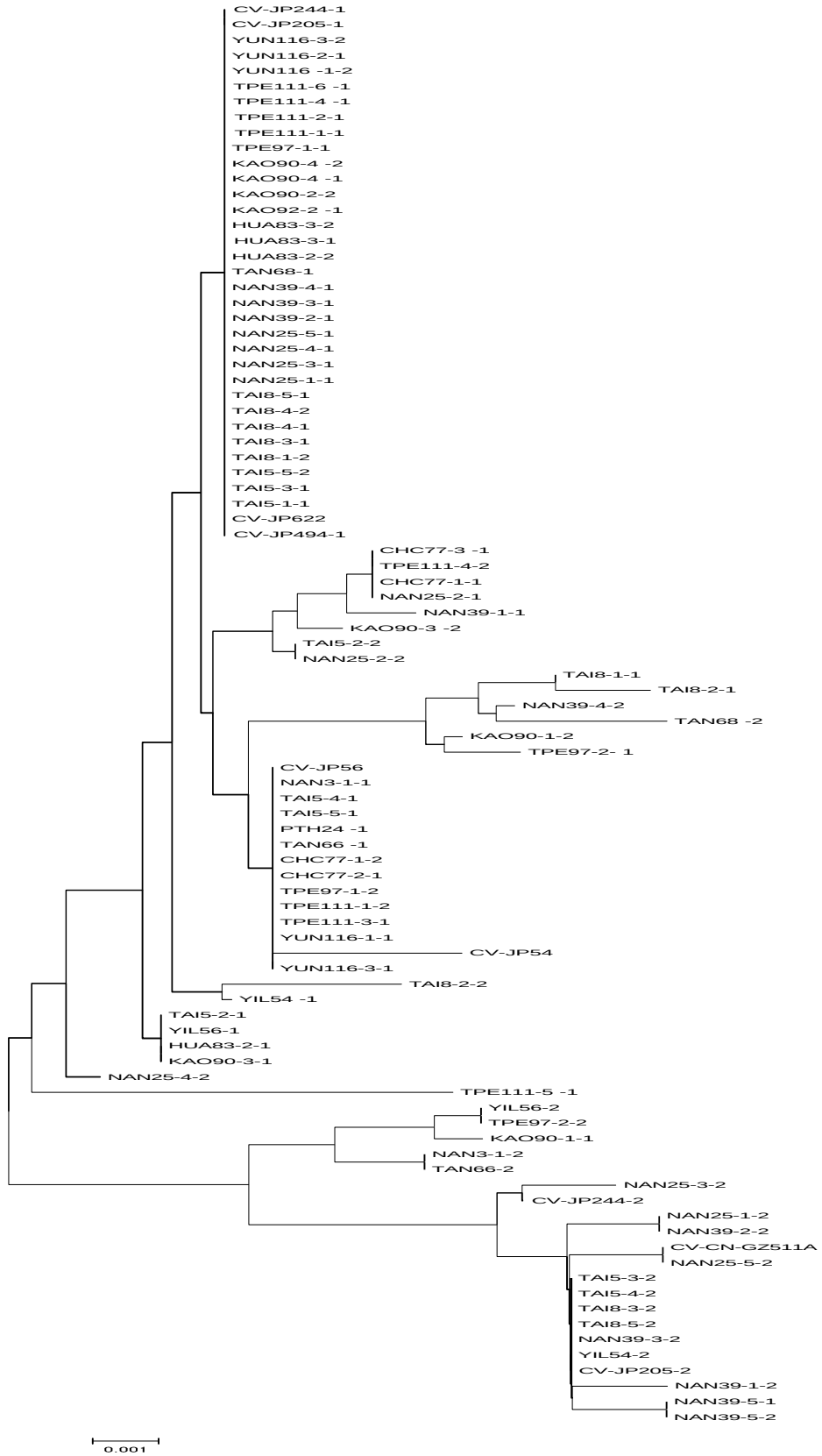

Fig.1 The phylogenetic analysis of *Cx. annulus* (Taiwan) and *Cx. vishnui* (Japan). All the sequence didn't form the specific group, but grouped with other sequence originated from Taiwan.

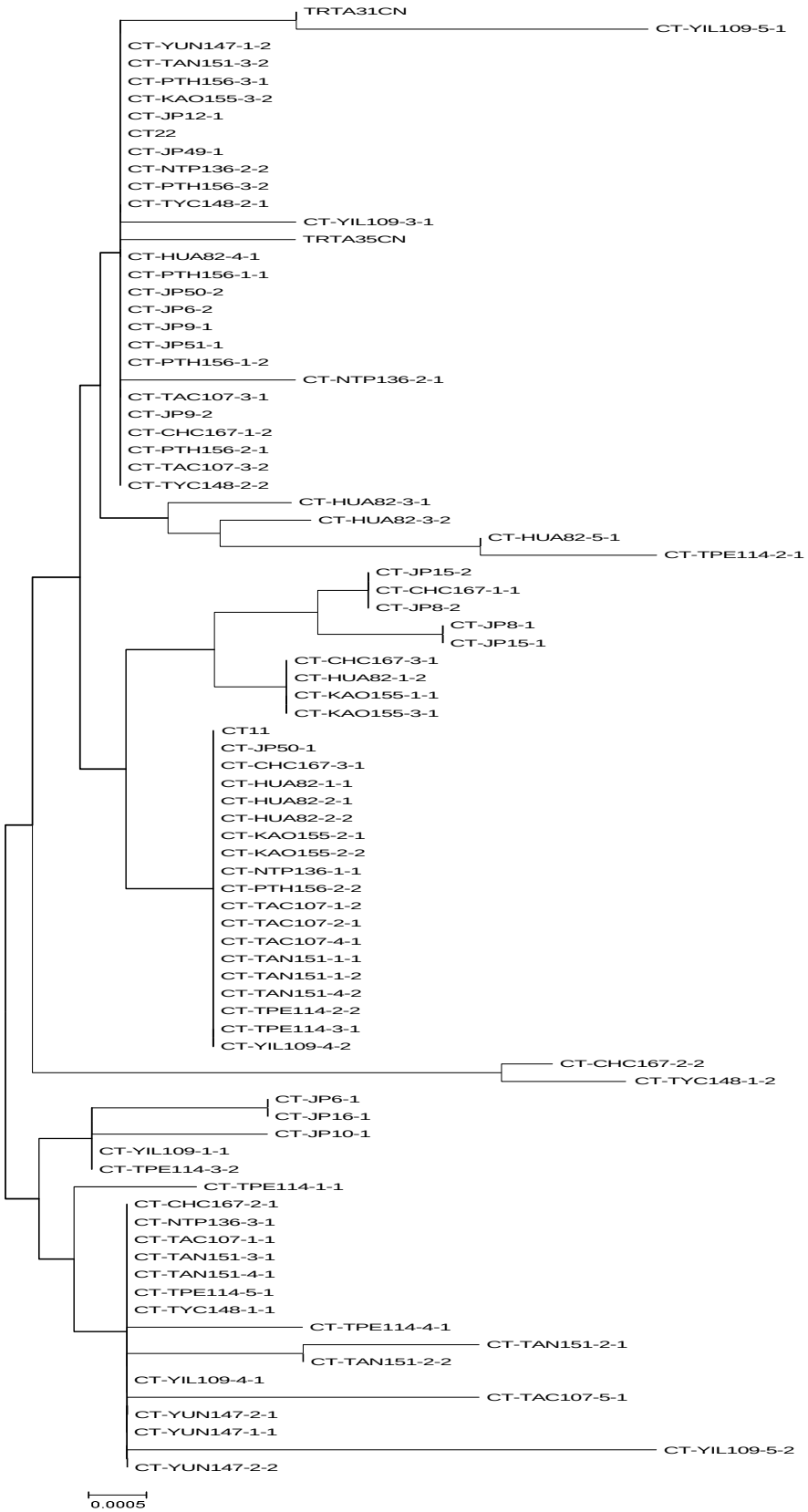

Fig.2 The phylogenetic analysis of *Culex tritaeniorhynchus* from Japan and Taiwan. All the sequence didn't form the specific group, but grouped with other sequence originated from Taiwan.

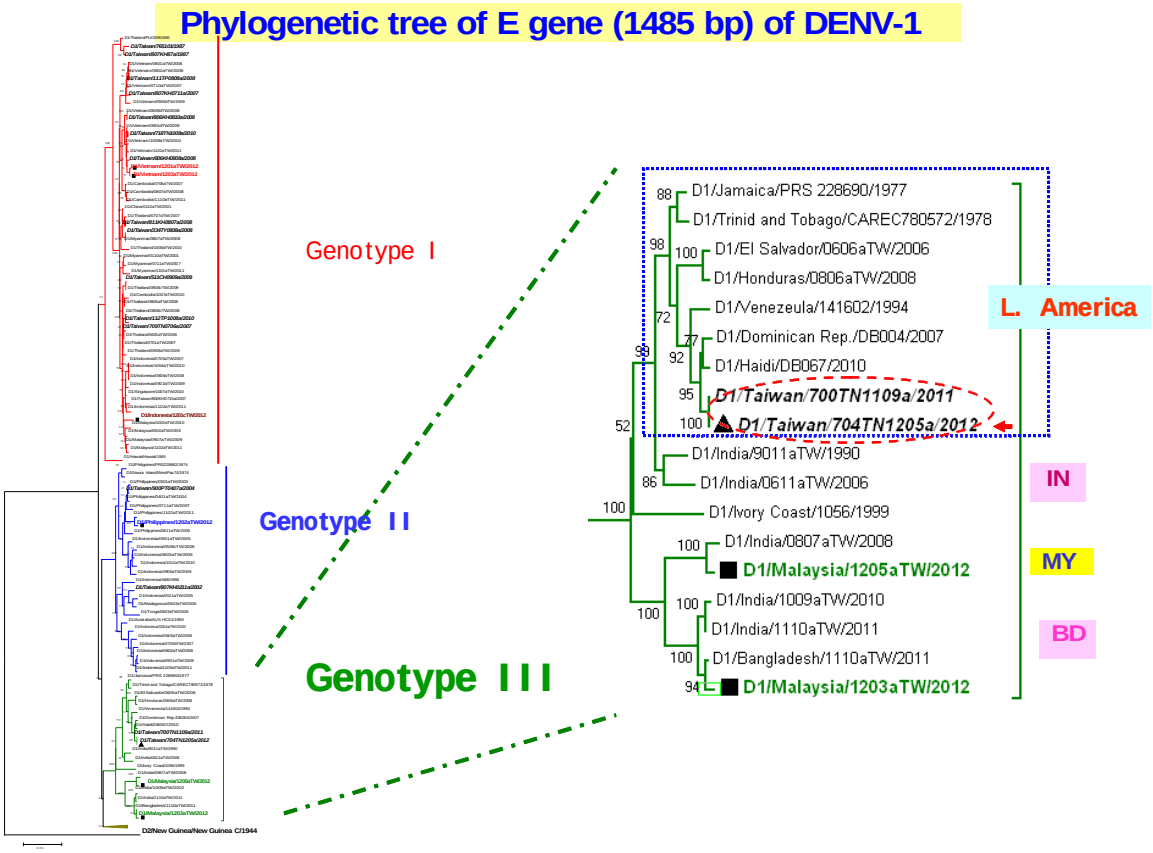

Figure 3. Phylogenetic trees derived from full-length E gene sequences of DENV-1. DENV-1 strains isolated from imported and indigenous cases in Taiwan, 2012, are designated in full squares and full triangles, respectively. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation

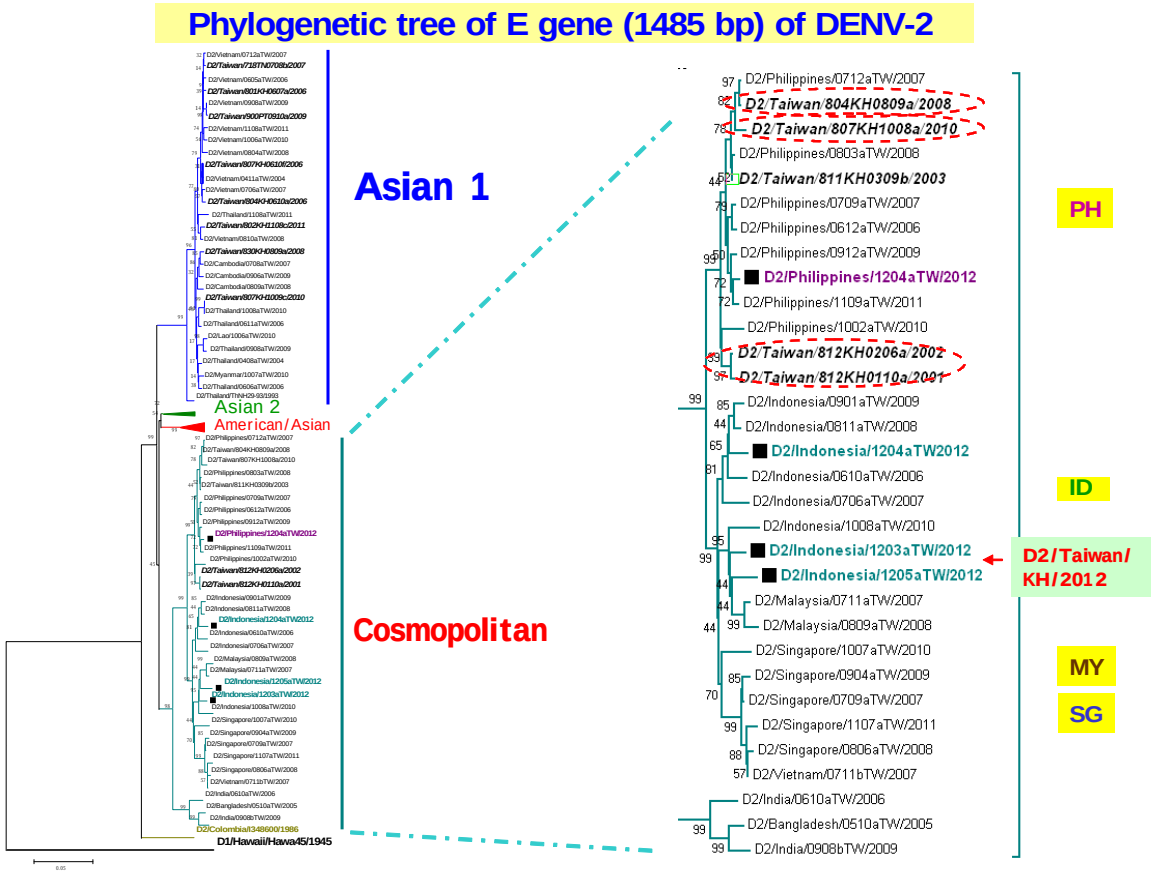

Figure 4. Phylogenetic trees derived from full-length E gene sequences of DENV-2. DENV-2 strains isolated from imported cases in Taiwan, 2012, are designated in full squares. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation

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Publication list for this work:

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