SURVEY AND CHARACTERIZATION OF MOSQUITO-BORNE FLAVIVIRUSES IN NATURAL POPULATIONS OF MOSQUITOES IN THAILAND

WORAWIT UAMCHAROEN

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……
Mr. Worawit Uamcharoen
Candidate

……
Assoc. Prof. Pattamaporn Kittayapong, Ph.D.
Major advisor

……
Prof. Jean-Paul Gonzalez, M.D., Ph.D.
Co-advisor

……
Lect. Pahol Kosiyachinda, Ph.D.
Co-advisor

……
Assoc. Prof. Rassmidara Hoonsawat
Ph.D
Dean
Faculty of Graduate Studies
Mahidol University

……
Assoc. Prof. Prayad Pokethitiyook
Ph.D.
Chair
Master of Science Programme
in Environmental Biology
Faculty of Science

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On
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.....
Mr. Worawit Uamcharoen
Candidate

.....
Assoc. Prof. Pattamaporn Kittayapong,
Ph.D.
Chair

.....
Prof. Jean-Paul Gonzalez,
M.D., Ph.D.
Thesis Defense Committee

.....
Assoc. Prof. Vijitta Lerdkamolkarn
Ph.D.
Thesis Defense Committee

.....
Lect. John R. Milne,
Ph.D.
Thesis Defense Committee

.....
Assoc. Prof. Rassamidara Hoonsawat
Ph.D
Dean
Faculty of Graduate Studies
Mahidol University

.....
Assoc. Prof. Prasert Sobhon
Ph.D.
Dean
Faculty of Science
Mahidol University

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Worawit Uamcharoen

SURVEY AND CHARACTERIZATION OF MOSQUITO-BORNE FLAVIVIRUSES IN NATURAL POPULATIONS OF MOSQUITOES IN THAILAND

WORAWIT UAMCHAROEN 4237510 SCEB/M

M.Sc. (ENVIRONMENTAL BIOLOGY)

THESIS ADVISORS: PATTAMAPORN KITTAYAPONG, Ph.D., JEAN-PAUL GONZALEZ, M.D., Ph.D., PAHOL KOSIYAJINDA, Ph.D.

ABSTRACT

Mosquitoes are of considerable medical and veterinary importance because they harm the host through their feeding action and vector many pathogens. In particular, there has been no comprehensive survey on mosquito-borne flaviviruses, which are implicated in dengue fever, Japanese encephalitis and other diseases. Mosquito-borne flaviviruses in Thailand were surveyed in this research. A total of 1,257 individuals representing 40 species of mosquitoes in 6 genera collected from 8 provinces of Thailand were examined for flavivirus by RT-PCR assay using universal flavivirus primers. One mosquito species, *Culex fuscocephala,* collected from Wang Thong District, Kampaengphet Province, Thailand, was flavivirus positive. Flavivirus infection rate in this species was 11.1% (2/18). Phylogenetic relationships among this virus and other flaviviruses were inferred from comparison of sequences of the partial NS5 gene. Results of phylogenetic analysis using maximum parsimony, neighbor-joining and maximum likelihood methods indicated that this virus represented a new flavivirus genotype closely related to The Cell Fusion Agent Virus (CFAV), found in *Aedes aegypti* mosquito cell line and The Kamiti River Virus (KRV) detected in the mosquito *Aedes macintoshi* from Kenya, respectively. This new flavivirus was designated as the Wang Thong Virus (WTV)

KEY WORDS: FLAVIVIRUS / CELL FUSION AGENT (CFAV) / CULEX / MOSQUITO / PHYLOGENY

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การสำรวจและการศึกษาคุณลักษณะของ Flavivirus ที่นำโดยยุงพาหะในประเทศไทย (SURVEY AND CHARACTERIZATION OF MOSQUITO-BORNE FLAVIVIRUSES IN NATURAL POPULATIONS OF MOSQUITOES IN THAILAND)

วรวิทย์ อ่วมเจริญ 4237510 SCEB/M
วท. ม. (ชีววิทยาสภาวะแวดล้อม)
คณะกรรมการควบคุมวิทยานิพนธ์: ปัทมาภรณ์ กฤตยพงษ์, JEAN-PAUL GONZALEZ, MD., Ph.D., พหล โกสิยจินดา, Ph.D.

บทคัดย่อ

ยุงจัดเป็นแมลงพาหะนำโรคที่สำคัญทางการแพทย์ เป็นสาเหตุสำคัญที่ทำให้สุขภาพของคนและสัตว์เสื่อมโทรมลง ไม่ว่าจะเป็นการก่อให้เกิดความรำคาญ การเกิดอาการแพ้ เป็นตุ่มคัน การสูญเสียเลือด หรือการเป็นโรคต่างๆ อันเนื่องมาจากยุงเป็นพาหะ ซึ่งอาจรุนแรงจนถึงขั้นเสียชีวิตได้ แม้ว่าในประเทศไทยจะมีการศึกษาการระบาดของโรค และการควบคุมโรคเป็นจำนวนมาก แต่ยังไม่มีประสิทธิภาพดีพอ ดังนั้นการศึกษาไวรัสที่ก่อให้เกิดโรคในยุงธรรมชาติ จึงเป็นเรื่องจำเป็น เพื่อเฝ้าระวังและป้องกันโรค อันอาจเกิดการระบาดได้ในอนาคต ดังนั้นการวิจัยนี้ได้ทำการศึกษาเชื้อไวรัสในตระกูลฟลาวิไวรัสในยุง โดยทำการเก็บตัวอย่างจากยุงจำนวน 1,257 ตัว 40 สปีชีส์ จาก 8 จังหวัดในประเทศไทย มาสุ่มตรวจหาเชื้อไวรัส ด้วยเทคนิค RT-PCR โดยใช้ primer ที่จำเพาะต่อไวรัสเหล่านั้น จากผลการทดลองพบว่าร้อยละ 11 ของยุงชนิด *Culex fuscocephala* จากตำบลวังทอง จังหวัดกำแพงเพชร มีการติดเชื้อไวรัสใหม่ 1 ชนิด เมื่อศึกษาความสัมพันธ์ทางวิวัฒนาการระหว่างไวรัสอื่นๆ ในตระกูลเดียวกันพบว่า ไวรัสที่พบใหม่นี้มีความใกล้เคียงกับไวรัส Cell Fusion Agent (CFAV) ซึ่งเป็นไวรัสที่พบในเซลล์ของยุง *Aedes agegypti* ที่เพาะเลี้ยงในห้องทดลอง และ Kamiti River (KRV) จากยุง *Aedes macintoshi* จากป่าในประเทศเคนยา และให้ชื่อไวรัสที่พบใหม่ชนิดนี้ว่าไวรัสวังทอง (Wang Thong Virus (WTV))

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CHAPTER 1
INTRODUCTION

There are over a million different species of insects, and individually they outnumber humans by more than 10^8 to 1. Some insects live in close association with both plants and higher animals and naturally exchange viruses with them. It has ever been speculated that viruses in general may have radiated through the plant and animal kingdoms from common insect origins. Be that as it may, since insects play pivotal role in the biosphere, both to the benefit and determent of mankind, they and the viruses that infect them are important subjects for study (22).

Mosquito is the vectors of many of the most debilitating parasites of man and his domesticated animals. They also transmit most of arboviruses to man and other animals. Culicine mosquitoes are most commonly involved as vectors, but anophelines do transmit a few viruses. The most important of the flavivirus transmitted are again infectious to man. These include dengue, which is largely an urban disease endemic in many parts of Southeast Asia and the Western Pacific, and also in the Caribbean. The major vector is *Aedes aegypti* with *Aedes albopictus* playing a subsidiary role in Asia (19).

In tropical countries especially, there have been changing patterns of disease transmission, frequently associated with economic development schemes and increased travel. Travel can involve just a few individuals or entail mass migrations, often in response to commercial, political, or religious pressures. Deforestation and colonization of cleared land as well as irrigation and resettlement have exposed people to the threat of "new" diseases. Population movements can either be responsible for introducing diseases into new areas or bringing non-infected susceptible people to disease-ridden areas. However, man's involvement in the dissemination of diseases is not new but rooted in antiquity. More recently, explorers

and colonist have been held responsible for spreading diseases through many parts of the world (30).

Thailand is a tropical country in Southeast Asia. There are many insects especially mosquitoes. Until now, there were 411 mosquito species found in Thailand. Many of them are vectors of diseases and cause serious problem of public health such as dengue haemorrhagic fever, Japanese encephalitis, malaria, filariasis. Report from the Department of Epidemiology of Thailand shown that there are many patients and people die every year (35). The study of flaviviruses in mosquitoes is required for understanding of flavivirus-borne diseases, this study designs to provide an information on mosquito distribution and flavivirus prevalence in mosquitoes in Thailand using RT-PCR, a powerful technique that especially useful for the detection of flaviviruses.

The objectives

The objectives of this research were:

(1) To survey mosquito-borne flaviviruses from field-collected mosquitoes in Thailand by RT-PCR technique using the non structural gene (NS5).

(2) To construct and compare the phylogeny of flaviviruses from mosquitoes and from other hosts using partial non-structural gene (NS5) sequences.

CHAPTER 2
LITERATURE REVIEW

2.1 Mosquitoes

2.1.1 General Characteristics

The mosquito in the Family Culicidae which are a family of about three and a half thousand species within the Order Diptera, the two-winged flies. They are one of the more primitive families of Diptera. Mosquitoes are found throughout the world except in places that are permanently frozen. Three quarters of all mosquito species live in the humid tropics and subtropics, where the warm moist climate is flavourable for rapid development and adult survival, and the diversity of habitats permitted the evolution of many species. Mosquito are classified into three subfamilies, the largest and most diversified of which is divided into a number of tribes:

Family	Culicidae:
Subfamily	Toxorhynchitinae
	Anophelinae
	Culicinae:
Tribes	Culicini, Aedini, Sabethini, Mansoniini, etc.

Like other true flies, culicids exhibit complete metamorphosis, i.e., the juvenile form passes through both larval and pupal stages. The larvae are anatomically different from the adults, live in a different habitat and feed on a different type of food. Transformation to the adult takes place during the non-feeding pupal stage (19, 40).

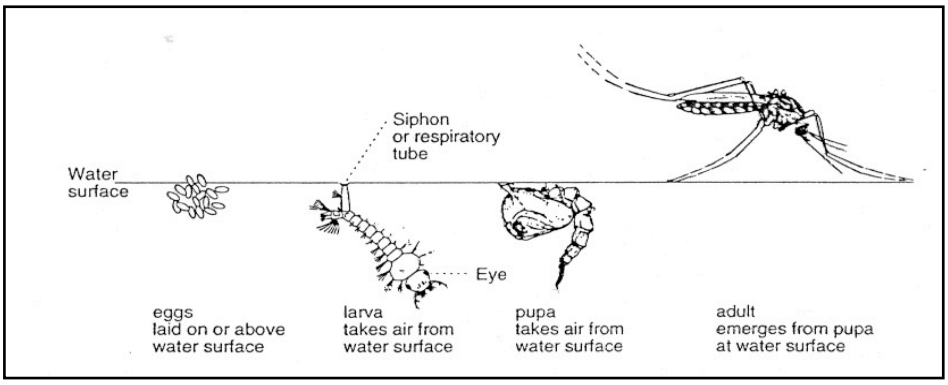


Figure 1. The mosquito life cycle showing developmental stages from eggs, larvae, pupae and adult mosquitoes.

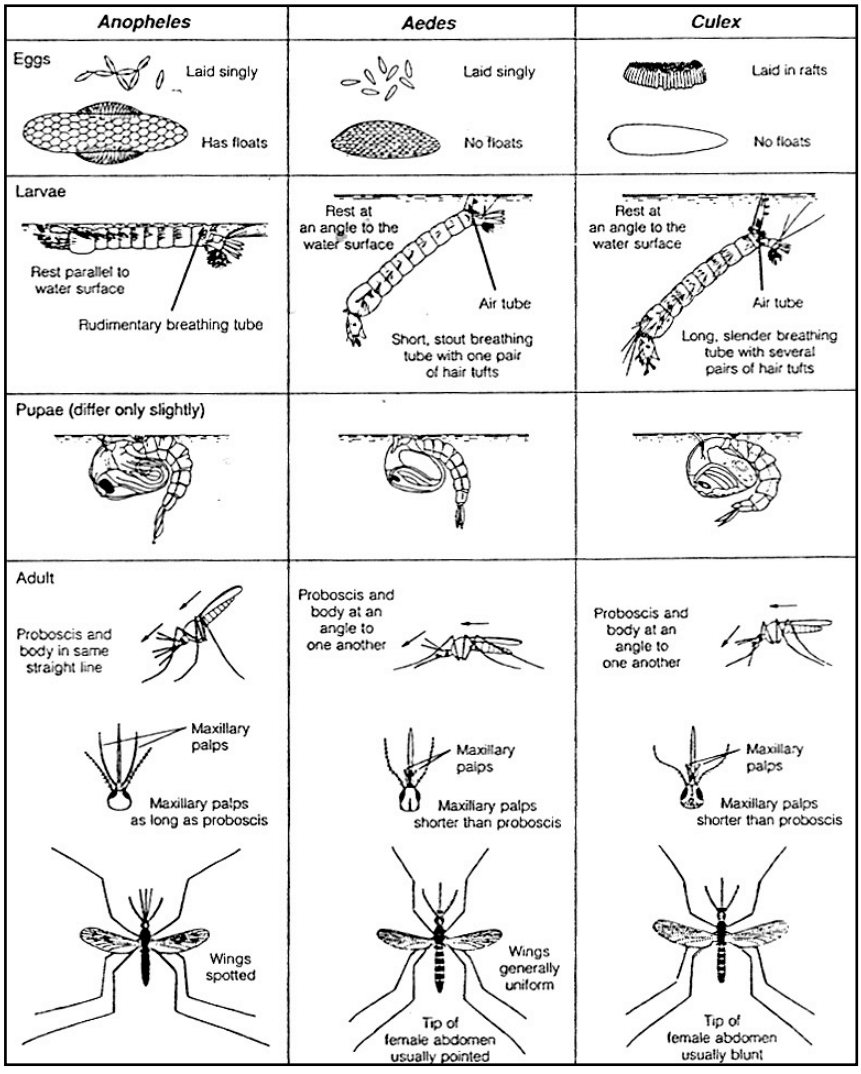


Figure 2. Characteristic stages of different mosquitoes of medical importance, i.e., *Anopheles*, *Aedes*, and *Culex*. (A) Eggs, (B) larvae, (C) pupae, and (D) adults.

2.1.2 The Eggs

Female mosquitoes lay some 50 to 500 eggs at one time, depositing them on water or on sites that will be flooded. Each egg is protected by an egg's shell, which in many species is elaborately sculpted. Spermatozoa stored by the inseminated female fertilize the oocytes as they are ovulated, and embryonic development starts almost immediately after the eggs have been laid. Within one or two days to a week or more, depending on temperature, the embryo develops into a fully formed larva. In most species the larvae hatches once it is formed, and can survive for a few days at most in the absence of water. Mosquitoes of the tribe Aedini have water-proofed egg shells capable of resisting desiccation, and fully-formed but unhatched aedine larvae can survive for months or even years in absence of free water. Aedine species lay their eggs in places that may not be flooded for days, weeks or months. A fall of rain that inundates oviposition site, or a high tide flooding a salt marsh, stimulates hatching and can lead to an apparent population explosion (19, 22, 40) (Fig. 2).

2.1.3 The Larvae

Mosquito larvae are legless, but they retain a well–formed head and so do not appear maggot-like. The larval habitats are small or shallow bodies of water with little or no water movement. Typically shallow pools, sheltered stream edges, marshes and water-filled tree holes, leaf axils or man-made containers. Most species live in fresh water but a few are adapted for life in brackish or saline water in salt marshes, rock pools or inland saline pools. All aquatic animals have problem of water and salt balance, whether they live in fresh or salt water. In addition to the usual internal organs of ion regulation, mosquito larvae have four external balloon-like anal papillae, which are capable of ion uptake from very dilute solution. When they hatch form the eggs the young mosquito larvae are fully adapted for living in water, and two features determine their manner of life: use oxygen for respiration and use of water-borne particle as food (19).

The air-breathing habit requires mosquito larvae either to live more or less permanently at the air/water interface, as most anopheline and some culicine larvae do, or to make frequent visit to water surface. The only functional respiratory opening are a pair of spiracles near the end of abdomen, from which air-filled tracheae extend to all parts of the body. The spiracles of culicine and toxorhynchitine larvae are situated at the end of tubes or siphons, and the larvae hang downwards from the surface membrane by their siphons with their spiracles open to the air. The spiracles of anopheline larvae are flush with the dorsal surface of the last abdominal segment, and the larvae lie horizontally below the surface membrane, their spiracles opening through it. Larvae of two culicine genera (*Mansonia* and *Coquillettidia*) are able to remain permanently submerged. They live with their respiratory siphons, which have modified saw-like tips, forced into the air-filled tissues that fill the stems and roots of certain aquatic plants (19, 22).

The characteristic food sources of mosquito larvae are 'particulate matters'. These include aquatic microorganisms, such as bacteria, diatoms and algae, and also, as an important component, particles of detritus that are largely derived from decayed plant tissues. Mosquito larvae, which live mainly in still water, are exceptional in not relying on natural water currents to bring particles to them. Through the regular beating of their 'mouth brushes', mosquito larvae generate water currents which flow towards the head, and in a manner that is not well understood they separate particles of a certain size from the water. Anopheline larvae typically feed at the water surface, in a particle-rich layer just below the surface membrane. Culicine larvae feed on particle suspended in the water column, and many supplement this feeding mode by abrading with their mouthparts the layers of organic matters that cover submerged surfaces, so generating new particles. Toxorhynchitine larvae are predated on small invertebrates, as are a very few species in the other two subfamilies (22).

2.1.4 The Pupae

The pupa remains an aquatic organism. It is has assumed that the form of an adult is largely concealed because the head and thorax, with their elongate

appendages, are cemented together in the form of cephalothorax. The abdomen, which now terminates in two large paddles, has retained the strong larval musculature and is an effective organ of propulsion. An air bubble, which is enclosed between the appendages, provides buoyancy, and the pupa floats at the water surface with the top of its thorax in contact with the surface membrane and its abdomen hanging down. The new form and posture preclude use of the terminal abdominal spiracles for respiration. That function is taken over by the mesothoracic spiracles, which open within large "respiratory trumpets". As the pupa floats at the air/water interface the hydrophobic rims of the respiratory trumpets protrude through the surface membrane.

During the pupal stage certain larval organs are destroyed, e.g. the alimentary canal, While replacement adult organs are constructed from undifferentiated embryonic cells. Other organs, including fat body, are carried over to the adult stage. These final stages of metamorphosis can be completed within one to two days if the temperature is sufficiently high. When the adult is fully formed within the pupal cuticle, the insect rests at the water surface and starts to swallow air. The consequent increase in internal pressure forces a split along the midline of the pupal thoracic cuticle, and the adult slowly expands out of the pupal cuticle and steps on to the water surface (19, 22).

2.1.5 The Adult

Adult mosquitoes have an elongate body and long wings and legs, which provide an aerodynamically stable form. The hind wings are modified as small oscillating sense organs, or halteres, which assist flight control. Like others in the Order Diptera, mosquitoes are fluid feeders. Exceptionally among the more primitive dipterans, their mouthparts have evolved into an elongate composite proboscis, half as long as the body suitable for probing nectaries and, in the case of the female, adapted for piercing skin and imbibing blood from peripheral blood vessels. The outer sheath-like part of the proboscis, the labium, encloses the remaining mouthparts, which have the form of needle-like stylets. The female's mandibles and maxillae, which are flattened and toothed, can be driven through tissue by the muscles at their bases,

making a channel for other styletized mouthparts which contain canals for the delivery of saliva and the removal of blood. Both males and females use the sugar in plant juices as a source of energy, usually obtaining it from nectaries but sometimes from other sources such as rotting fruits and honeydews. Anopheline and culicine females have a requirement for protein, from which to develop large batches of eggs, and they engorge on vertebrate blood for that purpose. Toxorhynchitine female feed only on plant juices (40).

Body odour and carbon dioxide, carried on the wind, stimulate sense receptors on the attennae and palps of female mosquitoes, alerting them to the presence of a host. The females respond by flying upwind, which take them toward the host. Close to the host, visual stimuli and the convection currents of warm moist air that rise from the host provide additional cues. The females of some species are able to detect individual vertebrate hosts at a distance. The female of all blood-feeding species show a degree of specificity in their choice of host, whether mammal, bird or cold-blooded vertebrate. Some species are highly specific, feeding predominantly on one or a few host species only, others are less specific. Individual human being differ in their attractiveness to mosquitoes; the cause of the difference has not been elucidated.

Once landed on appropriate part of the host, the female drives the styletized components of her mouthparts into its skin. The saliva that is injected as the mouthparts penetrate contains a substance that prevents haemostatsis, the aggregation of blood platelets that is the host's first defence against the laceration of small blood vessels. The saliva is also the source of immunogens that are responsible for the characteristic skin reactions to mosquito bites. If the female is undisturbed, feeding continues until abdominal stretch receptors signal repletion. Within a few minutes gorging mosquitoes can imbibe up to four times their own weight of blood. This provides the protein needed for egg production, but also inflicts upon the mosquito a water load, which renders flight difficult, and potentially toxic amount of sodium and potassium. The adult excretory system is capable of rapid elimination of water and salts, and diuresis commences while the female is still feeding (40).

Digestion of blood proteins yields amino acids which are reconstituted in the mosquito's fat body as proteinaceous yolk. This is transported to the ovaries and incorporated into the oocytes, which are matured in a number that matches the provision of yolk. It is a feature of mosquito biology that eggs are not matured continuously but in batches, following the periodic blood meals. This phenomenon has been a boon for reproductive physiologists, enabling them to probe the complex process of hormone secretion that regulates the conversion of blood protein into eggs. A small percentage of species are able to develop one batch of eggs from protein and lipid reserves carried over from the larval stage, i.e. without a blood meal. Species with this capability are termed autogenous. Male mosquitoes can be readily distinguished by their large and elaborate antennae, in which rings of fibrils encircle the shaft. These antennae resonate in response to a pure tone of a certain pitch. Female mosquitoes in flight produce a familiar whining sound, the pitch of which reflects the wing beat frequency of the species. That sound activates the antennae of conspecific males, and provides directional indicators which the massive sense organs at the base of the antennae can resolve (40).

The role of adult male mosquitoes is insemination of female, and when not resting the male are either feeding or exhibiting a behavior pattern that is likely to bring them into contact with females. One conspicuous manifestation of male behavior is swaming - the localized assembly of from two or three individuals to many thousands of individuals of a single species. Any conspecific female that enters a swarm will be seized immediately by a male. Mating also occurs outside swarms. Inseminated females store sufficient sperms in their spermathecae to fertilize a number of egg batches. A factor called "matrone" that is transferred in the semens renders females unreceptive to males and refractory to futher copulation, but its effectiveness may not persist throughout the life of the female.

The behavioral activities of adult mosquitoes – emergence, mating, feeding, oviposition – take place at particular times of day and night, which vary between species. For most mosquitoes, the oviposition site is a water body with particular characteristics: odour, taste, flow and shade are known to influence different species.

The eggs may be dropped individually to float on the water surface, as by female of *Anopheles*, or packed together to form a floating egg raft, as by *Culex*. Aedine species deposit their eggs on moist surfaces, often at the edge of a body of water or on an area of soil that will be flooded. By whatever means, females find the appropriate habitats, and the larvae hatch into conditions for which they are adapted. The passage of eggs from the ovarioles into the oviducts leaves permanent structural changes which form a record of a female's reproductive history, and which also provide a remarkably accurate guide to her age.

In the tropical region, life span of adult mosquitoes ranges from a few days to several weeks. In the temperate region, it is frequently longer; and in species that overwinter as adults the life span of females may approach one year. The females' behavioural responses and physiological processes follow a pattern of the gonotrophic cycle, which starts with response to the vertebrate hosts and feeding continues with the digestion of blood and formation of batch of mature oocytes and ends with oviposition. Within an hour of completing one gonotrophic cycle, a female may commence another. At warmer temperatures, tropical *Anopheles* oviposit regularly every two or three days (19, 22, 40).

2.2 The Flaviviruses

2.2.1 Background and Classification

The Genus Flavivirus consist of nearly 80 viruses, many of which are arthropod-borne human pathogens. Flaviviruses cause a variety of diseases including fevers, dengue fever, Japanese encephalitis, and Yellow fever. Other flaviviruses of regional or endemic concern include Kyasanur forest disease, Murray Valley encephalitis (MVE), St. Louis encephalitis, Tick-borne encephalitis (TBE), and West Nile (WN) viruses (1, 2, 3, 11, 13, 16, 22). Decrease in mosquito control efforts during the latter part of the 20th century, coupled with societal factors such as increased transportation and dense urbanization, have contributed to the reemergence of flaviviruses such as dengue (DEN) in South and Central America (40). In 1999,

WN virus was isolated for the first time in the western hemisphere during an outbreak in New York City that was several cases of encephalitis, including death (31).

Vaccination is available for Yellow fever (YF), using the live-attenuated 17D strain, and for TBE and Japanese encephalitis (JE) using inactivated viruses (43) Efforts to derive live-attenuated strains of other flaviviruses have met with only limited success. Development of effective DEN vaccines that exhibit cross-protection, thought to be important for preventing subsequent dengue-associated immunopathogenesis, are proving to be particularly challenging. The ability to genetically manipulate flaviviruses, is being used to develop novel approaches to vaccine design. One promising candidate live-attenuated vaccine is a chimeric Flavivirus created by replacing the structural glycoproteins of YF 17D with those of JE (42, 43) A similar approach to create a vaccine against DEN-1 is under investigation (45).

Viruses within the genus are categorized into antigenic complexes and subcomplexes based on classical serologic criteria or into clusters, clades, and species, according to molecular phylogenetics (6, 15). These latter methods have permitted the classification of viruses such as YF, which lacks close relatives. Mosquito-borne and tick-borne flaviviruses, although quite distinct, appear to have evolved via a common ancestral line that diverged from non-vector-borne viruses (15).

Flaviviruses can be culture in animals such as chick embryos, suckling mouse brain, and mosquitoes, as well as in primary or established cell lines of mammalian, avian, or insect origin. In vertebrate cells, dramatic cytopathic and ultrastructural effects can occur, including vacuolation and proliferation of intercellular membranes (44). Arthropod cells in culture may demonstrate cytopathic effects, most frequently manifested as cell fusion and syncytia formation (7, 29). However, infection of mosquito cells is often noncytopathic, and persistent infections can be established. Mosquitoes remain chronically infected for life and produce extremely high levels of infectious virus particles in the salivary glands (22).

2.2.2 Structure and physical properties of the virion

Flavivirus particles appear to be spherical, 40 to 60 nm in diameter, containing an electron dense core (about 30 nm in diameter) surrounded by a lipid bilayer. Mature virions sediment between 170 and 210S, have a buoyant density of 1.19 to 1.23 g/ml, and are composed of 6% RNA, 66% protein, 9% carbohydrate, and 17% lipid (46). Because of the lipid envelope, flaviviruses are readily inactivated by organic solvents and detergents (43). Three viral proteins are associated with virions: The E (envelope), M (membrane), C (capsid) proteins. The E protein is the major surface protein of the viral particles, probably interacts with viral receptors, and mediates virus-cell membrane fusion. Antibodies that neutralize virus infectivity usually recognize this protein, and mutation in E can effect virulence. M protein is a small proteolytic fragment of prM protein, which is important for muturation of the virus into an infectious form, as described later. Discrete nucleocapsids, composed of C protein and genomic RNA (120 to 140S, buoyant density 1.30 to 1.31 g/ml), can be isolated after solubilization of the envelope with non-ionic detergents (46, 47, 48).

2.2.3 Binding and entry

Flaviviruses attach to the surface of host cells through an interaction of E protein with one or more receptors, and many E-reactive antibodies have been shown to neutralize virus infectivity by interfering with virus binding. Several cell surface proteins have been described as candidate flavivirus receptors (61). In addition, recombinant E protein from DEN-2 virus has been shown to interact with highly sulfated glycosaminoglycans, and cell surface expression of heparan sulfate was required for efficient infection of mammalian cells by a laboratory-passaged strain of DEN-2 virus (46).

After binding it is generally believed that virions are taken up by receptor-mediated endocytosis, although direct fusion at the plasma membrane has also been observed. Ultrastructural studies have localized single virions and virions aggregated to clathrin-coated pits on the cell surface, and uptake of virus particles into coated

vesicles rapidly follows attachment. Virions are later found in uncoated prelysosomal vesicles, where an acid-catalysed membrane fusion is thought to release the nucleocapsid into the cytoplasm. Consistent with this, a conformational change in the viral E protein, which probably exposes a fusogenic domain, occurs at low pH. Acid pH can promote fusion of virions with liposomal membranes in vitro or at the plasma membrane of culture cells, although in the latter case mode of entry does not lead to productive infection. Following entry and fusion, nucleocapsid are presumably disassembled, genomic RNA is translated, and RNA is initiated (47, 48).

2.2.4 Genome structure

The genome of flaviviruses consists of a single-stranded RNA about 11 kilobases (kb) in length. This RNA constrains a 5' cap (m^7 G5'ppp5'A) at the 5' end and lacks a polyadenylate tail. Genomic RNA is the messenger RNA for translation of a single long open reading frame (ORF) as a large polyprotein. Surrounding the ORF are 5' and 3' noncoding regions (NCRs) of around 100 nucleotides (nt) and 400 to 700nt, respectively. These regions contain conserved sequences and predicted RNA structures that are likely to serve as cis-acting elememts directing the processes of genome amplification, translation, or packaging. Although it is not yet feasible to directly study the structures of flavivirus RNAs in vivo, the ability of RNA to adapt alternative folding could regulate these competing processes (57).

The 5' NCR sequence is poorly conserved between flaviviruses, but it appears to contain secondary structural elements that influence the translation of flavivirus genomes (57). However, the most significant function of the 5' NCR probably resides in its reverse complement, the 3' NCR of viral minus strands, which forms the site for initiation of plus-strand synthesis. Deletions engineered into the 5' NCR of DEN-4 were shown to have dramatic effects on the ability to recover live viruses, but they do not correlate with the translational efficiency of the mutant genomes. Interestingly, one of the resultant mutants exhibited a limited host-range growth phenotype, suggesting potential interactions of this region (or its reverse complement) with host-

specific factors. In keeping with this, cellular proteins were shown to bind specifically to a terminal stem-loop in the 3' NCR of WN virus minus strand (58).

The 3' NCR of the flavivirus genome, which presumably functions as a promoter for minus-strand synthesis, exhibits a great deal of sequence divergence and size heterogeneity. Nevertheless, computational analyses have revealed features that are common to all flaviviruses or that are conserved within specific taxonomic groups, and that tend to cluster in a region proximal to the 3' end (59). Mutations engineered within the 3' terminal stem-loop of the genome of DEN-2 virus greatly affected the ability to recover live virus, and one of the recovered viruses exhibited a host restricted-growth phenotype (60). The conserved portion of the 3' NCR also contains consensus sequences (CS1 and CS2) that are retained among all mosquito-borne flaviviruses, one of which has the potential to base-pair with a conserved region in the 5' region of the ORF, suggesting that cyclization of flavivirus genomes is possible (60). A DEN-4 genome engineered to contain a deletion in CS1 indicates that this element is required for virus viability (61). Other region in 3' NCR, which may be dispensable for virus replication in culture, nevertheless appear to be important determinants for growth in mammalian hosts, and further analysis of such region is likely to contribute greater insight into flavivirus pathogenesis and vaccine development (61, 62).

2.2.5 Translation and proteolytic processing

The flavivirus genome is translated as a large polyprotein that is processed co- and post-translationally by cellular protease and a virally encoded serine protease into at least 10 discrete products. The N-terminal on quarter of the polyprotein encodes the structural proteins, and the remainder contains the nonstructural (NS) proteins, in the following order: C-prM-E-NS1-NS2A-NS3-NS4A-NS4B-NS5 (15).

2.2.6 Features of the structural proteins

2.2.6.1 The C protein

C protein (about 11 kd) is highly basic consistent with its proposed role in forming ribonucleoprotein complex with packaged genomic RNA. Basic residues are concentrated at the N- and C-termini of C, and they probably act cooperatively to specifically bind genomic RNA. The central portion of C contains a hydrophobic domain that interacts with cellular membranes and may play a role in virion assembly. The nascent C protein (anchC) contains a C-terminal hydrophobic domain that acts as a signal sequence for translacation of prM into the lumen of the endoplasmic recticulum (ER). Mature C protein is generated by viral serine protease cleavage at a site upstream of this hydrophobic domain (62).

2.2.6.2 The prM protein

The N-terminus of prM (about 26 kd) is generated in the ER by host signal peptidase. This processing event appears to require prior processing of anchC by the cytoplasmic viral serine protease. However, in support of this model, signalase cleavage at this site can be made viral protease-independent by cleaving anchC with another protease or by improving the context of the signalase cleavage site. Interestingly, improvement of the prM signalase cleavage context was shown to be lethal for YF virion production (63). Thus, the availability of viral protease activity may regulate structural protein processing, and therefore virion assembly, over the course of an infection. During the egress of virions through the secretory pathway, prM is cleaved by the trans-Golgi resident enzyme furin, to form the structural protein M (about 8 kd) and the N-terminal "pr" segment which is secreted into the extracellular medium. The N-terminal "pr" segment is predominantly hydrophilic and contains one to three N-linked glycosylation sites and six conserved cysteine residues, all of which participate intramolecular disulfide bridges. The structural protein M, located in the C-terminal portion of prM, is present in mature virions and contains a

shortened ectodomain (about 41 amino acids) followed by two potential membrane-spanning domains. Antibodies to prM can mediate protective immunity (64).

2.2.6.3 The E protein

E protein (about 50 kd) is type I membrane protein, containing adjacent transmenbrane domain in the C-terminus that serve to anchor this protein to the membrane and as the signal sequence for NS1 translocation. E protein contains 12 highly conserved Cys residues that form intramolecular disulfide bonds, and it is often glycosylated (65). The three-dimensional structure of TBE E protein was determined by x-ray crystallography of soluble tryptic fragment obtained from purified virions (66). These data indicate that E protein is divided into three structural domains and forms head-to-tail homodimeric rods that are thought to lie parallel to the virion surface in a meshlike network. This structural model provides a framework for understanding the molecular basis of E protein interactions. Structural regions of the ectodomain contain putative receptor binding sites. A potential fusogenic domain, contact site for E homodimer formation, a region involved in prM-E interaction, and the trigger for low-pH-induced rearrangement (67). E protein homodimers disassociate at low pH, and each monomer reassociates with two adjacent E proteins. E trimer are thought to extend outward for the virion surface, presumably exposing the hydrophobic fusogenic domains. The stem-anchor region of E, for which structure was not determined, contains determinants for E trimer formation and for stabilization of prM-E heterodimers (68). Mutations in putative receptor binding site in TBE E protein gave rise to viruses with attenuated growth in culture and reduced virulence in mice (69). For some mosquito-borne flaviviruses, a similar region of E protein contain putative integrin binding motif Arg-Gly-Asp. Mutation of this sequence in E MVE virus produced viruses with decreased entry kinetics, increased reliance on glycoaminoglycans for entry, and attenuated virulence (63).

2.2.7 Features of nonstructural proteins

2.2.7.1 The NS1 protein

The NS1 glycoprotein (M_r of about 46 kd) exists in cell-associated, cell-surface, or extracellular nonvirion forms. NS1 is translocated into the ER lumen and released from the C-terminus of E by signal peptidase (70,71). It contains 12 highly conserved cysteines that form intracellular disulfide bonds, and it is rapidly glycosylated on two or three Asn residues. NS1 is cleaved from its downstream neighbor, NS2A, around 10 membrane-bound, ER-resident host protease. The eight C-terminal hydrophobic residues of NS1, and more than 140 amino acids of NS2A, are necessary determinants of this cleavage. Truncate and elongated forms of NS1, which presumably contain alternate C-terminal cleavage sites. Twenty to 40 minutes, after synthesis, NS1 forms homodimers that are resistant to denaturation with 6 M urea or 5 M guanidinium-Hcl, but that are unstable at high temperature or low pH. A point mutation has been identified in Kunjin NS1 that destabilizes NS1 dimers and confers a replication defect on the virus (72), indicating a functional role of NS1 dimerization. NS1 appears to be peripherally associated with membranes, as it does not contain any putative transmembrane domains or known lipid modification, and it can be released from membranes by sodium carbonate (pH 11.5), 8 M urea, or 5 M guandinium-HCl (73).

NS1 is slowly secreted from mammalian cells and is not secreted from mosquito cells. During secretion, one of the N-linked glycans is modified to contain complex sugars, and three NS1 dimers come together into a soluble hexameric form (74).

NS1 was first characterized as the soluble (non-virion associated) complement-fixing (SCF) antigen present in the serand tissues of experimentally infected animals. It is now understood that the extracellular forms of NS1 strongly elicit humoral immune responses, and immunization with purified or recombinant NS1 can be protective (75). The secretion of a viral NS protein that elicits protective

immune responses is one of the more curious aspects of flavivirus biology that await further inquiry.

Several lines of evidence implicate NS1 in the process of RNA replication. NS1 has been found to co-localize with markers of RNA replication in association with membrane structure that are presumed sites of replication. Mutations at the first or both N-linked glycosylation sites of NS1 dramatically impair virus replication. And demonstrate a decrease in viral RNA accumulation (76). Furthermore, a YF mutant containing a temperature-sensitive lesion in NS1 showed a profound decrease in RNA accumulation under nonpermissive conditions. NS1 can be supplied trans to a YF genome lacking a functional NS1 gene, and RNA replication is blocked at a very early stage in the absence of complementing NS1. Further genetic analysis has revealed that DEN NS1 does not productively interact with the YF replicase in trans. This block in replication can be suppressed by a mutation in NS4A, indicating that an interaction between NS1 and NS4A is critical for replicase function and suggesting a potential mechanism whereby NS1 participates in the cytoplasmic process of RNA replication (77).

2.2.7.2 The NS2A and NS2B proteins

NS2A is relatively small (about 22 kd), hydrophobic protein of unknown fuction. Cleavage of NS1-2A occurs in the ER, as previously mentioned, whereas the C-terminus is generated via cleavage at the NS2A/2B junction by the cytplasmic serine protease, indicating that this protein must be membrane spanning. An alternative cleavage within YF NS2A can also be utilized by the viral protease, leading to a C-terminally truncated form of this protein that is about 2 kd smaller in mass. Mutations that block cleavage at either site are lethal for some aspect of YF replication (78).

NS2B is a small (about 14 kd) membrane-associated protein containing two hydrophobic domain surrounding a conserved hydrophobic region (79). It forms a complex with NS3 and is a required cofactor for the serine protease function of NS3. Deletion analysis indicates that a 40-amino-acid region in the central conserved

domain of NS2B is required for NS2B 3 protease activity, and mutations in the central conserved domain that destabilize interaction with NS3 abolish protease activity (80).

2.2.7.3 The NS3 protein

NS3 is a large (about 70 kd) cytoplasmic protein that associates with membranes via its interaction with NS2B. NS3 contains several enzymatic activities that implicate this protein in polyprotein processing and RNA replication. The N-terminal one third of NS3 contains homology to trypsin-like serine protease. Mutation in NS3 that abolish protease activity also prevent the recovery of viable mutant viruses, substantiating a crucial role for this enzyme in the virus life cycle (81). Expression of the N-terminal 167 to 181 residues of NS3 together with NS2B, is sufficient to form the active protease. The NS2B-3 protease cleaves in both cis and trans configurations, and it mediates cleavages at the NS2A/NA2B, NS2B/NS3, NS3/NS4A and NS4/NS5 junctions, as well as cleavages that generate the C-termini of mature C and NS4A, and minor cleavages within NS2A and NS3 (82). Alignment of known cleavage sites and mutation of these residues have been used to characterize the substrate specificity of this enzyme, these data indicate that the NS2B-3 protease preferentially cleaves after pairs of basic residues and before an amino acid with small side chain (83). The structure of a recombinant DEN-2 NS3 protease domain, in the absence of cofactor NS2B, has recently been resolved to 2.1 A° by using X-ray crystallography (84). This model exhibits structural similarities to other serine proteases including the NS3-4A protease of HCV, provides a framework for the interpretation of NS3 mutagenesis studies, and suggests that NS2B could contribute to substrate binding specificity (84).

The C-terminal three quarters of NS3, which slightly overlaps the serine protease domain, has been implicated in RNA replication. This region has significant homology to RNA helicase containing the motif Asp-Glu-Ala-(Asp/His). These enzymes utilize the energy of nucleoside triphosphate (NTP) hydrolysis to power RNA unwinding. RNA-stimulated NTPase activity has been demonstrated for full-length and N-terminally truncated NS3 (86). Recently, DEN-2 NS3 has been shown

to contain an NTP-dependent RNA unwinding activity, consistant with the function of NS3 as an RNA helicase (87) is not known for flaviviruses, but it may help to dissociate nascent RNA strands from their template during RNA replication, or perhaps unwind secondary structures involved in template recognition or initiation of RNA synthesis (87).

2.2.7.4 NS4A and NS4B

NS4A and NS4B are relatively small (about 16 kd and 27 kd, respectively) hydrophobic proteins that are membrane associated. Based on its subcellular distribution and interaction with NS1, NS4A appears to function in RNA replication, perhaps by anchoring replicase components to cellular membranes. NS4B also localizes to presumed site of RNA replication, but it also appears to be dispersed throughout cytoplasmic membranes, and possibly the nucleus (88).

The N-terminus of NS4A is generated by the NS2B-3 protease, where as the C-terminus of NS4A contains a transmembrane domain that serves to translocate NS4B into the ER. The NS2B-3 protease cleaves at a site within NS4A, just upsteam of this signal sequence, and mutations that block this cleavage also block subsequent signal peptidase cleavage in the ER lumen to generate the N-terminus of NS4B (89). This unusual coordinated processing scheme, which is strikingly similar to processing at the C-prM junction, might serve to regulate replicase function. Furthermore, unprocessed NS3-4A and NS4A-4B have been detected in flavivirus-infected cells (90), suggesting that polyprotein cleavage in this region may be inefficient or controlled in additional ways. NS4B is post-translationally modified to a form that appears to be about 2 kd smaller than the nascent protein.

2.2.7.5 NS5

NS5, the largest (103 kd) and most conserved flavivirus protein, contains sequence homology to RdRPs of other positive-strand RNA viruses, including the invariant Gly-Asp-Asp (GDD) motif common to these enzymes (79). Purified

recombinant NS5 exhibits RdRP activity in primer extension reactions. And NS5 protein has been found to fractionate with RdRP activity in infected cell extracts (91). NS5 also shares homology with methyltransferase enzymes involved in RNA cap formation and is thus probably involved in methylation of the 5' RNA cap structure (92). Site-directed mutagenesis has confirmed that motifs implicated in polymerase and methyltransferase activities are essential for virus replication, and that the functions of NS5 can be supplied in trans. NS5 can be phosphorylated by an unknown cellular Ser/Thr kinase, a feature that is also conserved in the NS5A proteins of the pestiviruses and HCVs. The role of NS5 phosphorylation remains obscure, but it may regulate the interaction of NS5 and NS3 (94), or a subcellular redistribution of NS5 into the cell nucleus.

2.2.8 RNA replication

Following translation and processing of the viral proteins, a viral replicase is assembled from NS5 proteins, the viral RNA, and presumably some host factors. The replicase associates with membranes, probably through interactions involving the small hydrophobic NS proteins. Replication begins with the synthesis of a genome-length minus-strand RNA, which then serves as a template for the synthesis of additional plus-strand RNAs. The first round of minus-strand accumulation has been detected just over 3 hours after infection (95). Viral RNA synthesis appears to be asymmetric *in vivo*, with a plus-strand accumulation more than 10 times greater than that of minus strands. Viral minus strands appear to accumulate even late after infection and have been isolated exclusively in double-stranded forms. Flavivirus replication can be followed *in vivo* by metabolic labeling of virus-specific RNAs in the presence of actinomycin D, an inhibitor of DNA-dependent RNA polymerases. Three major species of labeled flavivirus RNAs have been described. One type sedimenting at 40S, another at 20S, and a heterogeneous population at 20S to 28S. 40S RNA is sensitive to RNase treatment and is identical to virion-associated RNA, consistent with its being genomic RNA (96). 20S RNA, frequently termed the replicative form (RF), is likely to be a transiently stable duplex of viral plus- and minus-strand RNAs based on RNase resistance and conversion to an RNase sensitive

form that comigrates with 40S RNA by heat denaturation (96). The 20S to 28S RNAs are partially sensitive to RNase treatment and are described as replicative intermediate (RI) RNAs that most likely contain duplex regions and recently synthesized plus-strand RNAs displaced by nascent strands undergoing elongation. This mode of replication, with minus strands serving as templates for the production of multiple plus strands, can be described as semiconservative and asymmetric (91)

2.2.9 Membrane reorganization and the compartmentalization of flavivirus replication

Several studies have described ultrastructural changes in membranes of flavivirus-infected cells, predominately in the perinuclear region. In general, the earliest event is the proliferation of ER membranes, followed by the appearance of smooth membrane structures around the time of early logarithmic virus production. Smooth membrane structures are small clusters of vesicles containing electron-dense material within the lumen of smooth ER. These structures continue to accumulate during later times of infection, when they become adjacent to newly form convoluted membranes. Convoluted membranes appear to be contiguous with the ER as randomly folded membranes or highly ordered structures that are sometime described as paracrystalline arrays (93) With the improved preservation of cellular membranes afforded by cryosectioning, demonstrated the appearance of vesicle packets, clusters of vesicles (each 100 to 200 nm in diameter), bound by a smooth membrane, during late times of infection. It seems likely that vesicle packets are related to or identical with smooth membrane structures, and they are often associated with smooth ER or Golgi-like membranes undergoing a morphologic process of wrapping. Such structures are also enriched for markers of the trans-Golgi (85,97)

The subcellular site of RNA replication have been probed by metabolic labeling of nascent RNAs, by immunolabeling with sera reactive to double-stranded RNA, which presumably recognizes RF and RI RNAs, and by *in situ* hybridization (98). Apart from one report of nuclear replication, all investigations concur that viral RNAs accumulate in association with cytoplasmic membranes in the perinuclear

region of mammalian cells and, in particular, with vesicle packets. NS1, NS2A, NS3, and NS4A have all been shown to localize to vesicle packets (97). Thus, it appears that along with double-stranded RNA, proteins implicated in RNA replication associate with vesicle packets. In contrast, the components of the viral serine protease, NS2B and NS3, colocalize with convoluted membranes. The membrane reorganization that occurs in infected cells might therefore give rise to adjacent, but distinct subcellular structures where viral polyprotein processing or RNA replication take place. It should however be emphasized that vesicle packets have been described only at late times after infection compared to the onset of RNA replication, and the sites of early RNA synthesis have not been defined. Furthermore, much remains to be learned about how interactions among NS proteins, between NS proteins and viral RNA, and between viral RNA and host factors (99) combine to form an active replicase.

2.2.10 Assembly and release of virus particles

Ultrastructural studies indicate that virion morphogenesis occurs in association with intracellular membranes. Electron microscopic studies of flavivirus-infected cells have consistently demonstrated morphologically mature virions within the lumen of a compartment believed to be the ER (87, 100). In many studies, virions appear to accumulate within disorderly arrays of membrane-bound vesicles. Budding intermediates and clearly distinguishable cytoplasmic nucleocapsids have not been frequently observed, suggesting that the process of assembly is rapid. Nascent virions are believed to be transported by bulk flow through the secretory pathway to the cell surface, where exocytosis occurs. Budding of the virions at the plasma membrane has been occasionally observed (101), and it does not appear to be a major mechanism for virion formation. These ultrastructure observations, together with studies on structural protein biosynthesis, oligomer formation, and the properties of intracellular and released virions, suggest the following model for virion assembly and maturation: the highly basic C protein interacts with viral genomic RNA in the cytoplasm to form nucleocapsid precursor. The orientation of C, prM, and E with respect to the ER membrane would suggest that nucleocapsids acquire an envelope by budding into ER

lumen. Cosynthesis of E and prM is necessary for proper folding of E. These protein have been shown to be associated as detergent-stable heterodimers that can form higher-order structure, which may represent virion-associated lattices (102).

Latter stages in virion maturation include glycan modification of E (for some viruses) and prM by trimming and terminal addition, implying that virions move through an exocytosis pathway similar to that used for synthesis of host plasma membrane glycoproteins. Although differences in the efficiency of prM cleavage have been noted, this cleavage generally distinguishes released virus from intracellular virus particles. Intracellular M-containing virions have not been detected, suggesting that prM cleavage occurs just prior to release of mature virions. This cleavage can be inhibited by elevating the pH in intracellular compartments, consistent with catalysis by furin, although inhibition of prM cleavage does not impair virus release. Studies on prM-containing particles suggest that this cleavage is required to generate highly infectious virus (103). Experimental data suggest that flaviviruses use oligomerization and prM cleavage to regulate the activation of E-protein-mediated fusion activity. The current hypothesis is that the function of prM in the prM-E heterodimer is to prevent E from undergoing an acid-catalyzed coformational change during transit of immature virions through an acid intracellular compartment. Upon cleavage of prM and release of mature viruses, the E-M interaction is destabilized (104). The hemagglutination activity exhibited by flaviviruses, which depends on low pH, probably results from activation of the fusogenic activity of E protein.

In addition to mature virions, slowly sedimenting non-infectious particles, which are also capable of agglutinating red blood cells at acid pH (called SHA, for slow sedimenting hemagglutinin are released from flavivirus-infected cells. SHA particles appear as 14 nm doughnut-like structures and are composed of E and M, with variable amounts of prM. Recent studies have shown that expression of prM and E is sufficient for release of SHA-like particles. These particles, which are fusogenic, provide an excellent model system for examining the function of E and prM, and they show promise as immunogens that elicit protective immunity (105).

2.3 The prevalence of flaviviruses

The earliest record of an illness thought to possibly be dengue found to date is in Chinese encyclopedia of disease symptoms and remedies, first published during the Chin Dynasty (265 to 420 A.D.) and formally edited in 610 A.D. (Tang Dynasty) and again in 992 A.D. The disease was called water poison by the Chinese and was thought to be connected with flying insects associated with water. Outbreaks of illness in the French West Indies in 1635 and in Panama in 1699 could also have been dengue. Thus, dengue or a very similar illness has wide geographic distribution before the 18th century, when the first known pandemic of dengue-like illness began. It is uncertain whether the epidemics in Jakarta, Indonesia, and Cairo, Egypt in 1779 were dengue but it is quite likely that the Philadephia epidemic of 1780 was dengue (41). DEN is a major public health problem in tropical regions of the world inhabited by over 2 billion people (Asia, Africa, Autralia, and the Americas). The four serotypes of the virus (types 1-4) each cause human disease and are transmitted between humans by *Aedes* mosquitoes. Up to 100 million cases of dengue fever and 450,000 cases of dengue hemorrhagic fever and dengue shock syndrome are reported annually (43, 54, 123, 127). JE virus is the most important cause of viral encephalitis in the Asia-Pacific region. In the last three decades, its geographical range has extended to previously unaffected parts of Asia and northern Australia. It is estimated that three billion people are at risk of infection. Approximately 35,000 JE cases with 5,000-10,000 deaths are reported each year. The natural transmission cycle of the virus involves vertebrate animals, domestic pigs as well as birds, and *Culex* mosquitoes. Tick-borne encephalitis is primarily endemic in Europe, Siberia, and the Far East of Russia. There are two subtypes of Tick-borne encephalitis: Central European encephalitis and Russian spring-summer encephalitis. In Europe the highest incidence of the diseases is observed in Austria, Czech Republic, Slovakia, Slovenia, and Hungary where several hundred cases are reported each year. In the former Soviet Union, up to 9,000 cases occur annually. The rate of transmission and number of human cases varies depend on fluctuating populations of rodent hosts and transmitting ixodid ticks (43).

During 1950s, the former principal vector of dengue viruses in Asia, *Aedes albopictus* , was replaced by *Aedes aegypti* in most major cities of the area. *Ae aegypti* is now considered the main vector of dengue viruses in Asia. Surprisingly, however, this mosquito has been described as having a relatively low oral receptivity for dengue viruses compared with *Ae. albopictus*. But in the one study, Vazeille compared the relative oral receptivities of *Ae. aegypti* and *Ae. albopictus* collected in southeast Asia from both sympatric and allopatric breeding sites. In all instances, the oral receptivity of *Ae. aegypti* to dengue viruse type 2 was significantly greater than *Ae. albopictus*. Male *Ae. albopictus* can transmit dengue transmit dengue virus sexually in the course of mating. And females can transmit it vertically more efficiently than can *Ae. aegypti* females (54). From the study of Armstrong, DEN-2 viruses with potential to cause dengue hemorrhagic fever have been shown to belong to the Southeast Asian genotype. They found that *Ae. aegypti* tends to be more susceptible to infection by Dengue 2 virus of the south-ease Asia genotype than to those of the American genotype and these viruses appear to be rapidly displacing the American genotype of DEN-2 in the Western Hemisphere (116).

Montana Myotis leukoencephalitis virus (MMLV) was first isolated in 1958 from mouse bitten by naturally infected little brown bat (*Myotis lucifugus*) captured in western Montana. The virus was subsequently isolated from saliva, brain and various other tissues from other bats of the same species. The biological and serological properties of the virus suggested that it belonged to the flaviviruses. Based on both antigenic and molecular relationships, it is classified in the Rio Bravo virus group within the genus flavivirus. In 1995, a virus tentatively named Alkhurma virus (ALKV) was isolated from the blood of several patients with severe hemorrhagic fever in Saudi Arabia. A total 16 cases were confirmed by virus isolation, of which 4 had a fatal outcome. The discovery of this virus was considered to be an important event because tick-borne flaviviruses responsible for hemorrhagic fever in human had been isolated previously only in Siberia (OmsK hemorrhagic fever virus, OHFV) and in India (Kyasanur Forest disease virus, KFDV). The complete coding sequence of ALKV, was determined to be 10,248 nucleotides long and encodes a single 3,416 amino acid poly protein. The sequence determination of ALKV NS5 gene suggest that

the virus was closely related to KFDV, one of the most pathogenic TB flaviviruses, causing hemorrhagic manifestations with a case fatality rate of 2 to 10% (129).

In August 1992, ThCAr105/92 was isolated from pooled specimens of the mosquito *Culex triteaniorhynchus*, collected in Chiang Mai, Thailand. The virus specimen and its antiserum were sent to the Division of Vector-Borne Infectious Diseases, Center for Disease Control and Prevention (CDC), USA. After examination, THCAr105/92 was determined to be a new flavivirus and identified as a distinct subtype of Tembusu virus (23). A new virus named Sitiawan virus (SV) was isolated from sick broiler chicks in chicken embryos, from a broiler farm, Sitiawan District, Perak State, Malaysia. The virus replicated well with cytophatic effects (CPE) in the chicken B-lymphocyte cell line LSSCC-BK3. The cDNA fragment of NS5 gene was amplified with primers corresponding to the NS5 gene of flaviviruses. The nucleotide sequenes were 92% homologous to Tembusu virus. In cross-neutralization tests with Tembusu virus, antiserum to SV did not neutralize Tembusu virus, and antiserum to Tembusu virus neutralized more weakly to SV than against the Tembusu virus. The results showed that SV is a new virus which can be differentiated serologically from Tembusu virus but is otherwise similar with respect to nucleotide sequence. This virus causes encephalitis, growth retardation, and increased blood glucose levels in inoculated chicks (44)

Kamiti River Virus (KRV) was a new flavivirus found in forest in Central Province, Kenya. Mosquito and larvae collection was done during the short rain season of 1999. Specimens were reared to adults, identified and pooled by species and were tested for the presence of virus. A total of 3,659 mosquitoes were collected of which 798 (21.8 %) were *Ae. macintoshi*. A total of 32 pools were subjected to newborn mice and C6/36 cell culture. There were 2 virus isolates from C6/36. This virus was identified as a new flavivirus closely related to the Cell Fusion Agent Virus (CFAV) (29). CFAV is an RNA virus originally isolated from a cell line of *Aedes aegypti* more than 25 year ago. CFAV was unlike the well-studied members of the Genus Flaviviruses, CFAV did not replicate in any of the several vertebrate cell lines tested (7).

A mosquito and avian survey of 13 sites in Bucharest and Ilfov district, Romania was conducted in 1996. Adult mosquitoes, collected by aspiration from resting sites, were identified and pooled, and ground suspensions were inoculated on to Vero cells. *Culex pipiens* was the predominant mosquito species in Bucharest. 3689 adult mosquitoes of seven taxa were collected, 94% of which were *Cx pipiens.* A virus recovered from a pool of *Cx pipiens* was identified antigenically as WN virus, which resulted in a minimum infection rate of 0.3/1000. Chicken and other domestic fowl were kept in Bucharest. Neutralising antibodies to WN virus were detected in 30 (41%) of domestic fowl and in one (8%) of 12 wild birds (33). Han et al. studied the risk factors for WN virus infection and Meningoencephalitis in Romania, they found mosquitoes in the home were associated with infection and, among apartment dwellers, flooded basements were a risk factor. Meningoencephalitis was associated with spending more time outdoors daily (11). In late summer 1999, an outbreak of WN virus occurred in New York, the first report of the WN virus outbreak in the northeastern United States. WN virus was isolated from American crows, and Chilean flamingo at the Bronx zoo (118). Native and exotic birds were detected with WN infection by using standard histologic and ultrastuctural methods, virus isolation, immunohistochemistry, in situ hybridizatrion and RT-PCR. WN caused severe pathologic changes and death in 27 birds representing 8 orders and 14 species. Virus was deteced in 23/26 brains (88%), 24/25 hearts (96%) 15/18 spleens (83%), 14/20 livers (70%), 20/20 kidneys (100%), 10/13 adrenals (77%), 13/14 intestines (93%), 10/12 pancreata (83%), 5/12 lungs (42%), and 4/8 ovaries (50%) (32). WN virus was isolated from mosquitoes, crows, and Cooper's hawk in Connecticut. A total of 3398 mosquitoes was collected and tested for WN. Virus was isolated from one pool of 12 *Culex pipiens* and one pool of 6 *Aedes vexans* and WN were isolated from 27 of 30 crows that died in New Haven Countries and from one brain of a Cooper's hawk. *Cx pipiens* is considered to be one of the principle vectors in Europe and possibly to be a reservoir for the virus during winter in Egypt. Another species of mosquito, *Ae vexans*, has been identified harboring WN virus in Senegal and in Russia (2). In 1999, WN virus was responsible for an outbreak of encephalitis in the northeastern United States, The origin of WN was examined by determination of WN infection in dead birds, Chilean flamingo, and crows. In July to September 1999, a widespread

outbreak of meningoencephalitis associated with WN virus occured in Southern Russia, with hundreds of cases and dozens of deaths. Two strains of WN virus were isolated from patient serum and brain-tissue samples. During July 25 to October 1, 1999, 826 patients were admitted to hospital in the Volgograd Region, Russia. Among them, there were 84 cases of meningocephalitis, 40 of which were fatal. Fourteen brain specimens confirmed the presence of WN virus and Kunjin virus (20, 26).

During late summer 2001 in Austria, a series of deaths in several species of birds occurred, similar to the beginning of the WN virus epidemic in the United Stated. Dead bird necropsy was examined. The investigations suggested a WN virus infection. Subsequently, the virus was isolated, identified, and partially sequenced and phylogenetic analysis was conducted. The isolate exhibited 97% identity to Usutu virus (USU), a mosquito-borne flavivirus of the JE virus group; USU virus has never previously been reported outside Africa nor associated with fatal disease in animals or humans. If established in Europe, this virus may have considerable effect on avian populations; whether USU virus has the potential to cause severe human disease is unknown (117). Powassan (POW) virus is responsible for central nervous system infection in humans in North America and the eastern part of Russia. Recently, a new flavivirus, Deer tick virus (DTV), related to POW virus was isolated in the United States. But neither its pathogenic potential in humans nor its taxonomic relationship with POW virus have been elucidated (130). JE is a mosquito-borne virus that is prevalent in some Southeast Asian countries and causes acute fever and encephalitis in humans, the incidence and geographic distribution of which are increasing in rural areas of tropical and temperate Asia. A total of about 45,000 to 50,000 clinical cases occur annually in these countries. The flavivirus responsible for the disease has pigs and chickens as amplifying hosts, and mosquitoes as vectors and man is a dead-end host (39).

From 1999 to 2000 in Singapore, *Aedes aegypti* female mosquitoes were screened for Dengue virus; 54 (6.9%) of 781 *Aedes aegypti* and 67 (2.9%) of 2256 *Aedes albopictus* were positive for dengue viruses, with a declining trend. The most common dengue virus type detected in *Aedes* mosquitoes was dengue-1 (120). There

is a report on dengue virus transmission and desease severity conducted in a cohort of 2,119 elementary school children in northern Thailand. A total of 717,106 person-school days were observed from 1998 to 2000. The incidence of inapparent and of symptomatic dengue virus infection was 4.3% and 3.6% in 1998, 3.2% and 3.3% in 1999, and 1.4% and 0.8% in 2000, respectively. Symtomatic dengue virus infection was responsible for 3.2%, 7.1%, and 1.1% of acute-illness school absences in 1998, 1999, and 2000, respectively (49). For Thailand, Vectors and potential vectors of mosquito-borne diseases shown on Table 1. Another the report on dengue virus transmission and disease severity in cohort of children showed a total of 108 dengue virus isolate were obtained from 167 acute dengue virus infection cases; 23% were DEN-1, 35% were DEN-2, 41% were DEN-3, and 1% were DEN-4 (50). Over the last 40 years, the incidence of dengue virus transmission and disease in Thailand has increased from a reported annual rate of 9/100,000 in 1959 to 189/100,000 in 1998. The largest reported outbreak of dengue occurred in 1987, with an incidence rate of 325/100,000. Dengue virus circulation and association with epidermis and severe dengue disease were studied in hospitalized children with suspected dengue at the Queen Sirikit National Institute of Child Health in Bangkok, Thailand, from 1973 to 1999, Acute dengue was diagnosed in 15,569 children and virus isolated from 4,846. DEN-3 was the most frequent serotype in primary dengue (49% of all isolates), DEN-2 in secondary dengue and in dengue hemorrhagic fever (37% and 35%, respectively). The predominant dengue serotype varied by year DEN-1 from 1990-92; DEN-2 from 1973-86 and 1988-89; DEN-3 in 1987 and 1995-99; and DEN-4 from 1993-94. Only DEN-3 was associated with severe outbreak years (51).

Table 1. Vectors and potential vectors of mosquito-borne diseases in Thailand and neighboring countries.

Diseases and vector species	Vectors in Thailand	Vectors elsewhere
Malaria		
An. aconitus	X	X
An. annularis	(X)	X
An. culicifacies	-	X
An. dirus	X	X
An. karwari	[X]	-
An. kochi	(X)	X
An. maculatus	[X]	X
An. minimus	X	X
An. nivipes	(X)	-
An. philippinensis	[X]	X
An. pseudowillmori	X	-
An. stephensi	-	X
An. subpictus	-	X
An. sundaicus	[X]	X
An. tesselatus	-	X
An. vagus	(X)	X
An. willmori	-	X
An. barbirostris group	[X]	X
An. hyrcanus group	(X)	X
An. umbrosus group	-	X
Dengue		
Ae. aegypti	X	X
Ae. albopictus	X	X
Japanese encephalitis		
Cx. fuscocephala	X	X
Cx. gelidus	X	X
Cx. pseudovishnui	X	X
Cx, tritaeniorhynchus	X	X
Cx. vishnui	X	X
An. annularis	-	X
An. vagus	-	X

Diseases and vector species	Vectors in Thailand	Vectors elsewhere
Filariasis		
Mn. annulata	X	X
Mn. annulifera	X	X
Mn. bonneae	X	X
Mn. dives	X	X
Mn. indiana	X	X
Mn. uniformis	X	X
Cq. crassipes	X	X
Ae. niveus subgroup	X	X
Ae. poicilius	-	X
Ae. togoi	-	X
Ae. vigilax	-	X
Ae. desmotesi	X	-
Ar. subalbatus	X	X
An. barbirostris group	X	X
An. umbrosus group	-	X
An. maculatus	-	X
An. tessellatus	-	X
An. subpictus	-	X
Cx. bitaeniorhynchus	-	X
Cx. quinquefasciatus	X	X
Cx. sitiens	X	-
Cx. whitmorei	-	X

(For Malaria only X = sporozite, [X] = oocysts, (X) = ELISA)

2.4 Reverse transcriptase polymerase chain reaction (RT-PCR) detection of flaviviruses in mosquitoes

Amplification of RNA by PCR can be performed by annealing a primer to RNA template and then synthesizing a cDNA copy using reverse transcriptase (RT), followed by PCR. Some DNA polymerases can be used for this step, for example the thermostable *T. thermophilus* (Tth) DNA polymerase in the presence of manganese can reverse transcribe RNA. Therefore, because Tth DNA polymerase can utilize both DNA and RNA templates, the whole procedure can be carried out in a single tube. For

mRNA that processes a poly(A) tract at the 3' end, oligo dT random hexamers or a gene-specific primer can be used to prime cDNA synthesis. Viral RNA templates (e.g. retroviruses, rhinoviruses, flavivirus, etc.) or nonpolyadenylated RNA can be copied using random hexamers or specific target primers. RNA (RT) PCR is a highly sensitive tool in the study of gene expression at the RNA level and, in particular, in the quantitation of mRNA or viral RNA levels (9,12,17,47,48,127).

Reverse transcriptase is usually used to synthesize first-strand cDNA from RNA. Reverse transcriptase can purified from several source (e.g. avian myeoblastosis virus (AMV) and Moloney murine leukemia virus (MMLV)). AMV reverse transcriptase is an RNA dependent DNA polymerase that uses single-stranded RNA as a template can synthesize a complementary DNA (cDNA) in the 5' to 3' direction if a primer is present. As well as DNA polymerase activity this enzyme also exhibits ribonuclease H activity, which is specific for RNA : DNA heteroduplex molecules (48).

Reverse transcriptase PCR has been developed for a number of RNA viruses in recent years and has the potential to revolutionize laboratory diagnosis. RT-PCR provides a rapid serotype specific diagnosis. The method is rapid, sensitive, simple and reproducible if properly controlled and can be used to detect viral RNA in human clinical samples, autopsy tissues, or mosquitoes (41, 120, 121). A semi-nested RT-PCR was evaluated for detection of JE virus infected mosquitoes. JE virus RNA was relatively stable in mosquitoes held for up to 14 days after death, viable virus was not detected after day1. RT-PCR can detect single laboratory-infected mosquitoes in pools of 200 mosquitoes and in pools diluted to 0.2/100 and 0.1/100 mosquitoes. However, the ability to detect live virus decreased as pool size increased. However, the semi-nested PCR was faster and more economical using larger pools. JE virus infected mosquito RNA was extracted using Trizol reagent protocal and RNA pellets were air-dried prior to resuspension in DEPC water. The primers used in the RT-PCR were forward primer FV-128 and reverse primer prMR3 and in the seminested PCR, the primers were FV-128 as the forward primer and FV-prM, JE659 were used as the reverse primer (124). Universal primers for Dengue virus detection (serotypes 1-4)

and WN, JE, YF, and Ziga virus were developed. These primers correspond to sequences in the 3' non coding region and the NS5 gene which are highly conserved among the mosquito-borne flaviviruses. Forward primer was EMF1 and VD8 (121). During 1997 to 2000 in Singapore, RT-PCR were developed for detection and typing of DEN virus RNA in field-caught *Aedes* mosquitoes to determine the infection rate in local *Aedes* mosquitoes and the dengue serotype circulating in Singapore (120). Dengue virus type 4 genotype IIA in Malaysia was found by AbuBakar. The virus was typed initially using specific monoclonal antibodies, and confirmed by performing multiplex RT-PCR using forward primer, DV1, and sets of four serotype-specific reverse primers DSP1, DSP2, DSP3, and DSP4 to amplify a portion of NS3 region from the different dengue virus serotypes. This primer set was developed for a single-step RT-PCR and followed by seminested PCR using upstream concensus primer and four type-specific primers (125). RT-PCR using specific primer for WN/Kunjin genome have been developed, WN11/WN2, WN1/WN2 primers were designed to hybridize to a relatively conserved region within the envelope (E) gene of WN/Kunjin viruses. The expected amplification product was 222 base pairs long. Another pair of primers, FLV1/FLV2, was designed to hybridize to conserved regions within the RNA replicase (NS5) gene of wide variety of flaviviruses, amplifying nearly a 220-bp flagment (26). In Thailand, ThCAr105/92 virus isolated from Chiang Mai, Thailand, was indicated a flavivirus using RT-PCR. However, the inability to amplify its genome by RT-PCR using JE-specific primers indicated it was different from existing strains of JE virus (23). A Sitiawan virus (SV), new flavivirus found in Malaysia, RNA was extracted using TRIzol reagent following the manufacture's recommendations. CDNA synthesis performed using RT-PCR (Takara RNA PCR kit) with random primers. Primers used for amplifying and sequencing the genomic regions (~1 kb long) are FU1 (F), cFD2 (R), cFD3 (R), cFD4 (R), and newly design B1 (F), 12 (F) (44).

For CFAV, reared in *Aedes albopictus* cell culture, RNA was extracted and cDNA was constructed using Avian myeloblsatosis virus reverse transcriptase and random primers. Certain CFA genome sequences were amplified by means of PCR using reverse transcriptase and ampli-Taq DNA polymerase. cDNA molecules, made

using random primers or PCR, were ligated into Smal-digest pUC19 plasmid and sequenced using a sequenase kit (United State Biochemical Coroperation) (7). KRV was inoculated into C6/36 cell culture and RNA was extracted from cell culture using QIAamp viral RNA Mini-kit. cDNA was constructed using Titan One Tube RT-PCR System (Roche). NS5 gene of Flavivirus isolate were sequence using FU1/ CFD2, FU2/CFD3 flavivirus universal primers (29, 119). Because DEN genome structural differences correlate with pathogenesis (DF and DHF), in 1999, a method for comparing DEN-2 genomes (6 fragments) directly from patient plasma was developed using RT-PCR. A total of six encoded amino acid changes between DF and DHF patients were seen in prM, E, NS4b, and NS5 genes, while sequence differences observed within the 5' nontranslated region (NTR) and 3' NTR were predicted to change RNA secondaty structures (122). During 2001 in Austria, USU virus RNA were extracted using the QIAamp viral RNA mini Kit (Qiagen). Reverse transcription and amplification were performed in a continuous RT-PCR method by using a QIAGEN Onestep RT-PCR Kit (Qiagen). RT-PCR with universal flavivirus primers resulted in clear PCR amplification products of the expected lengths. The PCR products are in the NS5 genomic region of mosquito-borne flavivirus (117). MMLV reverse transciptase acts in the same way as AMV reverse transcriptase; however, it lacks DNA endonuclease activity and has lower RNase H activity. Thus it has a greater chance of producting full-length copies of large mRNA species. There is also an RNase H- MMLV reverse transcriptase (Superscript II, Gibco) which is a modified form with point mutations in the RNase H coding sequence of a cloned MMLV reverse transcriptase gene. This form of the enzyme eliminates degradation of the RNA molecule during first strand synthesis, resulting in more full-length synthesis and greater yield of cDNA (47, 48). From the study of Kuno et al, the pair of flavivirus cross-reactive primers (FU1 and cFD3) proved to be highly efficient for generating about 1 kb long DNA templates near the 3' terminus of the NS5 gene for most of the viruses by RT-PCR (15).

2.5 Phylogenetics of flaviviruses

Phylogenetics is the study of evolutionary relationships. Phylogenetic analysis is the means of inferring or estimating these relationships. The evolutionary history inferred from phylogenetic analysis is usually depicted as branching (treelike) diagrams, which represent a sort of pedigree of the inherited relationships among molecules (gene trees), organisms, or both. Phylogenetics is sometimes called cladistics because the word "clade", a set of descendants from a single ancestor, is derived from the Greek word for branch. However, cladistics is a particular method of hypothesizing about evolutionary relationship (106). Phylogenetic analysis of DNA or protein sequences has become an important tool for studying the evolutionary history of organisms from bacteria to humans. Since the rate of sequence evolution varies extensively with genes or DNA segments, one can study the evolutionary relationships of virtually all levels of classification of organism by using different genes to DNA segments. Phylogenetic analysis is also important for clarifying the evolutionary pattern of multigene families as well as for understanding the process of adaptive evolution at the molecular level. Reconstruction of phylogenetic trees by using statistical methods was initiated independently in numerical taxonomy for morphological characters and in population genetics for gene frequency data. Some statistical methods developed for these purposes are still used for phylogenetic analysis of molecular data, but in recent years many new methods have been developed (107).

Now a day, the three major tree buildings criteria are distance, maximum parsimony (MP), maximum likelihood (ML). Distance trees use pairwise divergence estimates of all sequences in the data to determine tree topology and branch lengths. Maximum parsimony finds the tree that explains with the fewest number of discrete steps all the base differences in a multiple sequence alignment. Maximum likelihood finds the topology and branch lengths that have the highest probability of producing the observed multiple sequence alignment (106). Several techniques have been used to infer phylogenetic relationships from molecular data (107). Although there are a number of different methods of tree reconstruction, there is little agreement as to

which is best. No current method works well under all conditions and so it is useful to know the strength and weaknesses of each.Tree-building methods can be categorized in two different ways:

2.5.1 Algorithm-based vs. criterion-based.

Algorithm-based methods generate a tree according to a series of steps. The criterion-based approach is a means of evaluating alternative trees according to some optimizable functions. An example of a purely algorithmic method is neighbor joining (NJ), which derives a single tree and has the desirable property of being nearly or quite optimally distance-additive. A criterion-based distance approach evaluates the universe of possible trees according to the criterion that tree additivity is optimized.

2.5.2 Distance-based vs. character-based.

Much of the historical and current discussion in molecular phylogenetics dwells on the utility of various distance- and character-based methods (107,108). Distance-based methods compute pairwise distances according to some measures, then discard the actual data, using only the fixed distances to derive trees. Character-based methods derive trees that optimize the distribution of the actual data patterns for each character. Pairwise distances are thus not fixed, as they are determined by the tree topology. The most commonly applied character-based methods include maximum parsimony and maximum likelihood.

2.5.3 Distance-based methods

Distance-based methods use the amount of dissimilarity (the distance) between two aligned sequences to derive trees. A distance-based method would reconstruct the true tree if all genetic divergence events were accurately recorded in the sequences (109). However, divergence encounters an upper bound as sequences become mutationally saturated. After one sequence of a diverging pair has mutated at

a particular site, subsequent mutations in either sequences cannot render the sites any more “different”. In fact, subsequent mutations can make them again equal, thereby correcting for such “unseen” substitutions.

2.5.3.1 Neighbor Joining (NJ)

The neighbor-joining (110) algorithm is commonly applied with distance tree building, regardless of the optimization criterion. The fully resolved tree is “decomposed’ from a fully unresolved “star’ tree by successively inserting branches between a pair of closest neighbors and the remaining terminals in the tree. The closest neighbor pair is then consolidated, effectively reforming a star tree and the process is repeated. The method is comparatively rapid, i.e., requiring only a few seconds or less for a 50-sequence tree.

2.5.4 Character-based methods

Character-based methods have little in common besides the use of character data at all steps in the analysis. This allows the assessment of the reliability of each base position in an alignment on the basis of all other base positions. The most widely used character-based methods are maximum parsimony and maximum likelihood.

2.5.4.1 Maximum Parsimony (MP)

Maximum parsimony methods were first developed by Eck and Dayhoff (112) for amino acid sequence data and were later modified for use on nucleotide sequences by Fitch (111). These methods focus on the character values observed for each species, rather than working with the distances between sequences that summarize differences between character values. In these methods only shared and derived characters are viewed as phylogenetically informative.

The principle of maximum parsimony involves the identification of a topology that requires the smallest number of evolutionary changes to explain the observed differences among the operational taxonomic units (OTUs) under study. It is often said that the principle of maximum parsimony abides by William of Ockham's razor, according to which the best hypothesis is the one requiring the smallest number of assumptions. In maximum parsimony methods, we use discrete character states, and the shortest pathway leading to these character states is chosen as the best tree. Such a tree is called a maximum parsimony tree. Often two or more trees with the same minimum number of changes are found, so that no unique tree can be inferred. Such trees are said to be equally parsimonious.

Maximum parsimony methods minimize the numbers of changes in sequences between species over the tree, usually making the assumption that there have been approximately constant rates of changes In these methods, a nucleotide site is phylogenetically informative only if it favors some trees over others. The informative site is one where there are at least two kinds of nucleotides or amino acids, and each of them is represented in at least two sequences. The best tree from parsimony analysis is the tree with the smallest number of evolutionary changes to explain the observed differences. However, more than one tree with the same number of inferred changes is often found. The consensus tree must be calculated (106, 107).

There are restrictions on making a phylogenetic tree using maximum parsimony methods. One factor that makes maximum parsimony inefficient is the transition/transversion bias and the heterogeneity of substitution rates among different nucleotide sites, which will introduce noise into phylogenetic inference. One way to reduce this is to give higher weights to transversional changes or slowly evolving sites and lower weights to transitional changes or fast evolving sites (107)

2.5.4.2 Maximum Likelihood (ML)

The first application of maximum likelihood method to tree reconstruction was made by Cavalli-Sforza and Edwards for gene frequency data. Later, Felsenstein

developed maximum likelihood algorithms for amino acid and nucleotide sequence data (113). Maximum likelihood methods are statistically based. The likelihood, L, of a phylogenetic tree is the probability of observing the data (e.g. the nucleotide sequences) under a given tree and specified model of character state changes (e.g., the substitution pattern). This is usually written as L=P (data|tree). The aim of maximum likelihood methods is to find the tree (from among all the possible trees) with the highest L value (112). The likelihood is calculated in terms of the probability that the pattern of variation at a site would be produced by a particular substitution process given a particular tree and the overall base frequencies. The likelihood becomes the sum of the probabilities of each possible reconstruction of substitutions under a particular substitution process. Then the likelihood for all the sites are multiplied to give an overall "likelihood of the tree".

These methods are more difficult to compute and time consuming than the other two methods previously mentioned (113). Maximum likelihood methods assume the form of the tree and then choose the branch length to maximize the likelihood of the data given that tree. These likelihoods are then compared over different possible trees and the tree with the greatest likelihood is considered to be the best estimate.

2.5.5 Bootstrapping

The boostrap is a computational technique for estimating a statistic for which the underlying distribution is unknown or difficult to derive analytically. Since its introduction into phylogenetic study, the boostrap technique has been frequently used as means to estimate the confidence level of phylogenetic hypotheses. The statistical properties of this technique in the context of phylogenetics are quite complex, but theoretical studies have led to a better understrading of the technique. The boostrap belongs to a class of methods called resampling techniques because it estimates the sampling distribution by repeatedly resampling data from the original sample data set (114).

Bootstrapping is a resampling tree evaluation method that works with distance, parsimony, likelihood and just about any other tree derivation methods. It was invented in 1979 and introduced as a tree evaluation method in phylogenetic analysis by Joe Felsenstein (114, 115). The result of bootstrap analysis is typically a number associated with a particular branch in the phylogenetic tree that gives the proportion of bootstrap replicates that supports the monophyly of the clade.

Bootstrapping can be considered a two-step process comprising the generation of (many) new data sets from the original set and the computation of a number that gives the proportion of times that a particular branch appeared in the tree. That number is commonly referred to as the bootstrap value. While it has become common practice to include bootstrapping as part of a thorough phylogenetic analysis, there is some discussion on what exactly is measured by the method. It was originally suggested that the bootstrap value is a measure of repeatability. In more recent interpretations, it has been considered to be a measure of accuracy, a biologically more relevant parameter that gives the probability that the true phylogeny has been recovered (115).

Phylogenetic analysis of the Flavivirus genus, using either partial sequences of non-structural 5 gene (NS5) or the structural envelope gene, and amino acid sequence of envelope gene (E) revealed an extensive series of clades defined by their epidemiology and disease association. The phylogeny identified mosquito-borne, tick-borne and not known vector virus clades, which could be further subdivided into clades defined by their principle vertebrate host. the mosquito-borne flaviviruses revealed two distinct epidemiological groups: (i) the neurotropic viruses, often associated with encephalitic disease in humans or livestock, correlated with the *Culex* species vector and bird reservoirs and (ii) the non-neurotropic viruses, associated with hemorrhagic diseases in humans, correlated with the *Aedes* species vector and primate hosts. The tick-borne viruses also formed two distinct groups: one group associated with seabirds and the other, tick-borne encephalitis complex viruses, associated primarily with rodents. The not known vector flaviviruses formed three distinct group: one group, which was closely related to the mosquito-borne viruses, associated

with bats; a second group, which was more genetically distant, also associated with bats and a third group associated with rodents. Each epidemiological group within the phylogenies revealed distinct geographical clusters in either the old world or the New world, in which mosquito-borne viruses may reflect an old world origin (10) (Figure 3,4).

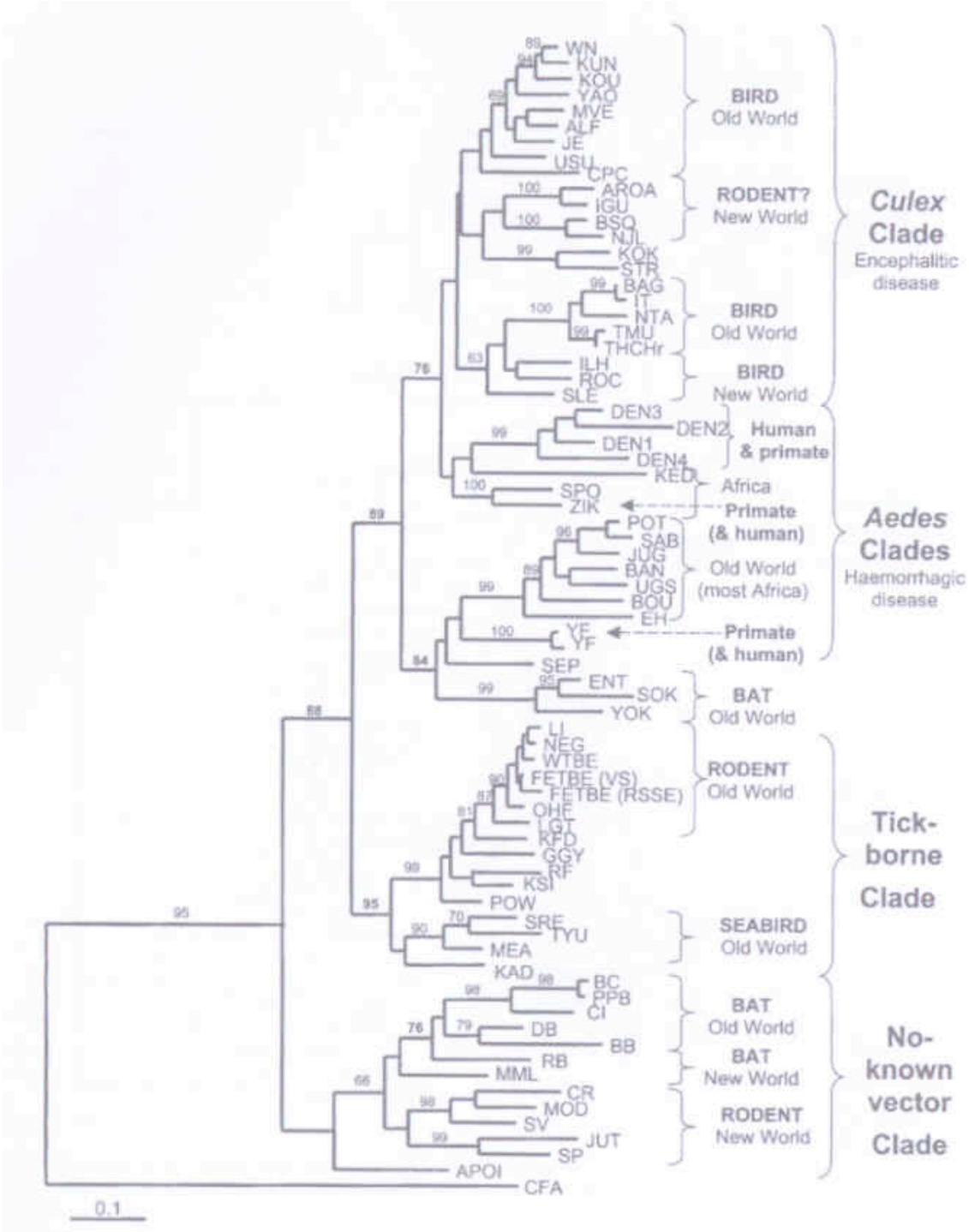


Figure 3. Phylogenetic tree of the Genus Flavivirus based on NS5 gene sequences constructed using an eight parameter model (GRT, Γ, and PINVAR). Brackets denote the principle vector clade or the principle vertebrate host clade for the viruses therein (10).

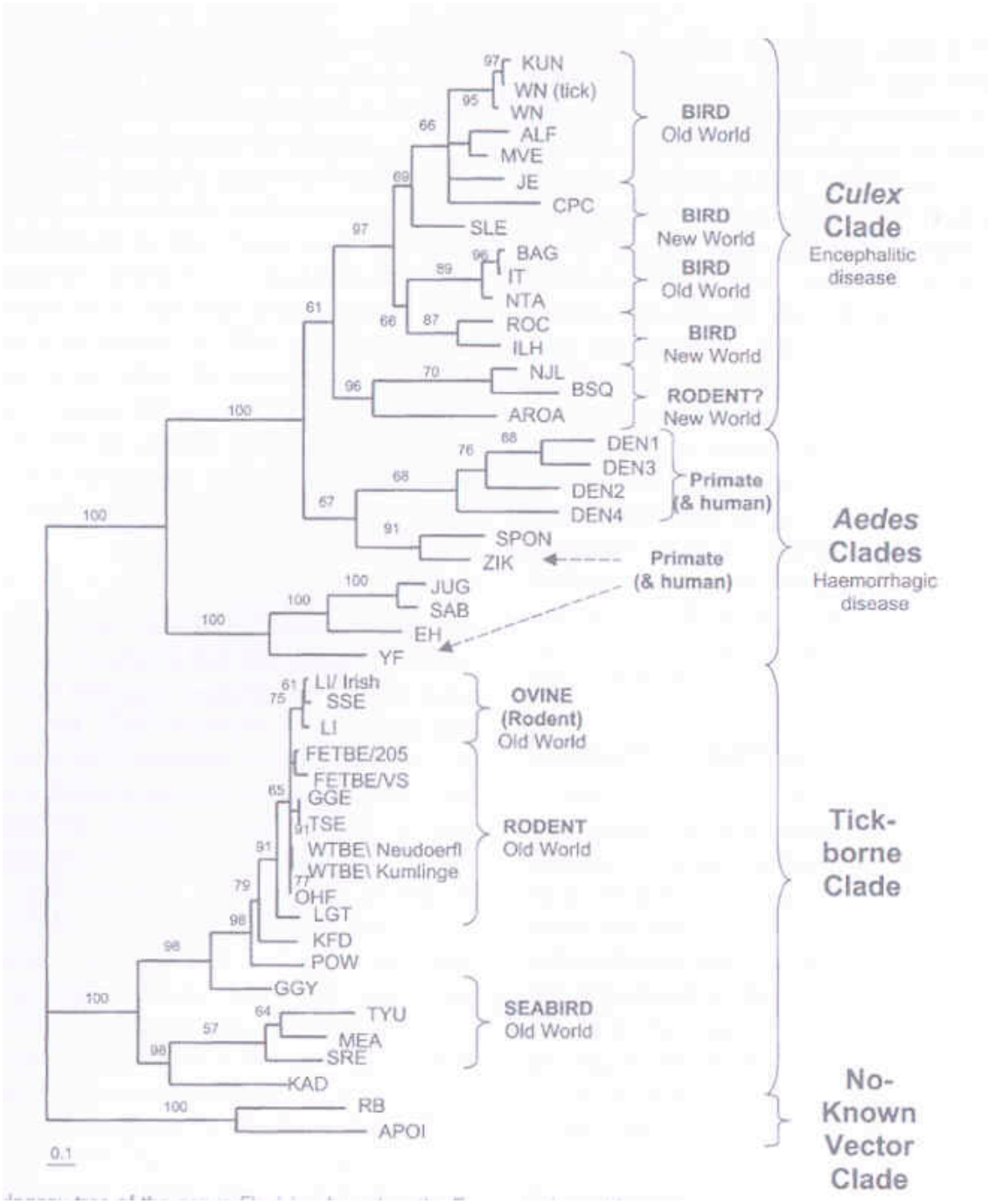


Figure 4. Phylogenetic tree of the Genus Flavivirus based on E gene amino acid sequences constructed using the JTT substitution model and incorporating an additional two parameters (Γ and PINVAR) (10).

Kuno et al. (1998) constructed a phylogeny of more than 70 viruses in the Genus Flavivirus. The multiple sequence alignment program Clustal W was used to obtain an optimal nucleotide or amino acid sequence alignment. Phylogram for the entire sequence (about 1Kb) between primers FU1 and cFD3 were obtained b MEGA or PHYLIP programs. The study revealed that from the putative ancestor of the genus Flavivirus, two major branches emerged, non-vector and vector-borne clusters, and that from the latter cluster emerged tick-borne and mosquito-borne clusters (15). A phylogeny of 123 complete envelope gene sequences was reconstructed in order to understand the evolution of tick and mosquito-borne flaviviruses. An analysis of phylogenetic tree structure revealed a continual and asymmetric branching process in the tick-borne flaviviruses, and an explosive radiation in the last 200 years in viruses transmitted by mosquitoes. The distinction between these two viral groups probably reflects differences in modes of dispersal, propagation, and changes in the size of host populations. The serious implication is that growing human populations are being exposed to an expanding range of increasingly diverse viral strains (38). In 1997, the sequences of an envelope protein gene fragment from 21 temporally distinct WN virus strains, isolates in nine African countries and in France were analyzed. Alignment of nucleotide sequences defined two groups of viruses, which diverged by up to 29%. The first group of subtypes was composed of nine WN strains from France and Africa. The Austral-Asian Kunjin virus was classified as a WN subtype in this first group. The second group inclued 12 WN strains from Africa and Madagascar. The distribution of virus subtypes into two lineages did not correlate with host preference or geographical origin. The isolation of closely related subtypes in distant countries is consistent with WN viruses being disseminated by migrating birds (4). The complete nucleotide sequences of eight WN virus strains (Egyp 1951, Romania 1996-MQ, Italy 1998-equine, New York 1999-equine, MD 2000-crow265, NJ 2000MQ5488, NY 2000-grouse3282, and NY 2000-crow3356) have been determined. The phylogenetic tree revealed the presence of two genetic lineages of WN viruses. Lineage 1 WN viruses have been isolated from the northeastern United States, Europe, Israel, Afriica, India, Russia, and Australia. Lineage 2 WN viruses have been isolated only in sub-Saharan Africa and Madagascar. Lineage 1 WN viruses can be furthur subdivided into three monophyletic clades (18). The

phylogenetic tree of USU virus isolated from Austria demonstrates close genetic relationships to South African viruses, therefore Austria USU virus was classified as part of the JE group of flaviviruses. At the amino acid level, the Austrian and the South African USU virus isolates proved to be 100% identical (117). For CFAV virus, isolated from *Aedes aegypti* cell line, the phylogeny of NS5 gene of CFAV to other flavivirus is even more distant than the relationship between TBE and mosquito-borne flavivirus. The relationship between CFA and either BVDV or HCV appears to be very distant (7). Phylogenetic analysis of NS5 gene of KRV showed KRV and CFAV to be more closely related to the not known vector flavivirus whereas prM-E analysis showed a closer relationship with the tick/vertebrate flaviviruses. It has been hypothesized that arthropod-borne viruses may have evolved from insect viruses. Comparisons of hydrophobicity plots based on amino acid sequences from KRV and CFAV, YF, TBE virus showed that, although sequence identity was low, structural homology based on predicted hydrophobicity/ hydrophobicity of individual proteins was potentially high. Conserved regions of hydrophobicity were observed in KRV proteins as in other flaviviruses, including C-terminal hydrophobic domains in the structural proteins, C, prM and E (119). From phylogenetic analysis of MMLV, three major branches were observed: (i) the mosquito-borne virus; (ii) the tick-borne virus; (iii) the not known vector virus. The results confirm the presence of MMLV in the group of the not known viruses, as predicted by Kuno et al. MMLV belongs to the Rio Bravo branch, which is consistent with the fact that both viruses have bats as their vertebrate host (126). In 2002, Leyssen et al, compared and analyzed an untranslated region (UTR) of Modoc virus, isolated from white-foot deer mouse, in California (1958). P)hylogenetic analysis of the entire coding regions confirmed the classification of Modoc virus within the flaviviruses with not known vector, which is in agreement with previous findings based on partial NS5 sequences (8). Aviles sequenced the C/prM and E/NS1 regions of 24 recent isolates of DEN-1 from South America, including 12 Argentinean and 11 Paraguayan DEN-1 strains isolated in 2000 plus a Paraguayan strain isolated in 1988. These sequences were compared with published sequences of DEN-1 isolated worldwide to determine the origin of these isolates. Pairwise comparison of strains from Paraguay and Argentina revealed a nucleotide divergence of 0-5% in the E/NS1 region and 0-3% in the C/prM region.

The results showed that these viruses belong to the same genotype, but can be separated into two clades. Interestingly, both clades circulated simultaneously in the same geographical area during the 2000 outbreaks (128). A phylogram for Sitawan virus (SV), a new flavivirus, of the NS5 gene (about 1kb between FU1 and cFD3) were obtained by the use of the UPGMA method showed that SV belongs to the mosquito-borne virus cluster, and grouped with Tembusu virus, from Kuala Lumpur, Malaysia, and THCAr, from Chiang Mai, Thailand (44). The nucleotide and amino acid sequence of the envelope (E) gene of Tyuleniy (TYU) and Saumeraz Reef (SRE) viruses have been determined. A phylogenetic tree obtained by maximum parsimony and distance methods for the 22 E gene showed that TYU and SRE viruses are a sister group of the TBE virus complex (21). The epidemiology of tick-borne encephalitis virus was investigated by comparative sequence analysis of virus strains isolated in endemic areas of Europe and Asia. Phylogenetic relationships were determined from nucleotide and amino acid sequences of the major envelope (E) protein of 16 newly sequenced strains and nine previously published sequences. Three genetic lineages could be clearly distinguished, corresponding to a European, a Far Eastern and Siberian subtype (131).

CHAPTER 3
MATERIALS AND METHODS

3.1 Collection and identification of mosquitoes

Mosquitoes were collected from Petchaburi, Kanchanaburi, Chantaburi, Rayong, Nakornsawan, Kampaengphet, and Tak Provinces in the years 2002-2003. They were collected manually by hand and by light traps from cattle, chickens, pig farms and from National Parks. We collected mosquitoes in both villages and National Parks. In the villages, we collected mosquitoes nearby animal farms and houses. Most people of these villages rear animals under their houses and nearby their toilets. For National Park, we collected mosquitoes around or nearby National Parks. Collected mosquitoes were kept in dry ice during transportation.

The technique that we use in this study is animal bias and light trapping. Animal biats is good method for animal like pigs, and cows. But researcher must be aware of mosquito biting when doing the work. The light trap is used for collect mosquito in chicken and pig farms. It is good for animal like chickens because they were always excited when we collected mosquitoes around the farms (figure 4). Light traps can collect a large number of mosquitoes but most of them were injured. We collected both death and live mosquitoes. Death mosquito were kept in tubes and stored under –70 °C.

Live mosquitoes were reared in cages before identification in Bangkok. Dr. Uruyakorn Chansaeng from Division of Entomology, Ministry of Public Health assisted me in identification of mosquitoes. Freezed mosquitoes were hard to identify because the body was blended from pressure and scales and hairs were lost. Incomplete parts of mosquitoes were not used. Only live mosquitoes were used in this study. Geographic Positioning System (GPS) was used to located collection sites

(figure 5) in Nakhonsawan, Kampaengphet, and Tak Provinces. In some locations, GPS could not be used because of heavy rain.

3.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

3.2.1 RNA extraction and cDNA synthesis

Mosquito was homogenized in 100 μl RPMI medium. A sample of 100 μl was prepared from 10 mosquitoes. A total amount of 10 μl. Sample were mixed with 1 ml Tri-reagent (RNA/DNA/Protein isolation reagent from Molecuar Research Center, Inc.) in 1.5 ml microcentrifuge tubes. And then vortexed at high speed 30 seconds before incubate at room temperature for 5 minutes. A total amount of 0.2 ml chloroform was added before vortexing at high speed. Samples were incubated at room temperature for 3 minutes and centrifuged at 14000 rpm 4 °C for 15 minutes. RNA on upper phase was then transferred to fresh tube. RNA was precipitated with 0.5 ml isopropanol, and incubated at room temperature for 10 minutes; then centrifuged at 14000 rpm 4 °C for 10 minutes. Supernatants were removed and RNA pellets were then washed with 1 ml 75% ethanol and mixed by vortex. Then the solution was centrifuged 1000 rpm 4 °C for 5 minutes and the supernatant was discarded and dried using speed vacuum for 10 minutes. RNA pellet was resuspended with 9 μl DEPC water (RNase free). A total amount of 2 μl random primer and 1 μl of 10 mM dNTPs were added. Mixture was heated at 65 °C for 5 minutes and then quick chilled on ice. The contents were collected by brief centrifugation. 4 μl 5X buffer strand buffer, 2 μl 0.1 M DTT, and add 1 RNase inhibitor were added respectively, mixed gently and incubated at 42 °C 2 minutes. 1 μl SUPERSCRIPT II was added and mixed gently, then incubated at 25 °C for 10 minutes and follow by 42 °C 50 minutes and cDNA was kept at –20 °C.

3.2.2 PCR amplification and gel electrophoresis

The oligonucleotide primers used for amplification are shown in Table 2. Primers were synthesized by a DNA Synthesizer (Bioservice Unit, Thailand). The

universal flavivirus primers (FLV1, FLV2) were used for detection of flavivirus in mosquitoes (26), the expected size was 220 base pairs length. These two primers amplified non-structural gene 5 (NS5) region of most flaviviruses. And mosquitoes samples were re-checked by using onestep semi-nested RT-PCR (Quigen OneStep RT-PCR kit) with PF1/PF2 and PF2/PF3 primers, the expected size is about 220 base pairs length. To generate longer sequence an other set of primers were used to amplify, commonly used primer is FU1PM (F)/cFD3PM (R) and FU1PM (F)/ cFD4PM (R) (15).

Table 2. Oligonucleotide primers used for PCR detection of flaviviruses (NS5 gene).

Primer	Nucleotide sequences (5'-3')
FLV1 (F)	GGI AGC AGI GCC ATI TGG T(A/T)C ATG TGG
FLV2 (R)	C(G/T)I GTG TCC CAI CCI GCI GTG TCA TC
F1 (F)*	TTC (G/A)TC GC(C/G) AAC GTT CG(A/G) AAT GA(C/T) GC
R1 (R)*	CGT GG(G/A) AAC ATG GC(A/C) AC(G/A) ATG TTC TG
FG1D (F)*	AA(C/T) (A/T)(C/G)C (A/T)CN (C/G)N(T/A) GA(G/A) ATG TA(C/T) T
cFD3D (R)*	A(G/A)C AT(G/A) TCN (T/C)(C/A)N GT(T/G) GTC ATC CA
EMF1 (F)$^{\alpha}$	GGG TCT CCT CTA ACC AG
VD8 (R)$^{\alpha}$	TGG ATG AC(C/G) AC(G/T) GA(A,G) GA(C,T) ATG
PF1$^{\alpha}$	TG(CT) (AG)T(GCT) TA(CT) AAC ATG ATG GG
PF2$^{\alpha}$	GTG TCC CA(AGT) CC(AGT) GC(AGT) GT(AG) TC
PF3$^{\alpha}$	AT(ACT) TGG T(AT)(CT) ATG TGG (CT)T(CGT) GG
FU1PM (F)	TACAACATGATGGGVAARAGWGARAA
cFD3PM (R)	ARCATGTCTTCYGTBGTCATCCA
cFD4PM (R)	ATNACRCARTCRTCTCCRCT

α = Primers provided by IRD, * = newly designed primers

3.2.2.1 PCR amplification using FLV1/FLV2 primers

PCR amplification were done in 20 μl reaction volumes containing:

14.1 μl distilled water

2.0 μl 10X buffer (Promega)

2.0 μl $MgCl_2$

0.5 μl dNTPs

0.5 μl 20 ρmol forward and reward primer

0.1 μl Taq DNA polymerase (Promega)

1.0 μl DNA template

The PCR cycling (Hybrid) was perform under condition optimized for each primer pair: 1 cycle of 94 °C 2 minutes, 32 cycles of 94 °C 20 seconds, 50 °C 30 seconds, 72 °C 30 seconds. Then 10 μl of each PCR products was electrophoresed in a 2% agarose gel, which was then stained with ethidium bromide.

3.2.2.2 Onestep RT-PCR for flaviviruses

For onestep RT-PCR, during RNA extraction, after air-dry, RNA was re-suspend with 10 μl of DEPC water, and first PCR amplification were done in 50 μl reaction volumes containing:

5X QIAGEN Onestep buffer	10 μl
5X Q-solution	10 μl
10 mM dNTP	2 μl
PF1 (F) primer	2 μl
PF2 (R) primer	2 μl
40 U RNase Inhibitor	1 μl
QIAGEN onestep enzyme mix	2 μl
RNase-free water	11 μl

The PCR cycling (Hybrid) was perform under condition optimized for each primer pair: pre run PCR machine at 50 °C for 1 hour. cDNA were synthesized at 50 °C 30 min and heat 95 °C for 15 minnutes 1 cycle, and follow by 40 cycles of 94 °C 30 seconds , 53 °C 90 seconds, and 72 °C 60 seconds. And 1 cycle of final extention 72 °C 10 minutes and keep 4 °C.

For semi-nested PCR, The first PCR product were used as template, DNA was diluted 1:200 in distilled water. PCR were done in 20 μl of reaction volume containing:

5X Q-solution	4 μl
10X PCR buffer	2 μl
10 mM dNTPs	0.4 μl
PF2 (R)	0.8 μl
PF1 (F) `	0.8 μl
Taq DNA polermerase	0.2 μl
DD water	9.8 μl
DNA template	2 μl

The PCR cycling (Hybrid) was perform under condition optimized for Each primer pair: heat 95 °C for 10 minnutes 1 cycle, and follow by 35 cycles of 94 °C 30 seconds, 53 °C 90 seconds, and 72 °C 60 seconds. And 1 cycle of final extention 72 °C 10 minutes and keep 4 °C, then 10 μl of each PCR products was electrophoresed in a 2% agarose gel, which was then stained with ethidium bromide.

3.3 Cloning and sequencing

3.3.1 Ligation

Ligation of the PCR product to the plasmid vector was performed by the protocol described in Promega's Technical Manual for the pGEM-T vector system. A total of 1 μl of PCR product was added to a reaction containing 5 μl of T_4 DNA ligase

buffer, 1 μl of pGEM-T vector and 1 μl of T_4 DNA ligase and dH_2O was added to make a final volume of 10 μl. The reactions were mixed well by pipetting, then incubated for 24 hours at 4°C.

3.3.2 Transformation

After ligation, *E. coli* DH5-α cells were transformed with the recombinant plasmid DNA. Transformation was accomplished by mixing 10 μl of each ligation reaction with 100 μl competent cells in a sterile microcentrifuge tube. The mixture was placed on ice for 20 minutes, then transferred to a water bath at 42°C for 1 minute, to heat shock the cells. Afterwards, the cells were quickly cooled on ice, 150 μl of SOC medium was added, then incubated at 37°C for 1 hour. Lastly, the cells were plated on LB agar containing 50 μg/ml of ampicillin; plates had also been overlaid with 20 μl of X-gal and 4 μl of IPTG. Plates were incubated overnight at 37°C.

Promega's pGEM-T plasmid vector carries a segment of DNA derived from the *lac* operon of *E. coli* that code for the amino-terminal fragment of β-galactosidase. Bacterial cells exposed to IPTG synthesize both fragments of the β-galactosidase enzyme, and form blue colonies when plated on to media containing X-Gal. Insertion of foreign DNA into the poly-cloning site of the plasmid inactivates the amino-terminal fragment of β-galactosidase, therefore giving rise to white colonies when plated with X-Gal. This property of the pGEM-T vector allows ease in screening colonies for the DNA insert of interest.

3.3.3 Screening for recombinant colonies

After overnight incubation, plates were screened for white colonies, which presumably had the foreign DNA insert. Replicate plates were made by streaking a small amount of each white colony on to a new LB agar/ampicillin plate. The remainder of the colony was dissolved in 20 μl STE, heated for 10 minutes at 95°C to

denature the DNA, and centrifuged at 14,000 rpm for 1 minute. A total of 1 μl of the resulting DNA extract was used in a standard 20 μl PCR reaction with T7/Sp6 primers which flank the cloning site in the vector. The temperature profile was as follows: DNA denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute, for 30 cycles. The size of the PCR product allowed detection of each DNA insert sequence. At least three independent clones were sequenced for each sample to identify polymerase errors.

3.3.4 DNA sequencing

The transformants with insert DNA were purified using a Qiaprep plasmid miniprep kit (Qiagen) according to the manufacturer's protocol. Sequencing of the inserts in both directions was carried out using T7 and Sp6 primers on an ABI 377 automated sequencer (Perkin Elmer, Norwalk, CT).

3.4 Phylogenetic analysis

Partial sequences of the nonstructural gene (NS5 gene) of flaviviruses were aligned (Table 3) using the clustal algorithm by MegAlign program (DNAstar, Lasergene). Phylogenetic relationships were inferred by using PAUP 4.0 b2. The data set was analyzed by maximum parsimony, neighbor-joining and maximum likelihood methods. The maximum parsimony trees were reconstructed by heuristic search and a bootstrap test was performed with 1,000 replicates. The characters were weighted equally. The neighbor-joining tree was constructed by heuristic search using the Tamura and Nei model and bootstrap analyses were done with 1,000 replicates. The maximum likelihood tree, the initial tree searching and model fitting were calculated using Modeltest 2.0. The estimated rates for six nucleotide substitution types, nucleotide frequencies and the proportion of invariable sites were used in later analyses. Rates of substitution at variable sites were assumed to follow a gamma distribution of four rates category represented by the mean. The shape parameter of gamma distribution was set to follow the previous calculation of Modeltest. The likelihood model used the model fitting from the previous calculation. Heuristic

search was conducted with a step-wise addition method using the model parameters mentioned previously. Bootstrap values using 100 replicates were determined.

Table 3. DNA sequences of flaviviruses used in the phylogenetic study (15).

Virus (strain)	Vector association	Distribution	Genbank accession no.
Aroa (VenA-1809)	N	Venezueala	AF013362
Batu Cave (P07-1459)	N	Malaysia	AF013369
Bouboui (DakAr B490)	M	Central and West Africa	AF013364
Bukalasa bat (UGBP-111)	N	Uganda	AF013365
Carey Island (P70-1215)	N	Malaysia	AF013368
Darka bat (209)	N	West and Central Africa and Madagascar	AF013371
Dengue type 1 (Singapore S275/90)	M	Tropical, semitropical areas	M87512
Dengue type 2 (Jamaica)	M	Tropical, semitropical areas	M20558
Dengue type 3 (H-87)	M	Tropical, semitropical areas	M93130
Dengue type 4 (814669)	M	Tropical, semitropical areas	M17255
Gadgets Gully (CSIRO 122)	T	Australia	AF013374
Japanese encephalitis (SA-14)	M	Asia and parts of the Pacific	U15763
Kunjin (MRM61C)	M	Australia, Asia	D00246
Langat (TP21)	T	Southeast Asia, Russia	M86650
Murray Valley encephalitis (original)	M	Australia, Papua New Guinea	AF013389
Negishi (original)	N	Japan and former Soviet Union	AF013391
Phnom Penh bat (CAMA-38D)	N	Cambodia, Malaysia	AF013394
Rio Bravo (M-64)	N	United States, Mexico	AF013396
Sal Vieja (38TWM-106)	N	Texas	AF013401
Saumarez Reef (CSIRO-4)	T	Australia	AF013403
St. Louis encephalitis (MSI-7)	M	Americas	AF013416
Tembusu (MM 1775)	M	Southeast Asia	AF013408
THCAr	M	Thailand	AF013409
Tyuleniy (LEIV-6C)	T	Russia, Oregon	AF013410
Uganda S (original)	M	Africa	AF013411
West Nile (EG-101)	M,T	Africa, Asia, Europe	M12294
Yellow fever (TN-96)	M	Tropical areas	AF013417
Cell fusion agent	N	Unknown	M91671

M = mosquito, T = Tick, N = Not kown vector

CHAPTER 4
RESULTS

4.1 Mosquito collection sites

Mosquitoes were collected from 8 provinces of Thailand. GPS (Geograpic Positioning System) were used to determine coordinates of collection sites in Nakornsawan, Kampaengphet, and Tak Provinces (Table 4.). In some areas, there was heavy raining and signal from satellite could not be received by GPS. For Chantaburi Province, I collected mosquito from Khao Soi Dao wildlife sanctuary and Takad-ngaw District. For Rayong Province, I collected mosquitoes from Khao Cha Mao national park. For Petchaburi Province, I collected mosquitoes from villages nearby Kaeng Krachan National Park. For Lopburi Province, mosquitoes were collected from Tasala District, Amphor Muang. For Karnchanaburi Province, mosquitoes were collected from Amphor Tri-Yok nearby Tri-Yok National Park.

Table 4. GPS coordinates of collection sites in Nakornsawan, Kampaengphet, and Tak Provinces

Collection site	GPS coordinates	Description
1. Nakornsawan	15° 46' 34.554" N	Nong Tai Siang, Tambon Nong Nam
	99° 54' 25.678" E	Woa, Amphoe Lat Yao
	NA	Klongwangchao National Park
	NA	Tablan National Park
	NA	Lansang National Park
2. Kamphaengphet	16° 17' 56.153" N	Moo 4, Tambon Wang Thong,
	99° 23' 45.258" E	Amphoe Muang
	Elevation 100 m	
3. Tak	16° 40' 42.371" N	Wat Phrathat Doi Hua Phai
	98° 37' 18.538" E	
	16° 46' 45.748" N	Taksin National Park
	98° 55' 54.236" E	
	Elevation 909 m	
	16° 40' 11.147" N	Ban Mai Village,
	98° 36' 07.870" E	Phrathat Phadaeng District
	Elevation 199 m	

NA = Non available

Figure 5. Mosquito collection using light trap

Figure 6. Geographic positioning using GPS (Geographic Positioning System)

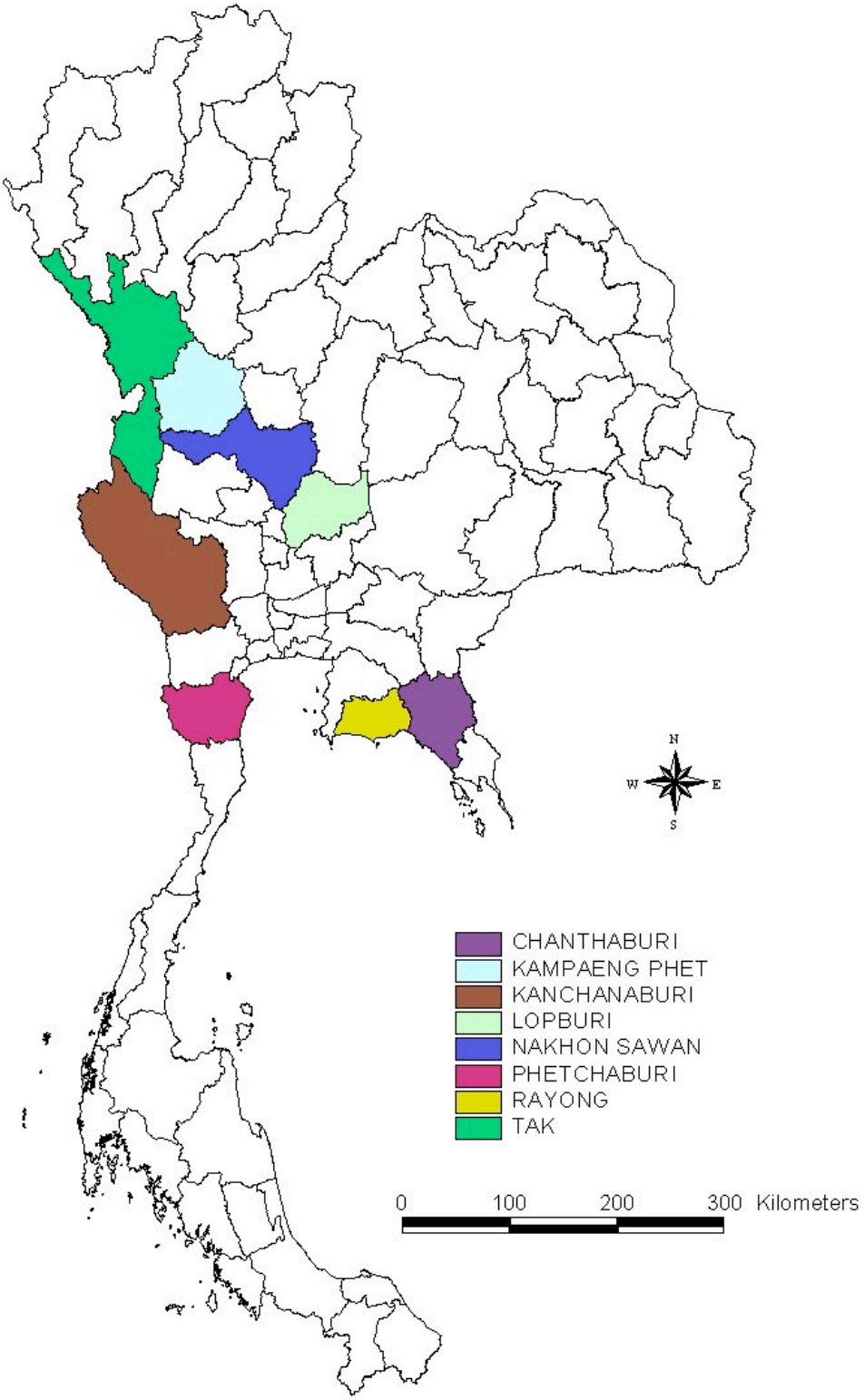


Figure 7 Map of Thailand showing provinces in which mosquitoes were collected.

4.2 Mosquito collections

A total of 1,257 individual mosquitoes representing 6 genera and 40 species were collected from 8 provinces of Thailand (Figure 7) using mosquito light trap (Figure 6), aspiration, and animal bias technique. The first genera, *Aedes* consisted of 9 species, *Ae. aegypti, Ae. albopictus, Ae. seatoi, Ae. niveus, Ae. vittatus, Ae. finlaya, Ae. gardnerii imitartor,* and *Ae. annandalei.* The second genus was *Culex*, which consisted of 6 species, *Cx. tritaeniorhynchus, Cx. vishnui, Cx. gelidus, Cx. fuscocephala, Cx. quinquefasciatus,* and *Cx whitmorei.* The third genus was *Mansonia*, which consisted of 6 species, *Mn. uniformis*, *Mn. bonneae*, *Mn. indiana, Mn. dives, Mn. annulata,* and *Mn. annulifera.* The fourth genus was *Armigeres*, which consisted of 8 species, *Ar. subalbatus, Ar. omissus, Ar. dentatus, Ar. cingulatus, Ar. longipalpi, Ar. annulitarsis, Ar. magnus,* and *Ar. flavus*. The fifth genus was *Anopheles*, which consisted of 11 species, *An. tessellates, An. vagus, An. minimus, An. campestris, An. balabacensis, An. magculatus, An. donaldi, An. subpictus, An. umbrosus, An. nigerrmus,* and *An. pampanai.* The sixth genus was *Coquinlettidia*, which consisted of 1 species, *Coquinlettidia spp*.

4.2.1 Collected mosquitoes in each province

Petchaburi (11 species 96 mosquitoes) *Culex quinquefasciatus(21), Culex vishnui(8), Culex whitmorei(1), Culex gelidus(19), Aedes seatoi(37), Aedes albopictus(3), Aedes aegypti(1), Aedes vittatus(1), Armigeres subalbatus(1), Mansonia uniformis(2), Culex fuscocephala(2).* **Chantaburi (19 species 293 mosquitoes)** *Mansonia uniformis(113), Culex triteaneorhynchus(69), Culex gelidus(17), Culex quinquefasciatus(3), Culex vishnui(10), Aedes seatoi(6), Armigeres subalbatus(29), Aedes sp.(10)*, Anopheles donaldi(4), Anopheles subpictus(2), Anopheles minimus(1), Anopheles umbrosus(1), Mansonia annulifera(6), Mansonia Indiana(4), Armigeres omissus(8), Armigeres dentatus(7), Armigeres cingulatus(1), Aedes annandalei(1), Aedes gardnerii(1).* **Nakhonsawan (10 species, 272 mosquitoes)** *Mansonia uniformis(1), Mansonia Indiana(3), Culex quinquefasciatus(2), Culex fuscosephala(13), Culex terteaniorhynchus(40), Culex*

gelidus(49), Culex vishnui(149), Armigeres dentatus(1), Armigeres annulitarsis(1), Aedes albopictus(13). **Kampaengphet (15 species, 168 mosquitoes)** *Mansonia dives(1), Mansonia uniformis(2), Mansonia Indiana(7), Aedes albopictus(3), Aedes aegypti(2), Anopheles umbrosus(1), Anopheles donaldi(1), Anopheles camprestris(2), Anopheles nigerrmus(4), Anopheles tessellates(4), Culex whitmorei(1), Culex fuscocephala(5), Culex vishnui(20), Culex gelidus(60), Culex triteaneorhynchus(55).* **Tak (9 species, 110 mosquitoes)** *Armigers subalbatus(14), Aedes albopictus(3), Aedes niveus(21), Culex quinquefasciatus(38), Culex tritaeneorhynchus(2), Anopheles tessellates(2), Anopheles vagus(1), Culex vishnui(17), Culex gelidus(12).* **Karnchanaburi (22 species 180 mosqiotes)** *Culex quinquefasciatus(47), Armigeres subalbatus(11), Armigeres dentatus(8), Armigeres annulitarsis(12), Armigeres magnus(1), Aedes niveus(34), Aedes seatoi(8), Aedes aegypti(5), Anopheles minimus(6), Anopheles campestris(3), Anopheles balabacensis(1), Anopheles magculatus(2), Culex vishnui(5), Culex gelidus(3), Culex tritaeneorhynchus(1), Aedes albopictus(16), Armigeres longipalpi(1), Mansonia annulata(1), Armigeres magnus(2), Aedes annandalei(4), Mansonia dives(1), Aedes gardnerii imitartor(2).* **Lopburi (12 species 66 mosquitoes)** *Mansonia uniformis(19), Culex tritaeneorhynchus(17), Culex vishnui(3), Culex gelidus(10), Culex quinquefasciatus(2), Aedes seatoi(1), Coquinlettidia spp.(4), Armigeres subalbatus(2), Aedes aegypti(1), Aedes finlaya(1), Aedes albopictus(2), Anopheles camprestris(4).* **Rayong (16 species 54 mosquitoes)** *Armigeres subalbatus(27), Armigeres flavus(1), Mansonia uniformis(4), Mansonia bonneae(1), Culex vishnui(1), Culex quinquefasciatus(1), Anopheles pampanai(4), Anopheles vagus(1), Anopheles umbrosis(1), Aedes albopictus(4), Armigeres flavus(1), Mansonia Indiana(4), Mansonia uniformis(1), Culex gelidus(1), Aedes niveus(1), Culex fascocephala(1).*

4.3 RT-PCR detection of flaviviruses in mosquitoes

Extracted RNA from 41 species of mosquitoes were examined for the presence of the flaviviruses by RT-PCR using random primers and universal flavivirus primers (Table 2). Amplification products of expected size (220 bp) were obtained only from one mosquito species, i.e., *Culex fuscocephala* (Figure 8). Samples that were positive with these universal primers yielded 213 bp fragments of PCR products. Amplified product of each *Culex fuscocephala* were done. The result showed that two *Culex fuscocephala* were positive to flaviviruses (Figure 9) RT-PCR using FLV1/FLV2 primer, results showed on Table 5 and semi-nested RT-PCR using PF1/PF2, PF2/PF3 primers, results showed on Table 6.

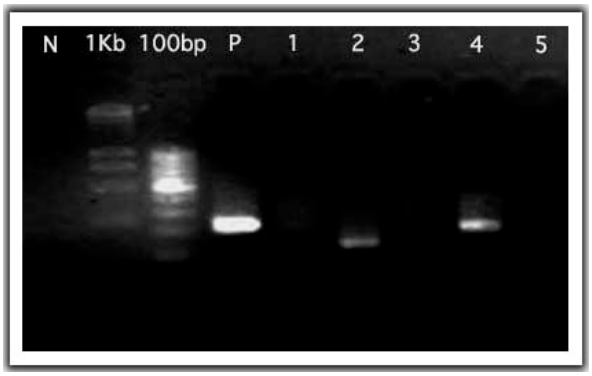


Figure 8. Electrophoretic separation of a 213 bp product of the NS5 gene amplified from 5 pooled mosquitoes of *Culex fuscocephala* shown in lane 4. Lanes 1-3 are *Culex tritaeniorhychus.* Lane 5 is *Culex vishnui*, N and P represent a negative control and positive control (DEN-1 virus), respectively. 1 Kb and 100 bp are DNA markers.

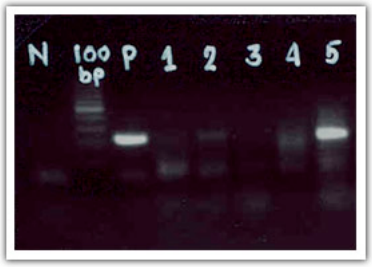


Figure 9. Electrophoretic separation of a 213 bp product of the NS5 gene amplified from *Culex fuscocephala* shown in lanes 2 and 5. Lanes 1-5 are *Culex fuscocephala*. N and P represent a negative control and positive control (DEN-1 virus), respectively. 100 bp are DNA markers.

Table 5. List of flavivirus-screened mosquitoes by RT-PCR. (FLV1/FLV2)

Mosquito species	Collection location	No. examined	RT-PCR results
1. *Ae. aegypti*	Khaeng Kra-Chan National Park, Petchburi	1	-
	Wang Thong, Kampaengphet	2	-
	Muang, Lopburi	1	-
2. *Ae. albopictus*	Khaeng Kra-chan National Park, Petchburi	3	-
	Maewong National Park, Nakornsawan	10	-
	Wang Thong, Kampaengphet	3	-
	Tak-Sin Nataional Park, Tak	1	-
	Ban Mai, Mae-Sod, Tak	2	-
	Muang, Lopburi	2	-
	Khao-Cha-Mao National Park, Rayong	4	-
3. *Ae. seatoi*	Khaeng Kra-Chan National Park, Petchburi	22	-
	Kitcha-Koot Nataional Park, Chantaburi	4	-
	Ta-Khad-Ngaw, Chantaburi	2	-
4. *Ae. niveus*	Tak-Sin Nataional Park, Tak	6	-
	Khao-Cha-Mao National Park, Rayong	1	-
5. *Ae. vittatus*	Khaeng Kra-Chan National Park, Petchburi	1	-
6. *Ae. finlaya*	Muang, Lopburi	1	-
7. *Ae. gardnerii imitartor*	Kitcha-koot Nataional Park, Chantaburi	1	-
8. *Ae. annandalei*	Kitcha-Koot Nataional Park, Chantaburi	1	-
9. *Ar. subalbatus*	Khaeng Kra-Chan National Park, Petchburi	1	-
	Kitcha-Koot Nataional Park, Chantaburi	27	-
	Ta-Khad-Ngaw, Chantaburi	2	-
	Tak-Sin National Park, Tak	1	-
	Ban Mai, Mae-Sod, Tak	10	-

Mosquito species	Collection location	No. examined	RT-PCR results
	Khao-Cha-Mao National Park, Rayong	10	-
10. *Ar. omissus*	Kitcha-Koot Nataional Park, Chantaburi	8	-
11. *Ar. dentatus*	Kitcha-Koot Nataional Park, Chantaburi	7	-
	Maewong National Park, Nakornsawan	1	-
12. *Ar. cingulatus*	Kitcha-Koot Nataional Park, Chantaburi	1	-
13. *Ar. longipalpi*	Kitcha-Koot Nataional Park, Chantaburi	1	-
14. *Ar. annulitarsis*	Maewong National Park, Nakornsawan	1	-
15. *Ar. magnus*	Kitcha-Koot Nataional Park, Chantaburi	1	-
16. *Ar. flavus*	Khao-Cha-Mao National Park, Rayong	2	-
17. *Cx. quinquefasciatus*	Khaeng Kra-chan National Park, Petchburi	21	-
	Ta-Khad-Ngaw, Chantaburi	3	-
	Nakhonsawan	2	-
	Ban Mai, Mae-Sod, Tak	35	-
	Muang, Lopburi	2	-
	Khao-Cha-Mao National Park, Rayong	1	-
18. *Cx. vishnui*	Khaeng Kra-Chan National Park, Petchburi	8	-
	Ta-Khad-Ngaw, Chantaburi	10	-
	Nakhonsawan	40	-
	Maewong National Park, Nakornsawan	3	-
	Wang Thong, Kampaengphet	21	-
	Ban Mai, Mae-Sod, Tak	10	-
	Muang, Lopburi	3	-
19. *Cx fuscocephala*	Khaeng Kra-Chan National Park, Petchburi	2	-
	Nakhonsawan	10	-
	Wang Thong, Kampaengphet	5	+(2)

Mosquito species	Collection location	No. examined	RT-PCR results
	Khao-Cha-Mao National Park, Rayong	1	-
20. *Cx gelidus*	Khaeng Kra-Chan National Park, Petchburi	19	-
	Ta-Khad-Ngaw, Chantaburi	17	-
	Nakhonsawan	20	-
	Wang Thong, Kampaengphet	50	-
	Ban Mai, Mae-Sod, Tak	10	-
	Muang, Lopburi	8	-
	Khao-Cha-Mao National Park, Rayong	1	-
21. *Cx. whitemorei*	Khaeng Kra-chan National Park, Petchburi	1	-
	Wang Thong, Kampaengphet	1	-
22.*Cx. tritaeniorhynchus*	Ta-Khad-Ngaw, Chantaburi	70	-
	Nakhonsawan	20	-
	Wang Thong, Kampaengphet	45	-
	Ban Mai, Mae-Sod, Tak	2	-
	Muang, Lopburi	10	-
23. *Mn. uniformis*	Khaeng Kra-chan National Park, Petchburi	2	-
	Ta-Khad-Ngaw, Chantaburi	20	-
	Wang Thong, Kampaengphet	2	
	Muang, Nakhonsawan	1	-
	Muang, Lopburi	10	-
	Khao-Cha-Mao National Park, Rayong	5	-
24. *Mn. bonneae*	Khao-Cha-Mao National Park, Rayong	1	-
25. *Mn. indiana*	Soi-Dao wildlife sanctuary, Chantaburi	4	-
	Nakhonsawan	1	-
	Wang Thong, Kampaengphet	7	
	Khao-Cha-Mao National Park, Rayong	4	
26. *Mn. dives*	Wang Thong, Kampaengphet	1	
27. *Mn. annulata*	Tha-kra-daan, srisawadi, Karnchanaburi		
28. *Mn. annulifera*	Soi-Dao wildlife sanctuary, Chantaburi	6	-

Mosquito species	Collection location	No. examined	RT-PCR results
29. *An. tessellatus*	Wang Thong, Kampaengphet	4	-
	Ban Mai, Mae-Sod, Tak	2	-
30. *An. vagus*	Ban Mai, Mae-Sod, Tak	2	-
	Khao-Cha-Mao National Park, Rayong	1	
31. *An. minimus*	Thongbhabhumi, Karnchanaburi	1	-
32. *An. campestris*	Thongbhabhumi, Karnchanaburi	2	-
	Muang, Lopburi	4	-
33. *An. balabacensis*	Thongbhabhumi, Karnchanaburi		-
34. *An. magculatus*	Thongbhabhumi, Karnchanaburi		-
35. *An. donaldi*	Ta-Khad-Ngaw, Chantaburi	4	-
	Wang Thong, Kampaengphet	1	-
36. *An. subpictus*	Ta-Khad-Ngaw, Chantaburi	2	-
37. *An. umbrosus*	Ta-Khad-Ngaw, Chantaburi	1	-
	Wang Thong, Kampaengphet	1	-
	Khao-Cha-Mao National Park, Rayong	1	-
38. *An. nigerrmus*	Wang Thong, Kampaengphet	4	-
39. *An. pampanai*	Khao-Cha-Mao National Park, Rayong	4	-
40. *Coquinlettidia spp.*	Muang, Lopburi	4	-
Total		692	+ (2)

Table 6 List of flavivirus-screened mosquitoes by nested RT-PCR (PF1, PF2, PF3).

Mosquito species	Collection location	No. examined	RT_PCR Result
1. *Ae. aegypti*	Khaeng Kra-Chan National Park, Petchburi	1	
	Wang Thong, Kampaengphet	2	
	Muang, Lopburi	1	-
2. *Ae. albopictus*	Maewong National Park, Nakornsawan	10	-
	Khaeng Kra-chan National Park, Petchburi	3	
	Wang Thong, Kampaengphet	3	
	Tak-Sin Nataional Park, Tak	1	-
	Ban Mai, Mae-Sod, Tak	2	
	Muang, Lopburi	2	
	Khao-Cha-Mao National Park, Rayong	4	-
3. *Ae. seatoi*	Khaeng Kra-Chan National Park, Petchburi	22	
	Kitcha-Koot Nataional Park, Chantaburi	4	
	Ta-Khad-Ngaw, Chantaburi	2	-
4. *Ae. niveus*	Tak-Sin Nataional Park, Tak	6	
	Khao-Cha-Mao National Park, Rayong	1	-
5. *Ae. vittatus*	Khaeng Kra-Chan National Park, Petchburi	1	-
6. *Ae. finlaya*	Muang, Lopburi	1	-
7. *Ae. gardnerii imitartor*	Kitcha-koot Nataional Park, Chantaburi	1	-
8. *Ae. annandalei*	Kitcha-Koot Nataional Park, Chantaburi	1	-
9. *Ar. subalbatus*	Khaeng Kra-Chan National Park, Petchburi	1	-
	Kitcha-Koot Nataional Park, Chantaburi	10	-
	Ta-Khad-Ngaw, Chantaburi	2	-
	Tak-Sin National Park, Tak	1	-
	Ban Mai, Mae-Sod, Tak	10	-
	Khao-Cha-Mao National Park, Rayong	6	-

Mosquito species	Collection location	No. examined	RT_PCR Result
10. *Ar. omissus*	Kitcha-Koot Nataional Park, Chantaburi	8	-
11. *Ar. dentatus*	Kitcha-Koot Nataional Park, Chantaburi	7	-
12. *Ar. cingulatus*	Kitcha-Koot Nataional Park, Chantaburi	1	-
13. *Ar. longipalpi*	Tha sao, Triyok, Karnchanaburi	1	-
14 *Ar. annulitarsis*	Maewong National Park, Nakornsawan	1	-
15. *Ar. magnus*	Tha-kra-daan, Triyok, Karnchanaburi	1	-
16. *Ar. flavus*	Khao-Cha-Mao National Park, Rayong	2	-
17. *Cx. quinquefasciatus*	Khaeng Kra-chan National Park, Petchburi	10	-
	Ta-Khad-Ngaw, Chantaburi	3	-
	Nakhonsawan	2	-
	Khao-Cha-Mao National Park, Rayong	1	-
	Muang, Lopburi	2	-
	Ban Mai, Mae-Sod, Tak	10	-
18. *Cx. vishnui*	Khaeng Kra-Chan National Park, Petchburi	8	-
	Ta-Khad-Ngaw, Chantaburi	10	-
	Nakhonsawan	10	-
	Maewong National Park, Nakornsawan	9	-
	Ban Mai, Mae-Sod, Tak	10	-
	Wang Thong, Kampaengphet	10	-
19. *Cx fuscocephala*	Khaeng Kra-Chan National Park, Petchburi	2	
	Khao-Cha-Mao National Park, Rayong	1	
	Nakhonsawan	10	-
20. *Cx gelidus*	Khaeng Kra-Chan National Park, Petchburi	10	-
	Ta-Khad-Ngaw, Chantaburi	10	-
	Nakhonsawan	10	-
	Wang Thong, Kampaengphet	10	-

Mosquito species	Collection location	No. examined	RT_PCR Result
	Ban Mai, Mae-Sod, Tak	10	-
21. *Cx. whitemorei*	Khaeng Kra-chan National Park, Petchburi	1	-
22.*Cx. tritaeniorhynchus*	Ta-Khad-Ngaw, Chantaburi	10	-
	Nakhonsawan	10	-
	Wang Thong, Kampaengphet	10	-
	Ban Mai, Mae-Sod, Tak	2	-
23. *Mn. uniformis*	Ta-Khad-Ngaw, Chantaburi	10	-
	Khaeng Kra-chan National Park, Petchburi	2	-
	Wang Thong, Kampaengphet	2	-
	Muang, Nakhonsawan	1	-
	Khao-Cha-Mao National Park, Rayong	4	-
	Muang, Lopburi	10	-
24. *Mn. bonneae*	Khao-Cha-Mao National Park, Rayong	1	-
25. *Mn. indiana*	Soi-Dao wildlife sanctuary, Chantaburi	4	
	Nakhonsawan	1	
	Khao-Cha-Mao National Park, Rayong	3	
	Wang Thong, Kampaengphet	2	-
26. *Mn. dives*	Wang Thong, Kampaengphet	1	-
27. *Mn. annulata*	Tha-kra-daan, srisawadi, Karnchanaburi	1	-
28. *Mn. annulifera*	Soi-Dao wildlife sanctuary, Chantaburi	6	-
29. *An. tessellatus*	Wang Thong, Kampaengphet	4	
	Ban Mai, Mae-Sod, Tak	2	-
30. *An. vagus*	Ban Mai, Mae-Sod, Tak	1	
	Khao-Cha-Mao National Park, Rayong	1	-
31. *An. minimus*	Thongbhabhumi, Karnchanaburi	5	-
32. *An. campestris*	Thongbhabhumi, Karnchanaburi	3	
	Muang, Lopburi	4	-
33. *An. balabacensis*	Thongbhabhumi, Karnchanaburi	1	-
34. *An. magculatus*	Thongbhabhumi, Karnchanaburi	2	-

Mosquito species	Collection location	No. examined	RT_PCR Result
35. *An. donaldi*	Ta-Khad-Ngaw, Chantaburi	4	
	Wang Thong, Kampaengphet	1	-
36. *An. subpictus*	Ta-Khad-Ngaw, Chantaburi	2	-
37. *An. umbrosus*	Ta-Khad-Ngaw, Chantaburi	1	
	Khao-Cha-Mao National Park, Rayong	1	-
38. *An. nigerrmus*	Wang Thong, Kampaengphet	2	-
39. *An. pampanai*	Khao-Cha-Mao National Park, Rayong	4	-
40. *Coquinlettidia spp.*	Muang, Lopburi	4	-
Total		390	0

After I have sequenced of this flavivirus I tried to amplify with another set of primers, to obtain longer sequences in order to determined the relationship of this flavivirus to others. PCR amplifications were done with different primers and PCR conditions. PCR conditions are shown in an appendix A. FU1/cFD3PM and FU1/cFD4PM degenerated primers are commonly used to determine flavivirus sequences (15). EMF1/VD8 provided from IRD, F1 and R1 were newly designed from CFAV and KRV, which are viruses that are closely related to this flavivirus according to their sequence alignment. And FG1 and cFD3D were designed to be compatible with FLV1 and FLV2, the primers that can amplify cDNA of flaviviruses. The results showed that these primers can not amplify this cDNA (Table 7).

Table 7. Results of PCR amplification of cDNA of flaviviruses using flavivirus primers

Primers	Result
FU1/cFD3PM	-
FU1/cFD4PM	-
EMF1/VD8	-
F1/R1	-
FG1/FLV2	-
FLV1/cFD3D	-

4.4 Flavivirus infection rates

Flavivirus was detected in 1 out of 40 species that were collected, namely *Culex fuscocephala*. *Culex fuscocephala* were collected from Wang Thong District, Kampaengphet Province. The infection rate of flaviviruses in *Culex fuscocephala* were 11% (2/18) and 1.2% (2/168) of mosquitoes collected from Wang Thong District, Kampaengphet Province.

4.5 Phylogenetic relationships of flaviviruses

In order to establish the relationship of the flavivirus strains of this study with those of other flaviviruses, I conduct phylogenetic analysis. I cloned and sequenced partial fragments of NS5 gene. One NS5 positive sample from *Culex fuscocephala* was used. Figure 10 showed maximum parsimony (MP) for NS5 gene, 1035 bp, of 30 taxa of flavivirus obtained from GenBank (Table 4). The result showed that flaviviruses have been divided into three major clusters, namely the not known vector, mosquito-borne vector, and tick-borne vector clades. Cell Fusion Agent Virus (CFAV) was closely related to Kamiti River Virus (KRV) with very highly boostrap (100%) and also related to the not known vector clade with high bootstap (91%) but tick-borne virus group was related to mosquito-borne virus group with low bootstrap support (67%). Percent identity of sequence distance between groups was shown on Figure 11. Amino acid sequences of flaviviruses were used to construct MP tree (Figure 12). MP tree of amino acid sequences showed the result as same as DNA sequences, there were three major clusters, Not known vector, mosquito-borne vector, tick-borne vector clades. CFAV still closely related to KRV with very highly bootstrap (100%) and grouped in the not known vector clade with highly bootstrap (86%) support.

MP tree of a new flavivirus from *Culex fuscocephala* and other flaviviruses was shown in Figure 13. This virus was designated as Wang Thong Virus (WTV). The phylogeny tree classified 30 flaviviruses into three clusters, i.e., not known vector, tick-borne and mosquito-borne viruses, containing 10, 5 and 15 viruses,

respectively. WTV was only distantly related to the other mosquito-borne flavivirus clade and was clustered with CFAV and KRV with high bootstrap support (85%). The WTV has pair-wise nucleotide sequence identities with viruses of the designated clades as follows: 62.3% with CFAV, 60.4% with KRV, 45 to 53% with clade I, 46 to 50% with clade III, 46% with clade IV, 40 to 54% with clade V, and 43% with the Yellow fever virus. Phylogenetic tree was still be divided into three major clades as same as 1-Kb phylogenetic tree. But there was a little bit shift of taxon in that clade. MP tree of amino acid sequences of WTV was constructed (Figure 14), the result showed WTV was closely related to CFAV and KRV with very highly bootstrap (100%)

Figure 15 shows the neighbor-joining tree (NJ) based on the Tamura and Nei method with shape parameter of gamma distribution = 1.6296 (TrN+G). WTV was in the same clade as CFAV and KRV with high boostrap support (81%). Maximum likelihood (ML) tree of WTV (Figure 16) showed the same result as NJ and MP that KWV was in the same clade with CFAV and KRV with highly bootstrap support (86%). These three phylogenetic tree, i.e., MP, NJ, ML, all showed that WTV was closely related to CFAV and KRV.

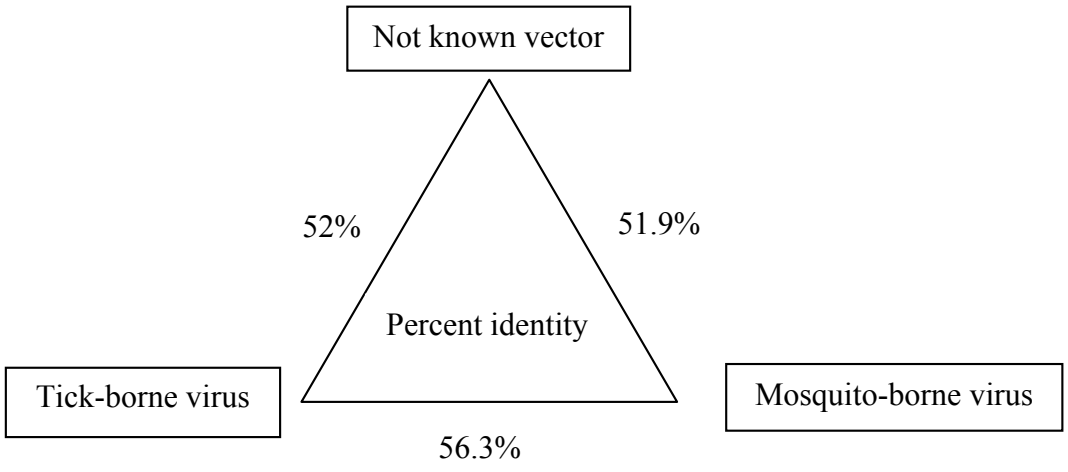


Figure 10. Percent identity of sequence distance between groups of flaviviruses.

Figure 11. The maximum parsimony tree (MP) for NS5 gene of flaviviruses, all sequence obtained from GenBank. Branch lengths are propotional to the scale. Numbers at node are bootstrap percentages obtained from 1,000 resamplings. Only bootstrap percentages which are greater than 50% are shown.

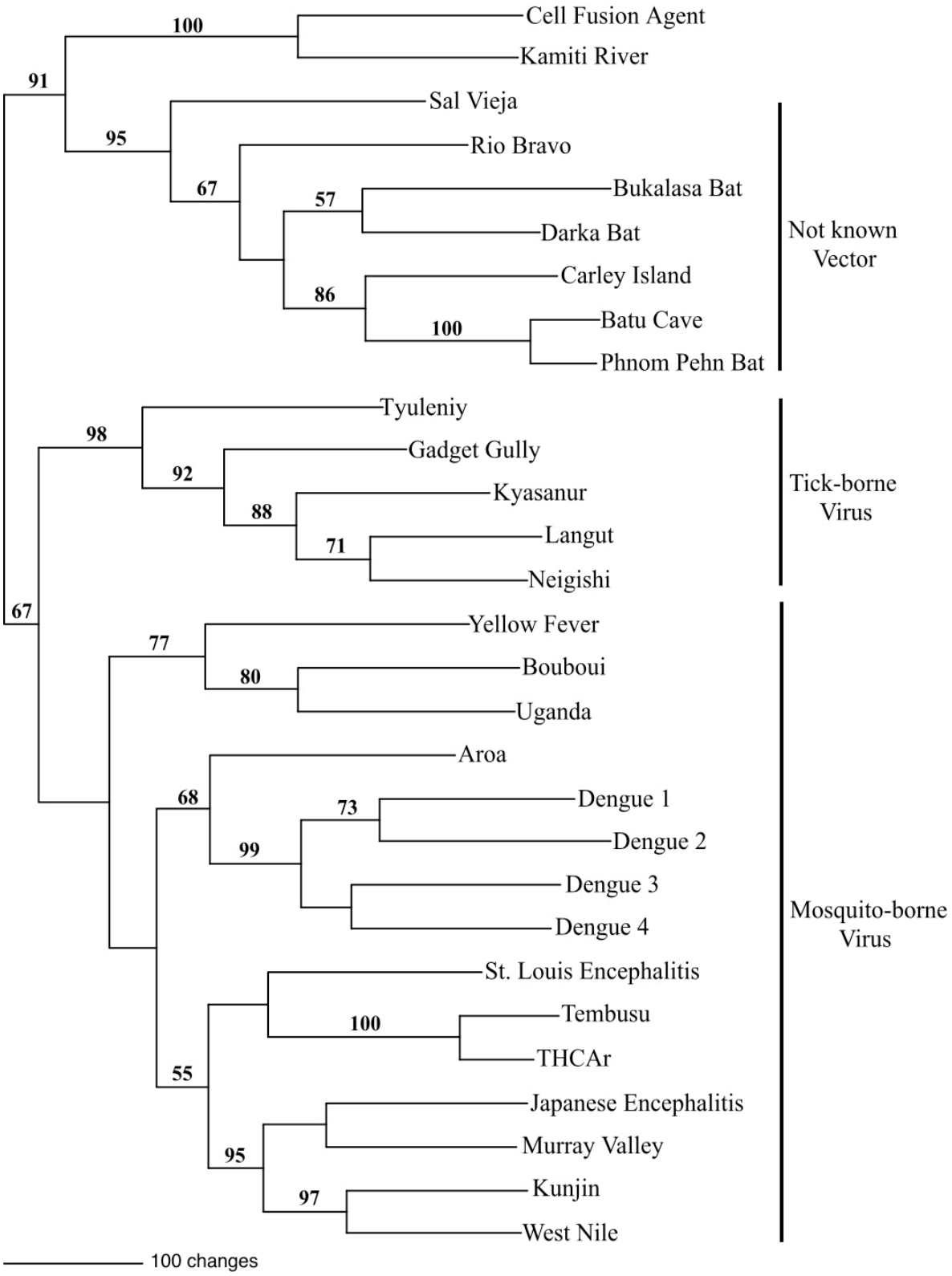

Cell Fusion Agent
Kamiti River
Sal Vieja
Rio Bravo
Bukalasa Bat
Darka Bat
Carley Island
Batu Cave
Phnom Pehn Bat
Not known Vector
Tyuleniy
Gadget Gully
Kyasanur
Langut
Neigishi
Tick-borne Virus
Yellow Fever
Bouboui
Uganda
Aroa
Dengue 1
Dengue 2
Dengue 3
Dengue 4
St. Louis Encephalitis
Tembusu
THCAr
Japanese Encephalitis
Murray Valley
Kunjin
West Nile
Mosquito-borne Virus
100
91
95
67
57
86
100
98
92
88
71
67
77
80
68
73
99
55
100
95
97
100 changes

Figure 12. The maximum parsimony tree (MP) for amino acid sequences from the NS5 gene of flaviviruses, all sequences obtained from GenBank. Numbers at nodes are bootstrap percentages obtained from 100 resamplings. Only bootstrap percentages which are greater than 50% are shown.

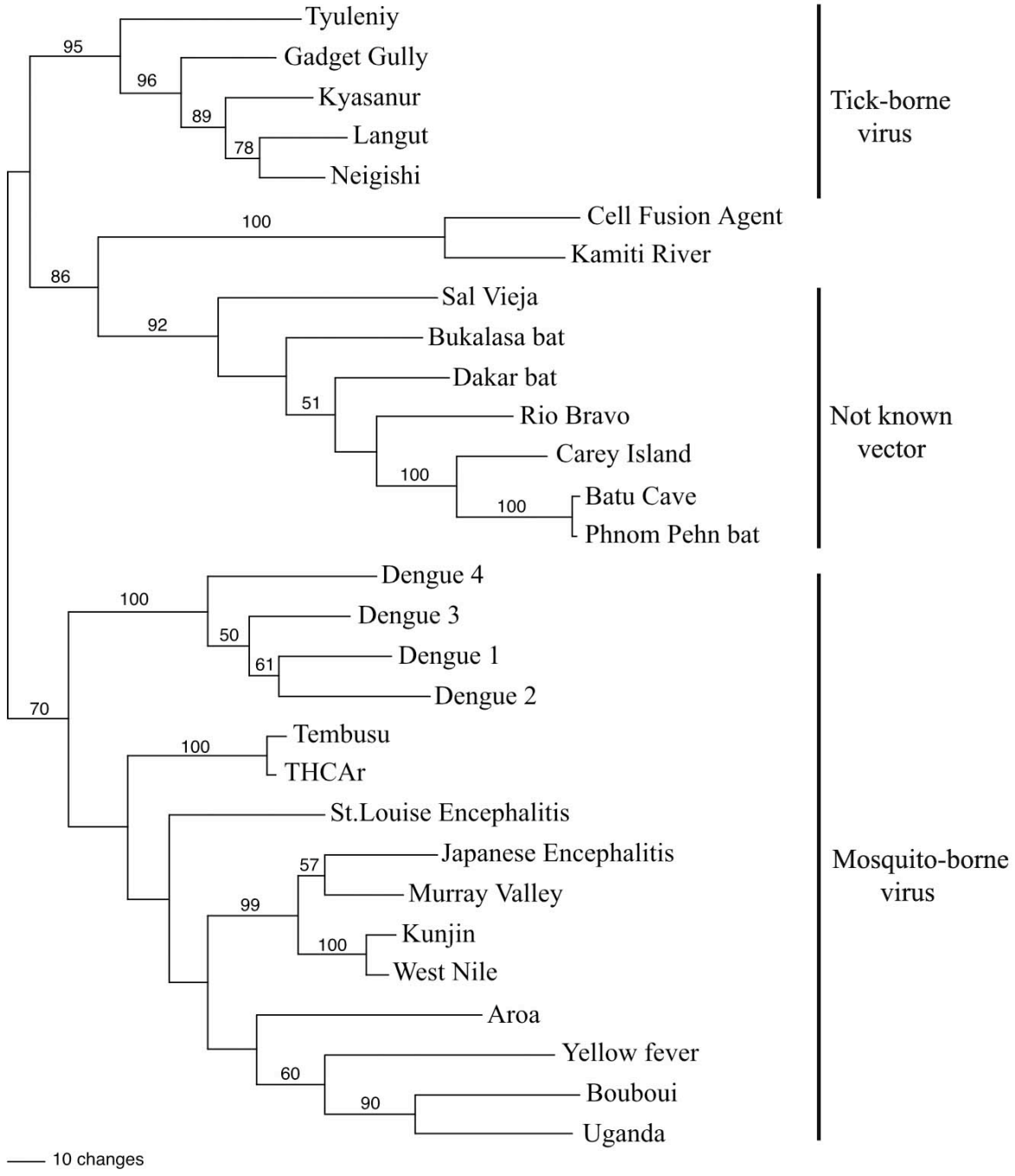

Tyuleniy
Gadget Gully
Kyasanur
Langut
Neigishi
Cell Fusion Agent
Kamiti River
Sal Vieja
Bukalasa bat
Dakar bat
Rio Bravo
Carey Island
Batu Cave
Phnom Pehn bat
Dengue 4
Dengue 3
Dengue 1
Dengue 2
Tembusu
THCAr
St.Louise Encephalitis
Japanese Encephalitis
Murray Valley
Kunjin
West Nile
Aroa
Yellow fever
Bouboui
Uganda
95
96
89
78
100
86
92
51
100
100
100
50
61
70
100
57
99
100
60
90
Tick-borne virus
Not known vector
Mosquito-borne virus
10 changes

Figure 13. The maximum parsimony tree (MP) for NS5 gene sequence of a new flavivirus, Wang Thong Virus (WTV) from *Culex fuscocephala,* collected in Wang Thong District, Kampaengphet Province. Branch lengths are proportional to the scale. Numbers at node are bootstrap percentages obtained from 1,000 resamplings. Only bootstrap percentages which are greater than 50% are shown.

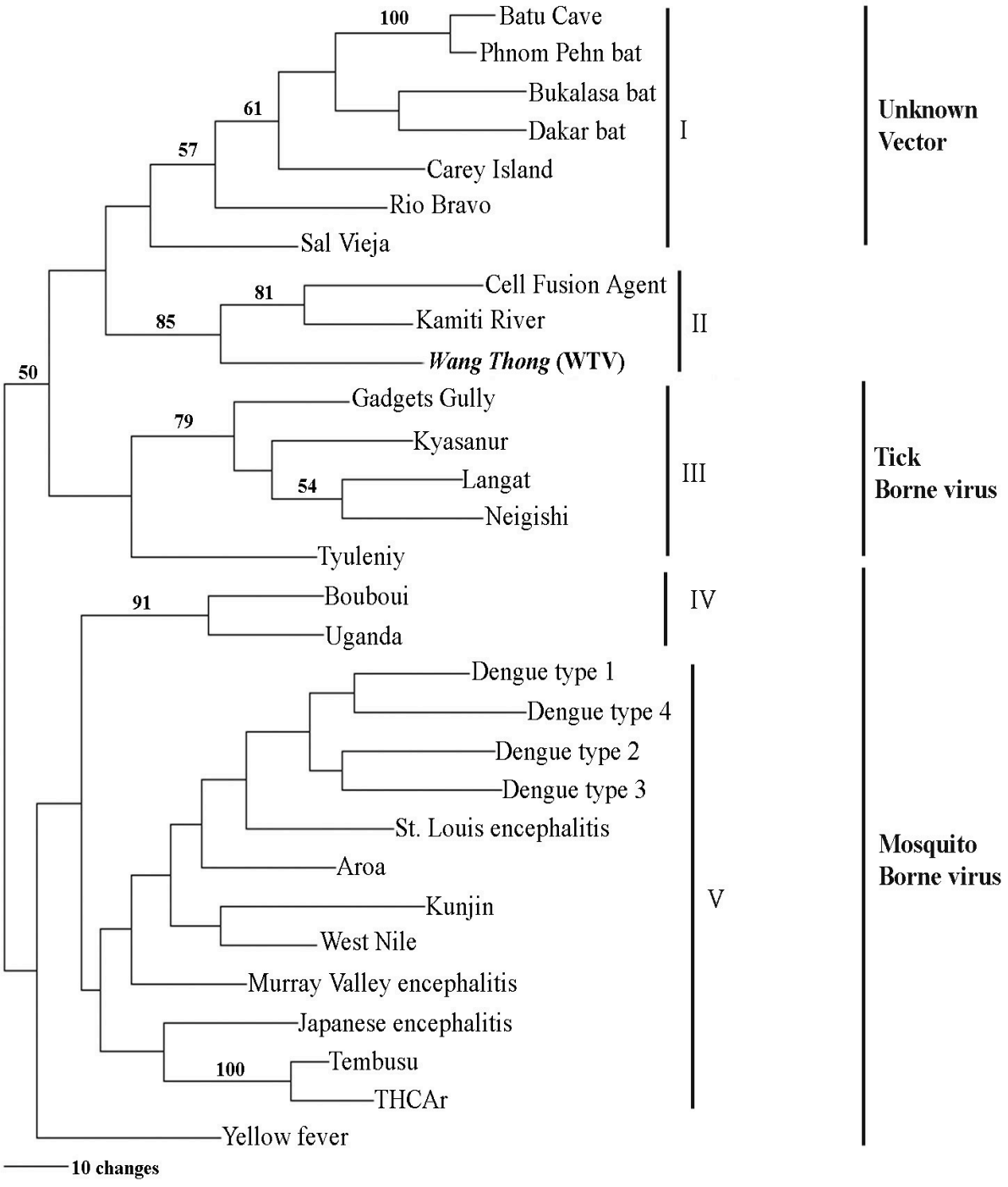

100
Batu Cave
Phnom Pehn bat
Bukalasa bat
61
Dakar bat
57
Carey Island
Rio Bravo
Sal Vieja
I
Unknown Vector
81
Cell Fusion Agent
85
Kamiti River
Wang Thong (WTV)
II
50
Gadgets Gully
79
Kyasanur
54
Langat
Neigishi
Tyuleniy
III
Tick Borne virus
91
Bouboui
Uganda
IV
Dengue type 1
Dengue type 4
Dengue type 2
Dengue type 3
St. Louis encephalitis
Aroa
Kunjin
West Nile
Murray Valley encephalitis
Japanese encephalitis
100
Tembusu
THCAr
Yellow fever
V
Mosquito Borne virus
10 changes

Figure 14. The maximum parsimony tree (MP) for amino acid sequence of the NS5 gene of a new flavivirus, Wang Thong Virus (WTV) from *Culex fuscocephala ,* collected in Wang Thong District, Kampaengphet. Branch lengths are proportional to the scale. Numbers at nodes are bootstrap percentages obtained from 100 resamplings. Only bootstrap percentages which are greater than 50% are shown.

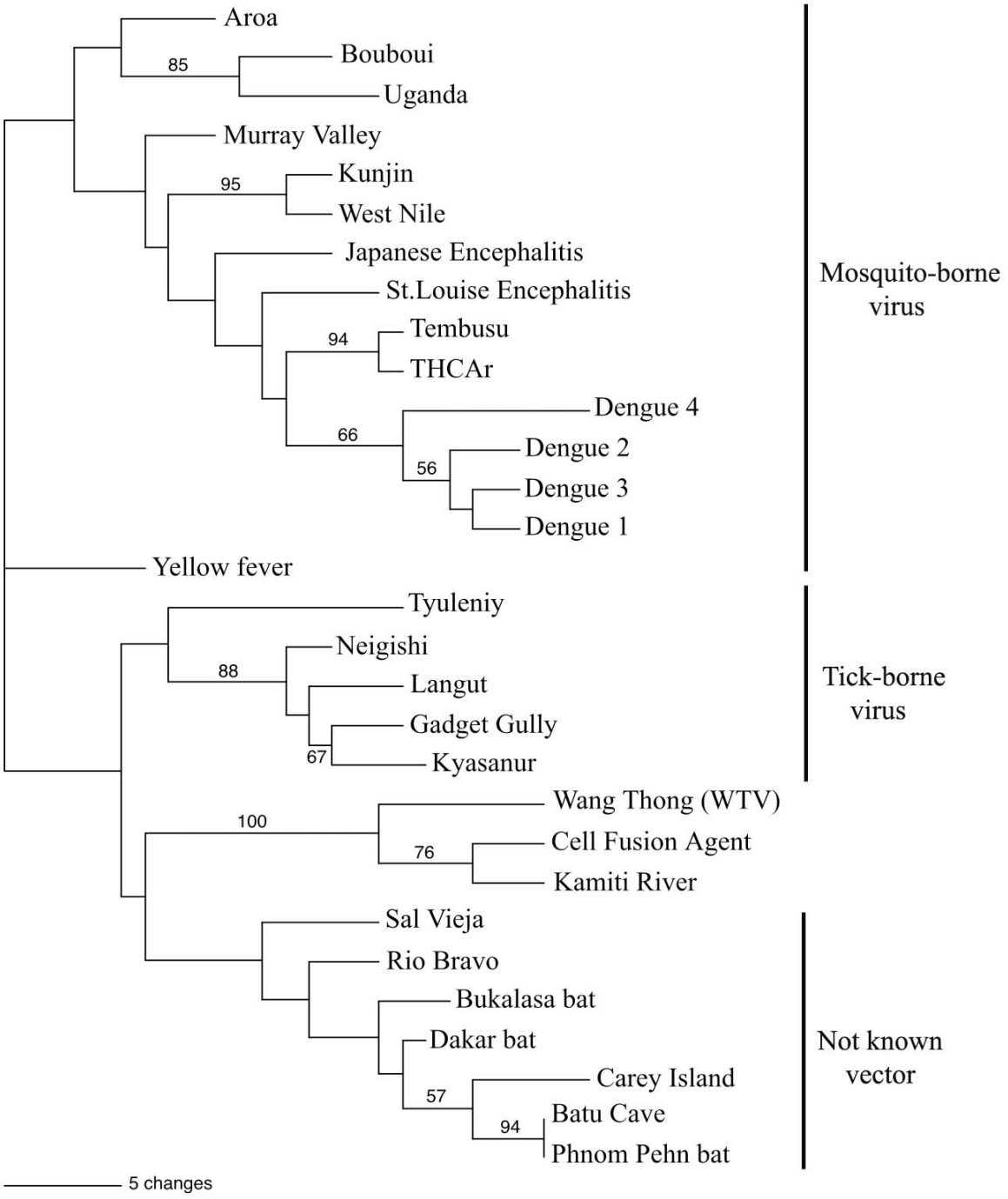

Aroa
Bouboui
Uganda
85
Murray Valley
Kunjin
West Nile
95
Japanese Encephalitis
St.Louise Encephalitis
Tembusu
THCAr
94
Dengue 4
Dengue 2
Dengue 3
Dengue 1
66
56
Mosquito-borne virus
Yellow fever
Tyuleniy
Neigishi
Langut
Gadget Gully
Kyasanur
88
67
Tick-borne virus
Wang Thong (WTV)
Cell Fusion Agent
Kamiti River
100
76
Sal Vieja
Rio Bravo
Bukalasa bat
Dakar bat
Carey Island
Batu Cave
Phnom Pehn bat
57
94
Not known vector
5 changes

Figure 15. The neighbour-joining tree of a new flavivirus, Wang Thong Virus (WTV) from *Culex fuscocephala,* collected in Wang Thong District, Kampaengphet Province, inferred by comparisons of NS5 gene sequences. The evolutionary distance values are determined by the Tamura and Nei method and shape parameter of gamma distribution. These values are used to construct a dendrogram by the neighbor-joining method. Numbers at nodes are bootstrap percentages obtained from 1,000 resamplings, only bootstrap percentages which are greater than 50% are shown

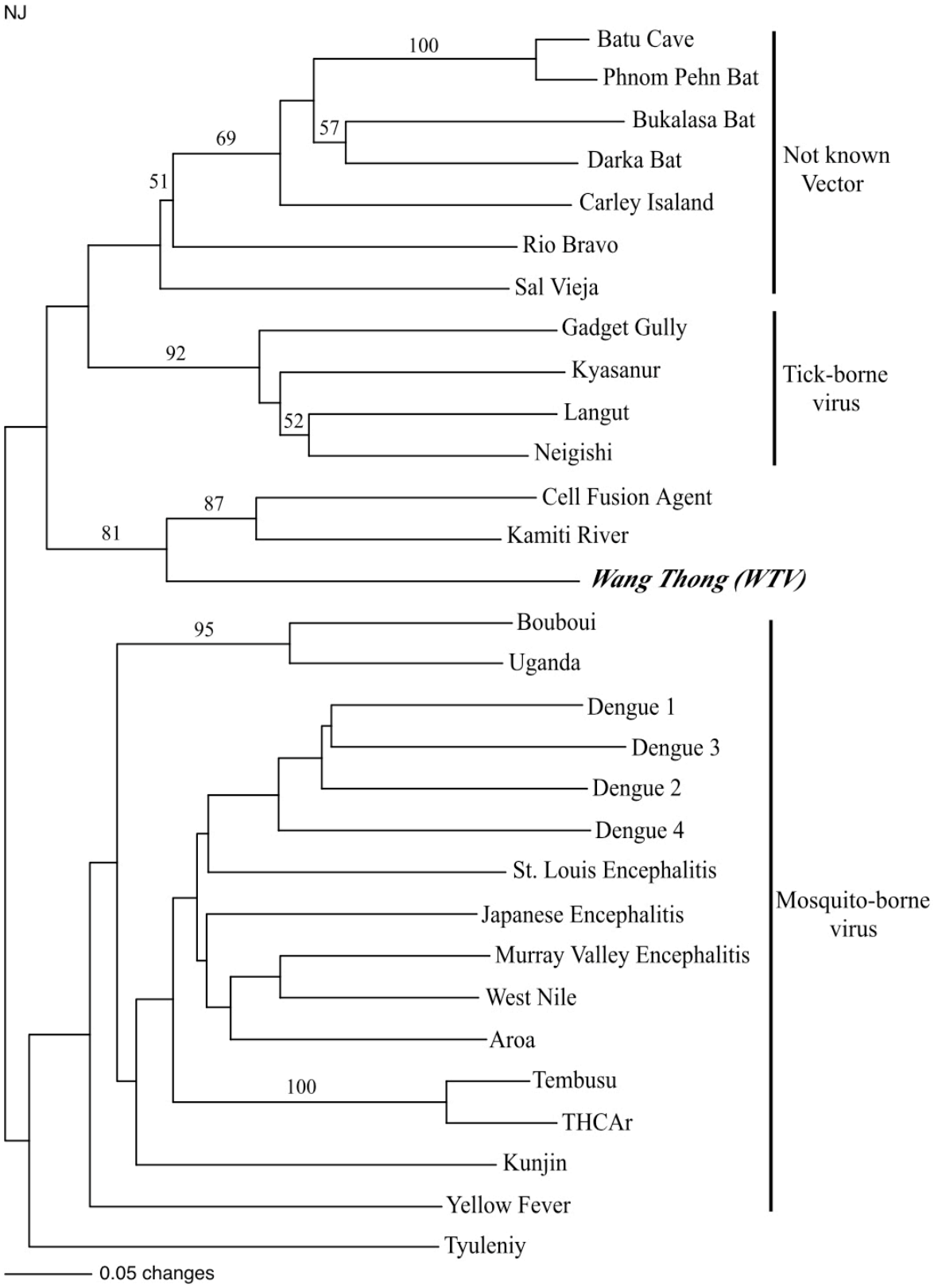
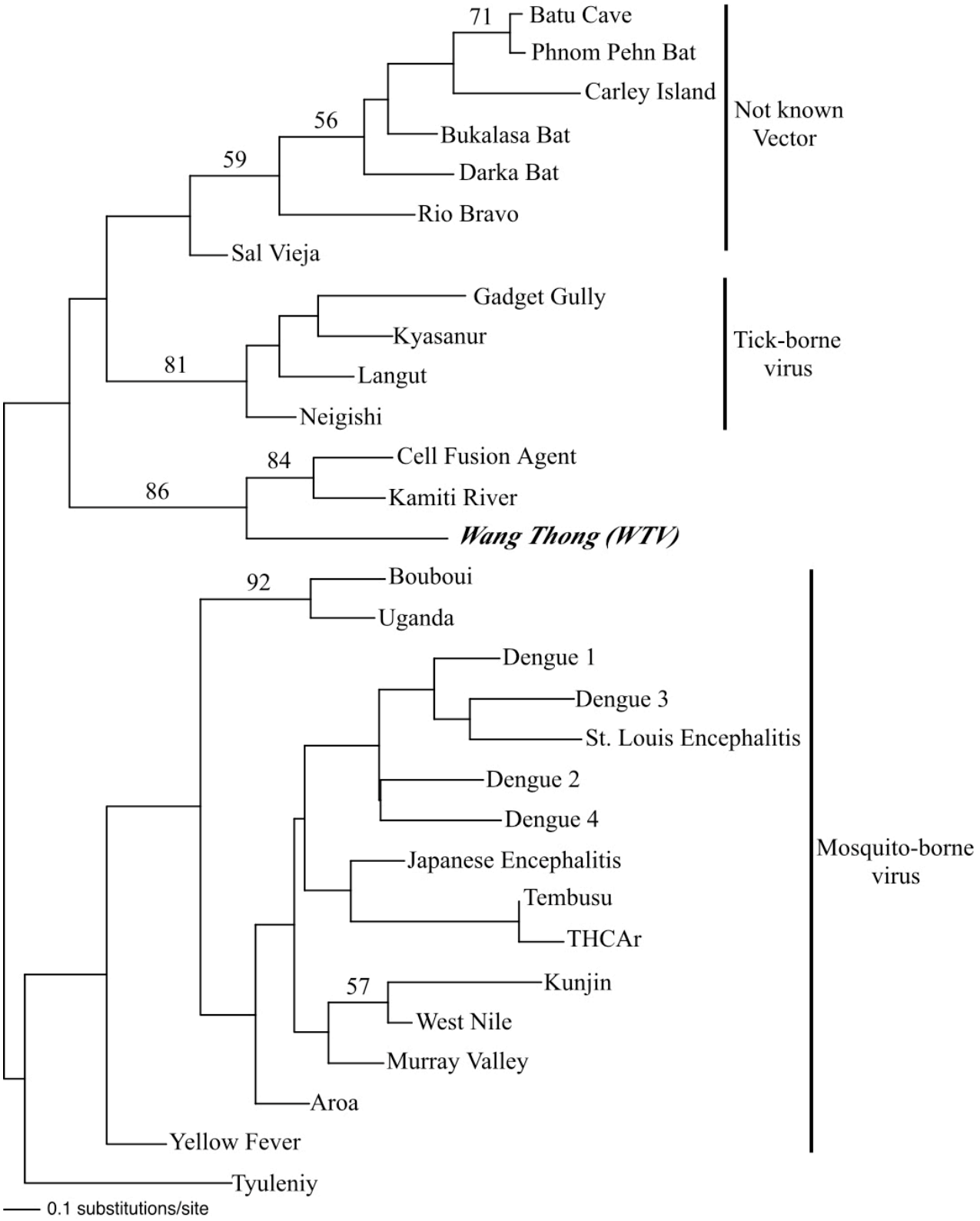

NJ
100
Batu Cave
Phnom Pehn Bat
57
Bukalasa Bat
69
Darka Bat
51
Carley Isaland
Rio Bravo
Sal Vieja
Not known Vector
Gadget Gully
92
Kyasanur
52
Langut
Neigishi
Tick-borne virus
87
Cell Fusion Agent
81
Kamiti River
Wang Thong (WTV)
95
Bouboui
Uganda
Dengue 1
Dengue 3
Dengue 2
Dengue 4
St. Louis Encephalitis
Japanese Encephalitis
Murray Valley Encephalitis
West Nile
Aroa
100
Tembusu
THCAr
Kunjin
Yellow Fever
Tyuleniy
Mosquito-borne virus
0.05 changes

Figure 16. Maximum likelihood tree (ML) for NS5 gene sequences of a new flavivirus, Wang Thong Virus (WTV) from *Culex fuscocephala,* collected in Wang Thong District, Kampaengphet Province. The ML tree was obtained by using the general time reversible model, which allows different rate parameters for codon positions. Numbers at nodes are bootstrap percentages obtained from 100 resamplings. Only bootstrap percentages which are greater than 50% are shown


71
Batu Cave
Phnom Pehn Bat
Carley Island
56
Bukalasa Bat
59
Darka Bat
Rio Bravo
Sal Vieja
Not known Vector
Gadget Gully
Kyasanur
81
Langut
Neigishi
Tick-borne virus
84
Cell Fusion Agent
86
Kamiti River
Wang Thong (WTV)
92
Bouboui
Uganda
Dengue 1
Dengue 3
St. Louis Encephalitis
Dengue 2
Dengue 4
Japanese Encephalitis
Tembusu
THCAr
57
Kunjin
West Nile
Murray Valley
Aroa
Yellow Fever
Tyuleniy
Mosquito-borne virus
0.1 substitutions/site

CHAPTER 5
DISCUSSION

We chose the study area by using an annual report from the Ministry of Public Health, Thailand. We collected mosquitoes in both villages and national parks. In the villages, we collected mosquitoes nearby animal farms and houses. Most people rear animals under their houses and nearby their toilets. For national parks, we collected mosquitoes in villages around or nearby national parks. We also found *Anopheles spp.* in the villages nearby national parks. In Khoa Kitchakood Waterfall National Park, most of the mosquitoes were *Armigeres spp*. The collecting techniques that we used in this study were animal baits and light trapping. Animal baits is a good collecting methods for animals like pigs and cows. But researchers must be aware of mosquito biting when doing their work. Light traps were used for collecting mosquitoes in chicken and pig farms. This collecting method was good for animals like chickens, because they were always excited when we collected mosquitoes. Light trap can collect a large number of mosquitoes but most of the specimens were injured. For Rayong and Kanchanaburi Provinces, mosquitoes were collected using an animal bait technique. For Lopburi, mosquitoes were collected using net traps. We collected both dead and live mosquitoes. Dead mosquitoes were kept in tubes under –80 °C. Live mosquitoes were reared in cages before identification in Bangkok.

Dr. Uruyakorn Chansaeng from Division of Entomology, Ministry of Public Health, helped me with mosquito identification. Freezed mosquitoes were hard to identify because the shape of the body was blended from pressure and scales and hairs were lost. Incomplete parts of mosquitoes were discarded. Only live mosquitoes were used in this study. Geographic positioning system (GPS) was used to located collection sites. I started using GPS in Nakhonsawan, Kampaengphet, and Tak Provinces. In some location I did

not use GPS because of heavy raining. Results showed that only Kampaengphet Province had positive samples. Those mosquitoes were *Culex fuscocephala*. Mosquitoes were collected from Moo 4, Wang Thong Subdistrict, Muang District, Kamphaengphet Province, located 358 km northwest of Bangkok. According to the year 2000 census, Muang District had a total population of 198,943. Kamphaengphet Province had a 332-bed public health hospital that is the major source of health care for the province (49, 50). When I was collecting mosquitoes, I noticed that most of children and elders got sick with red eyes.

Parts of homogenated mosquitoes in RPMI medium were used to do RT-PCR. The rest were kept at –80 °C for purified and propagation. The cDNA was constructed by using random primers and then amplified by FLV1 and FLV2 (26), degenerated primers, amplify a wide variety of flaviviruses. These primers used Inosine base, (I). Inosine is a rare natural purine base which can base-pair with the four bases; A, C, G, and T. Deoxyinosine is most widely used universal base which can base pair with A, C, G or T but with different affinities (17). In addition, onestep semi-nested RT-PCR, using PF1/PF2, and PF2/PF3 primers, were done in the same mosquito groups but using different number of mosquitoes. However, there was no positive sample.

To generate longer sequences (~1 Kb) (15), cDNA of positive samples were further amplified by another set of primers, i.e., FU1PM/cFD3PM, FU1PM /cFD4PM, but these primers cannot amplify NS5 gene of this virus. I designed primers from CFAV and KRV sequence alignment, F1 and R1. This primer designed to prime covering upstream and downstream of FLV1 and FLV2. The expected size is 500 base pairs, but these primers cannot amplify the cover this region. It implied that the sequence identity of WTV was different from CFAV and KRV. For EMF1/VD8, universal primers for flaviviruses provided by IRD, the expected size was 500-700 base pairs. These primers cannot amplify cDNA of WTV. FG1D and cFD3D, highly degenerated primers, were designed to amplify FLV1 and FLV2. FG1D/FLV2, and FLV1/cFD3D cannot amplify

cDNA of WTV, I hypothesized that these primers were highly degenerated, so there was a small number of bases that can prime to cDNA of WTV. On the other hand, cDNA of WTV may be unique and different from many other flaviviruses.

PCR products were purified and cloned into plasmid pGEM-T Easy kit. Plasmid were purified and sequenced using ABI automated sequencer by BioService Unit (BSU), Ministry of Science and Technology. DNA sequences were blasted in GenBank. The result showed that sequences are closely related to the flavivirus group, especially, Cell Fusion Agent Virus (CFAV) (7) and Kamiti River Virus (KRV). CFAV is a member of the Genus *Flavivirus* that was originally isolated from *Aedes aegypti* mosquito cell lines and was subsequently found to grow only in mosquito cells but not in vertebrate cells (7, 21). Recently, KRV, the only virus related to CFAV, was isolated from *Aedes macintoshi* mosquitoes collected in western Kenya (29). The RT-PCR is a highly sensitive method for flavivirus detection. So, unspecific band may cause problem. In our study, unspecific bands occur in some species of mosquitoes, some bands were lower than 200 bp and equal to positive control. The PCR products that equal to positive control were also sequenced. However, after doing blast search in GenBank, I found that they were not flaviviruses.

Most of flavivirus phylograms created in the past were based on the sequences of only about one-third of fewer of the numbers and thus provided only partial information. In 1997, Kuno established a comprehensive phylogeny of the Genus *Flavivirus*, using genomic sequences of a 1-kb segment at the 3' terminus of NS5 gene from all viruses. His study revealed that from the putative ancestor of the Genus *Flavivirus*, two major branches emerged, non-vector and vector-borne clusters, and that from the latter cluster emerged tick-borne and mosquito-borne cluster (15). MP tree of WTV using both DNA sequences and amino acid sequences revealed that WTV still related to CFAV and KRV with highly bootstrap support. Even though, there are a little bit shift in some taxons, and the tree still divided into three clades, not known vector, mosquito-borne vector, tick-

borne vector. Our 1-kb nucleotide sequence phylogram was made as a standard for comparison with our WTV tree because our tree was made from short nucleotide sequences and thus data may not be reliable.

Although the flavivirus phylograms produced in the past were primarily based on envelope gene sequences, it has been reported that the topologies based on envelope and NS5 gene showed perfected agreement. The envelope gene of flavivirus was less conserved than the NS5 gene, and the difference was reflected in greater different in the amino acid sequences (4,,10,15).

Furthurmore, with respected to vector association, some viruses in the mosquito-borne cluster, have been some time isolated from ticks. It is noteworthly that none of the members of non-vector cluster replicated in mosquito cell culture. Thus, there was a casual association of mosquito-borne virus with ticks before adaptation to mosquitoes. The observations provide evidences to support of the second possibility that the viruses of this genus evolved from non-vector group to tick-borne and then to mosquito-borne group. Exception to the above speculation is Aroa, which is placed in the mosquito–borne cluster in our phylograms despite the absence of arthropod vectors. This may be partly due to the lack of in-depth field investigations to search for arthropod vectors of these viruses. In fact, all of them are known to replicate in the mosquito cell cultures.

The phylogram generated based on short sequences (<300 bases) are sometimes different from those generated on much longer sequences. In this study, phylogram based on short sequences was different to those based on 1-kb sequences and boostrap supports at some nodes were much lower or disappeared, rendering phylograms unreliable. Thus, a caution was voiced against the use of such short sequences for phylogenetic studies of flaviviruses. (15)

CHAPTER 6
CONCLUSION

In year 2002-2003, I collected 1,257 mosquitoes including 40 species of 6 genera from 8 provinces of Thailand. We collected from villages, national parks, and wildlife sanctuaries by using an animal bait technique and light trapping. And I found that animal baits was a better method for capture mosquitoes when compared to light trapping because light trapping caused injury to mosquitoes.

This survey was intended to survey flaviviruses distributed in Thailand as well as to determine the phylogenetic relationships among flaviviruses. From RT-PCR assays for flaviviruses in 692 individual mosquitoes, I found that 11.1 % of *Culex fuscocephala* were infected with flaviviruses. Other mosquito species were not infected. Further survey with higher numbers of mosquito samples and greater areas of mosquito collection, may increase the possibility of finding mosquitoes infected with flaviviruses.

Phylogentic relationships among the new flavivirus and other flaviviruses was studied by using partial sequences of the non-structural protein gene (NS5). I found that these new flavivirus was closely related to two flaviviruses, i.e., CFAV (7), and KRV (29). This new flavivirus was designated to be Wang Thong Virus (WTV). WTV formed clade with CFAV and KRV but were separated from the other group of flaviviruses. The phylogenetic trees of WTV indicated that WTV was a new strain of flavivirus and formed a separate cluster among mosquito-borne virus, tick-borne virus, and not known vector virus clades. However, to identify as a new flavivirus, virus isolation, and molecular characterization should be performed.

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APPENDIX

APPENDIX A
EXPERIMENTAL REAGENTS

1. PCR amplification using EMF1 and VD8 primers

PCR Master mix (20 μl)

Deionized Water	13.1
10X buffer ($MgCl_2$ included)	2.0
dNTPs	0.4
EMF1 (F)	1.2
VD8 (R)	1.2
Taq	0.5
cDNA	1.6
Total	20

PCR program

1. Denature	94	°C	5 mins
2. Denature	94	°C	30 sec
Annealing	50	°C	90 sec
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	10 min

2. PCR amplification using F1 and R1 primers

PCR Master mix (20 μl)

Deionized Water	14
10X buffer	2.0
$MgCl_2$ buffer	1.0
dNTPs	0.5
F1 (F)	0.5
R1 (R)	0.5
Taq	0.5
cDNA	1.0
Total	20

PCR program 1

1. Denature	94	°C	2 mins
2. Denature	94	°C	30 sec
Annealing	58	°C	30 sec
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

PCR program 2

1. Denature	94	°C	5 mins
2. Denature	94	°C	30 sec
Annealing	50	°C	30 sec
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

3. PCR amplification using FG1D and FLV2 primers

PCR Master mix (20µl)

Deionized Water	14
10X buffer	2.0
$MgCl_2$ buffer	1.0
dNTPs	0.5
FG1D (F)	0.5
FLV2 (R)	0.5
Taq	0.5
cDNA	1.0
Total	20

PCR Master mix (50µl)

Deionized Water	32
10X buffer	5.0
$MgCl_2$ buffer	5.0
dNTPs	1.0
FG1D (F)	2.5
FLV2 (R)	2.5
Taq	1.0
cDNA	1.0
Total	50

PCR program 1

1. Denature	94	°C	2 mins
2. Denature	94	°C	30 sec
Annealing	50	°C	1 min
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

PCR program 2

1. Denature	94	°C	5 mins
2. Denature	94	°C	30 sec
Annealing	45	°C	1 min
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

4. PCR amplification using FLV1 and cFD3D primers

PCR Master mix (20µl)

Deionized Water	14
10X buffer	2.0
$MgCl_2$ buffer	1.0
dNTPs	0.5
FLV1 (F)	0.5
cFD3D (R)	0.5
Taq	0.5
cDNA	1.0
Total	20

PCR Master mix (50µl)

Deionized Water	32
10X buffer	5.0
$MgCl_2$ buffer	5.0
dNTPs	1.0
FLV1 (F)	2.5
cFD3D (R)	2.5
Taq	1.0
cDNA	1.0
Total	50

PCR program 1

1. Denature	94	°C	2 mins
2. Denature	94	°C	30 sec
Annealing	50	°C	1 min
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

PCR program 2

1. Denature	94	°C	5 mins
2. Denature	94	°C	30 sec
Annealing	45	°C	1 min
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

5. PCR amplification using FU1PM and cFD3PM primers

PCR Master mix (50µl)

Deionized Water	32
10X buffer	5.0
$MgCl_2$ buffer	5.0
dNTPs	1.0
FU1PM (F)	2.5
cFD3PM (R)	2.5
Taq	1.0
cDNA	1.0
Total	50

PCR program 1

1. Denature	94	°C	2 mins
2. Denature	94	°C	30 sec
Annealing	50	°C	1 min
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

6. PCR amplification using FU1PM and cFD4PM primers

PCR Master mix (50µl)

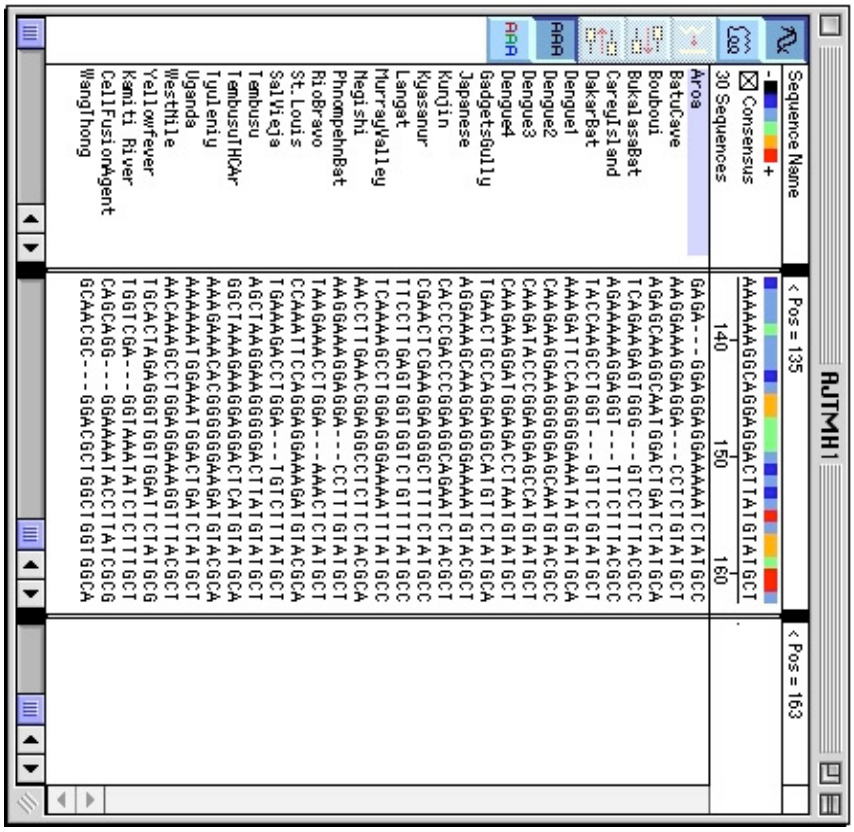
Deionized Water		32
10X buffer		5.0
$MgCl_2$ buffer		5.0
dNTPs		1.0
FU1PM (F)		2.5
CFD4PM (R)		2.5
Taq		1.0
cDNA		1.0
	Total	50

PCR program 1

1. Denature	94	°C	2 mins
2. Denature	94	°C	30 sec
Annealing	50	°C	1 min
Extension	72	°C	1 min 30 cycles
3. Extension	72	°C	1 min

APPENDIX B
DNA SEQUENCES ALIGNMENT OF EXPERIMENTAL FLAVIVIRUSES

AJTMH1
Sequence Name
Consensus
30 Sequences
Aroa
BatuCave
Bouboui
BukalasaBat
CareyIsland
DakarBat
Dengue1
Dengue2
Dengue3
Dengue4
GadgetsGully
Japanese
Kunjin
Kyasanur
Langat
MurrayValley
Negishi
PhnompenhBat
RioBravo
St.Louis
SalVieja
Tambusu
TambusuTHCAr
Tyuleniy
Uganda
Westhile
YellowFever
Kamiti River
CellFusionAgent
WangThong


RJTMH1
Sequence Name
Consensus
30 Sequences
< Pos = 135
< Pos = 163
140
150
160
Arboa
BatuCave
Bouboui
BukalasaBat
CareyIsland
DakarBat
Dengue1
Dengue2
Dengue3
Dengue4
GadgetsGully
Japanese
Kunjin
Kyasanur
Langat
MurrayValley
Negishi
PhnompehnBat
RioBravo
St.Louis
SalVieja
Tembusu
TembusuTHCAr
Tyuleniy
Uganda
WestNile
YellowFever
Kamiti River
CellFusionAgent
WangThong

AMINO ACID SEQUENCES ALIGNMENT OF EXPERIMENTAL FLAVIVIRUSES

BIOGRAPHY

NAME Mr. Worawit Uamcharoen

DATE OF BIRTH 27 November 1977

PLACE OF BIRTH Chantaburi, Thailand

INSTITUTIONS ATTENDED

Silpakorn University, 1994-1997

Bachelor of Science (Biology)

Mahidol University, 1999-2003

Master of Science (Environmental Biology)

RESEARCH GRANT

Institute de researche pour le developpement (IRD)

HOME ADDRESS

3 Moo 6, Bangkrachaw Subdistrict,

Phrapradaeng District, Samutprakarn Province, 10130

Tel. 02-461-0350