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Molecular Evolution of Dengue Viruses: Contributions of Phylogenetics to Understanding the History and Epidemiology of the Preeminent Arboviral Disease

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Abstract

Dengue viruses (DENV) are the most important arboviral pathogens in tropical and subtropical regions throughout the world, putting at risk of infection nearly a third of the global human population. Evidence from the historical record suggests a long association between these viruses and humans. The transmission of DENV includes a sylvatic, enzootic cycle between nonhuman primates and arboreal mosquitoes of the genus *Aedes*, and an urban, endemic/epidemic cycle between Aedes aegypti, a mosquito with larval development in peridomestic water containers, and human reservoir hosts. DENV are members of the genus Flavivirus in the Family Flaviviridae and comprise of 4 antigenically distinct serotypes (DENV-1-4). Although they are nearly identical epidemiologically, the 4 DENV serotypes are genetically quite distinct. Utilization of phylogenetic analyses based on partial and/or complete genomic sequences has elucidated the origins, epidemiology (genetic diversity, transmission dynamics and epidemic potential), and the forces that shape DENV molecular evolution (rates of evolution, selection pressures, population sizes, putative recombination and evolutionary constraints) in nature. In this review, we examine how phylogenetics have improved understanding of DENV population dynamics and sizes at various stages of infection and transmission, and how this information may influence pathogenesis and improve our ability to understand and predict DENV emergence.

1. Dengue, the preeminent human arboviral disease

Arthropod-borne viruses (arboviruses) comprise a taxonomically diverse group of viruses that are transmitted by arthropod vectors (Calisher and Karabatsos, 1988). Nearly all arboviruses have RNA genomes, probably a reflection of the genetic plasticity required to maintain transmission cycles requiring replication in disparate arthropod and vertebrate hosts. However, arboviruses are found in several RNA virus families, all of which also include members that do not rely on arthropod transmission. This suggests that the arthropod-borne transmission cycle probably arose at least several times during the course of virus evolution. All currently recognized arboviruses are found in 5 RNA virus families, including the flaviviruses (genus *Flavivirus*, one of 3 genera in the family *Flaviviridae*) (Hanley and Weaver, 2008). More than 50% of all known flaviviruses are associated with human disease, including some of the most important human pathogens, such as dengue (DENV), yellow fever, Japanese encephalitis, West Nile and tick-borne encephalitis viruses.

Among all arboviruses, the dengue viruses (DENV) are by far the most important human pathogens. An estimated 50-100 million DENV infections occur each year in tropical and subtropical regions, where more than 2.5 billion people are at risk (nearly a third of the global population)(Gubler, 1994; Gubler, 2002). Unlike many flaviviruses, DENV are

highly restricted in their natural vertebrate host range, generally utilizing primates as their amplification and reservoir hosts. They are also among the most widely distributed of the flaviviruses, and all 4 DENV serotypes can be found nearly throughout the tropics where the mosquito vector *Aedes aegypti* is abundant, putting at risk of infection nearly a third of the global human population. DENV comprise 4 antigenically distinct serotypes (DENV-1-4), which, though epidemiologically nearly identical, are genetically quite distinct. Infection with one DENV serotype leads to lifelong protection against homologous challenge, but only brief protection against heterologous infection with a different serotype (Kurane and Ennis, 1992; Sabin, 1952). All DENV cause dengue (DEN) fever (DF), a self-limited febrile illness lasting 2-10 days. However, some patients progress to develop life threatening syndromes including dengue hemorrhagic fever (DHF) characterized by thrombocytopenia and hemorrhage, and dengue shock syndrome (DSS) due to excessive plasma leakage (Gubler, 1994; Monath, 1994). Most DHF and DSS occur following a secondary infection, and 2 principal hypotheses explain this epidemiological pattern: 1) the immune enhancement theory maintains that hemorrhage occurs in secondary infections when DENV-specific antibodies and memory T cells, resulting from primary infection with a different serotype, enhance the binding of heterologous DENV-IgG complexes to Fcγ receptors on monocytic cells; 2) the virulence hypothesis suggests that some DENV strains are intrinsically more virulent than others, and cause higher viremia leading to more severe disease. An Asian genotype of DENV-2 recently introduced into the New World may be associated with increased risk for hemorrhagic fever and shock during secondary infections (Rico-Hesse et al., 1997). Also, endemic transmission on the South Pacific islands of Tonga, involving more highly susceptible vectors than Ae. aegypti, may have resulted in less severe dengue because selection for high viremia was relaxed due to the high degree of mosquito susceptibility (Gubler et al., 1978).

2. Dengue viruses and their genomes

All DENV are members of the DEN antigenic complex in the genus *Flavivirus*, family *Flaviviridae* (Calisher et al., 1989). The inclusion of DENV in this genus is based on antigenic cross-reactivity with other flaviviruses, as well as genomic organization and sequence homology. The 4 DENV serotypes are defined based on limited cross-reactions in various serological tests. Initial genetic characterizations of DENV in all serotypes identified “topotypes” or geographic variants by T1 RNase fingerprinting (Repik et al., 1983; Trent et al., 1990). Later, nucleic acid sequencing confirmed the homology of the 4 serotypes as well as their conserved genetic organization, and allowed for the more precise and broad classification of DENV into genetically distinct groups or genotypes within each serotype (Rico-Hesse, 1990). Rico-Hesse defined DENV “genotypes” as clusters of DENV with sequence divergence not greater than 6% within the chosen genome region (in this case the E/NS1 junction), which was based on the clustering of strains for which associations could be inferred on epidemiological grounds (Rico-Hesse, 1990).

Like that of other flaviviruses, the DENV genome is a single stranded RNA molecule of positive polarity or messenger sense and about 11 kB in length that encode a single open reading frame (Lindenbach and Rice, 2003). The genomic RNA includes 5′ (ca 100 nt) and 3′ (ca. 400-800 nt) untranslated regions (UTRs)(Fig. 1). Translation of the single ORF results in a single polyprotein that is cleaved by host and virus-derived proteases to produce the structural (C-prM-E) and non-structural (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) proteins. The envelope (E) protein is comprised of 3 domains; domain III is believed to interact with cellular receptors for entry, and includes most important epitopes that bind to neutralizing antibodies. The 5′ and 3′ termini of the DENV genomes include untranslated sequences important for viral RNA replication and translation, and probably interact with cellular factors involved in these functions.

The capsid protein (C), an 11 kDa homodimer protein with an unusually high net charge (Trent, 1977), is essential for RNA genome encapsidation (Chang et al., 2001). The membrane protein (prM), a 27-31 kDa N-glycosylated protein cleaved in the trans-Golgi network (TGN) by a cell-encoded furin-like protease during the late stages of virus assembly, to release mature virions (Kuhn et al., 2002; Li et al., 2008; Yu et al., 2008). The envelope (E) protein is a 53 kDa class II N-glycosylated dimeric membrane fusion protein that mediates virus binding and fusion to host cell membrane (Johnson, Guirakhoo, and Roehrig, 1994; Modis et al., 2004; Rey et al., 1995), as well as confers protective immune responses by eliciting neutralizing, antifusion, and replication-enhancing antibodies (Chen, Maguire, and Marks, 1996; Roehrig, Bolin, and Kelly, 1998). As mentioned above, each of the monomer subunits of the E protein is composed of three distinct domains: domain I, a β-barrel structure oriented parallel to the viral membrane; domain II, a finger-like structure composed of a pair of discontinuous loops one of which is highly conserved among all flaviviruses functioning as an internal fusion peptide and is stabilized by three disulfide bridges; and the C-terminal domain III (aa 303 – 395), located in the outer lateral surface of the dimer. Domain III has been suggested to contain residues that are responsible for the determination of host range, tropism and virulence among flaviviruses (Rey et al., 1995). Mutations within domain III, such as N390D which is located within the putative glycosaminoglycan binding motif (386L-411M) responsible for the binding of DENV onto the host cell membrane via a non-Fc receptor (Chen, Maguire, and Marks, 1996), have been implicated as a potential virulence determinant in American DENV genotypes (Leitmeyer et al., 1999), and has been shown to alter virulence in mice (Sanchez and Ruiz, 1996).

Among the nonstructural proteins, NS1 is a 46 kDa dimeric N-glycosylated glycosyl-phosphatidylinositol (GPI) anchored protein that exists in both intra- and extracellular forms (Jacobs et al., 2000; Winkler et al., 1989). NS2A, is a 22 kDa protein involved in the coordination of change between RNA packaging and replication (Khromykh et al., 2001) and possibly antagonism of interferon (IFN) (Jones et al., 2005; Munoz-Jordan et al., 2003). Several recent analyses have indicated that the gene encoding for NS2A are under weak selection pressures, a process that influences virus evolution during natural transmission (Bennett et al., 2003; Vasilakis et al., 2007a; Zhang et al., 2005). NS2B, is a membrane-associated 14 kDa protein which associates with NS3 to form the viral protease complex and serves as a cofactor in the structural activation of the DENV serine protease of NS3 (Erbel et al., 2006; Leung et al., 2001). NS3, is a 70kDa multifunctional protein with trypsin-like serine protease, helicase, and RNA triphosphatase (RTPase) enzyme activities (Gorbalenya et al., 1989; Li et al., 1999) and is involved in the processing of the viral polyprotein, as well as RNA replication. NS4A and NS4B, are small hydrophobic proteins of 16 and 27 kDa, respectively, with the latter functioning as an interferon (IFN)-signaling inhibitor (Jones et al., 2005; Munoz-Jordan et al., 2003). Recent evidence demonstrated a concentration of putative positive selection in NS4B of sylvatic DENV (Vasilakis et al., 2007a), which suggests a possible role in distinguishing endemic and sylvatic DENV genotypes. Lastly, NS5 is a large multifunctional, well-conserved protein of 103 kDa with RNA cap-processing, RNA-dependent RNA polymerase (RdRp) (Ackermann and Padmanabhan, 2001; Egloff et al., 2002), interleukin-8 induction (IL-8) (Medin, Fitzgerald, and Rothman, 2005) and nuclear localization (Pryor et al., 2007; Uchil, Kumar, and Satchidanandam, 2006) activities.

3. The history of dengue disease

Although DEN reached dramatic levels of incidence that brought increased awareness in many tropical and neotropical locations following the cessation of World War II, the disease has a long history of human interaction. It is known from the historical record that a DEN-like illness with similar clinical description occurred in China as early as the 3nd Century

during the Chin Dynasty [Common Era (CE) 265-420]. Similar reports were described during the 7th and 10th Century [Tang Dynasty (CE 610) and Northern Sung Dynasty (CE 992), respectively] (Gubler, 1997b). These reports described a disease called 'water poison,' due to its association with water-associated flying insects and whose clinical description included fever, rash, arthralgia, myalgia and hemorrhagic manifestations. After a long absence in the historical record, reports of a similar illness appeared almost seven centuries later, describing an acute illness with prolonged convalescence in the French West Indies and Panama during 1635 and 1699, respectively (Gubler, 1997b). A century later (1779-1788), several reports of DEN from Batavia (present day Jakarta), Cairo, Philadelphia, and Cadiz and Seville, Spain suggested the possibility of a DEN pandemic (Bylon, 1780; Christie, 1881; Hirsch, 1883; Pepper, 1941; Rush, 1789). These accounts described for the first time evidence for a widespread DENV geographic distribution (or at the very least of an illness very similar to DEN), reaching pandemic proportions by 1788. Interestingly, this widespread geographic distribution of DEN prevalence coincided with the increase in global commerce aided by sailing ships.

The historical record also indicates that a second series of DEN or DEN-like pandemics criss-crossed the globe, from Africa to India to Oceania to the Americas, from 1823 to 1916 (Brown, 1977; Christie, 1881; Hirsch, 1883; More, 1904), each lasting for 3-7 years. Although it is impossible to know which serotype was involved, these outbreaks were probably caused by the same DENV serotype and were transported among geographic regions by the slave trade and commerce (Brown, 1977; Christie, 1881; Gubler, 1997b). Further credence for commerce-fueled spread comes from Leichtenstern, who first recognized DEN as a disease of seaports and coastal regions that could also spread inland along rivers, like the Ganges and the Indus in India, or the Mississippi in the United States (Leichtenstern, 1896). As described below (see DENV transmission Cycles and Control of Disease), the increased prevalence of DEN was also facilitated by the invasion of the tropics by the African *Ae. aegypti* mosquito vector, most likely due to the movement of people (i.e. slave trade, commerce, migration) and their water storage containers by sailing ships (Daniels, 1908; Smith, 1956; Stanton, 1920; Steadman, 1828; White, 1934). Subsequently, these factors (commerce and new vector invasion of the tropics) altered dramatically DENV behavior in Southeast Asia, the Indian subcontinent and the Philippines from the sudden onset of urban epidemics to endemicity.

A new relationship between DENV and humans dawned with the onset of World War II, which brought immense ecologic, demographic, and epidemiologic changes leading to the establishment of scientific commissions to study the disease, its etiologic agent and development of diagnostic tests (Hota, 1952; Sabin, 1952; Sabin and Schlesinger, 1945). However, the cessation of World War II led to uncontrolled urbanization, where inadequate housing, water distribution systems, as well as sewer and waste management, allowed for the vector (Ae. aegypti) to reach high densities and facilitated dispersal of the DENV serotypes among diverse geographic regions. Although rare occurrences of severe and fatal hemorrhagic disease associated with DEN were reported as early as the 1780 Philadelphia (Rush, 1789) and 1927-29 Greek epidemics (Copanaris, 1928), the ecologic and demographic changes of the 20th Century created ideal conditions for the emergence of DHF in Southeast Asia (Hammon, Rudnick, and Sather, 1960; Hammon et al., 1960).

The initiation of an *Ae. aegypti* eradication program to combat urban epidemics of yellow fever under the auspices of the Pan American Health Organization (PAHO), led to a quiescence of DEN in the Americas for the next 20 years. However, abandonment of this program during the early 1970s allowed for the gradual reinfestation by Ae. aegypti, a process that continued well into the 1990s. An important characteristic of DEN epidemics in the Americas during the 1960s and 1970s was the circulation of a single serotype at any

given time within a given region (hypoendemicity). However, during the 1960's, a dramatic increase in DENV activity was observed in many tropical locations throughout the rest of the world (Carey et al., 1971; Ehrenkranz et al., 1971; Russell et al., 1966; Saugrain et al., 1970), with a series of epidemics associated with an increased incidence of disease severity (Balaya et al., 1969; Basaca-Sevilla and Halstead, 1966; Halstead et al., 1965; Halstead and Yamarat, 1965). By the end of the 1960's, it was evident all 4 DENV serotypes shared overlapped in a spatiotemporal manner (hyper-endemicity) throughout Southeast Asia and the Indian subcontinent. Subsequent, prospective field studies by Halstead and colleagues in Thailand suggested an inherent association between secondary infections and the severity of DEN disease (Halstead et al., 1967; Russell, Udomsakdi, and Halstead, 1967), which formed the basis for the development of the antibody-dependent enhancement theory (ADE) of DEN pathogenesis (Halstead, Chow, and Marchette, 1973).

Although the historical record indicated the presence of DEN in Africa as early as the 19th Century (Christie, 1881; Hirsch, 1883), relatively little was known about DEN prevalence (Edington, 1927; Kokernot, Smithburn, and Weinbren, 1956) until a surveillance program established by the Rockefeller Foundation at the University of Ibadan, Nigeria in 1964, documented endemic transmission of DENV-1 and DENV-2 in humans (Anonymous, 1969; Carey et al., 1971). Even today, a major limitation in our understanding of DEN prevalence and disease burden in Africa is the absence of effective surveillance, although there is evidence for increased DEN incidence fueled by an increase in circulation of all 4 serotypes (Botros et al., 1989; Fagbami and Fabiyi, 1976; Fagbami, Monath, and Fabiyi, 1977; Gonzalez et al., 1985; Gubler et al., 1986; Hyams et al., 1986; Johnson et al., 1982; Nogueira et al., 1991; Rodier et al., 1996; Saleh et al., 1985; Saluzzo et al., 1986). Curiously, this increased incidence has not been associated with any increase in disease severity, except on rare occasions (Gubler et al., 1986).

However, the introduction of DENV genotypes from Southeast Asia into the Americas in 1981 (Kouri et al., 1983; Rico-Hesse, 1990) was associated with a significant increase in DEN severity during subsequent epidemics (Alvarez et al., 2006; Cabrera-Batista et al., 2005; Kouri et al., 1989; Nogueira, de Araujo, and Schatzmayr, 2007; Rigau-Perez, Vorndam, and Clark, 2001; Uzcategui et al., 2001). Retrospective observations from the Cuban epidemic of 1981 were instrumental in inferring the putative role of host genetics (Bravo, Guzman, and Kouri, 1987; Kouri, Guzman, and Bravo, 1987), gender and age (Guzman et al., 1984) in influencing the severity of disease. Later studies also implicated nutritional status (Kalayanarooj and Nimmannitya, 2005) and immune enhancement (Halstead, 2003).

A new era in human-DENV relationships began by the 1990s and the beginning of the 21st Century, when the global distribution of all DENV serotypes had been nearly completed. This process was facilitated by the gradual convergence of several factors, including expanding urban populations, increased vector density due to unsustainable control programs, and the increase in commercial air travel facilitating the rapid movement of viremic humans. These factors facilitated the rapid and dramatic re-emergence of DEN associated with increased disease severity throughout the tropics. Although the intensity of several epidemics peaked globally in 1998 (Aziz et al., 2002; Corwin et al., 2001; Cunha et al., 1999; Ha et al., 2000; Harris et al., 2000; Thomas, Strickman, and Vaughn, 2003), the incidence rates of severe DEN have increased significantly throughout all hyperendemic regions (Cordeiro et al., 2007; Kusriastuti and Sutomo, 2005; Ooi, Goh, and Gubler, 2006; PAHO, 2007; WHO, 2006).

Although the information presented above suggests a long relationship between humans and DENV due to infections from strains circulating in urban settings throughout the tropics,

little is known about the interaction of sylvatic DENV and humans. The pioneering studies of Smith (Smith, 1956) presented for the first time serological evidence for the existence of an ecologically distinct transmission cycle. Smith proposed a connecting link (*Ae. albopictus)* to the prevalence of mostly subclinical or mild DEN among the rural population of peninsular Malaysia. Subsequent studies by Rudnick and colleagues (Rudnick, 1986; Rudnick and Lim, 1986) confirmed the existence of an ecologically distinct DENV transmission cycle with the isolation of DENV-1, -2, and -4 from sylvatic habitats.

Retrospective serologic studies of isolated, forest-inhabiting human populations first suggested an interaction between sylvatic DENV and humans (Rudnick, 1986; Rudnick and Lim, 1986). Currently, our understanding of human illness after infection with sylvatic DENV comes from 5 documented case histories of human DENV infections by an ecologically and genetically distinct sylvatic genotype of DENV-2 (Monlun et al., 1992; Robin et al., 1980; Saluzzo et al., 1986). Conducting similar DEN seroprevalence studies among nonhuman primates and human communities within rainforests, Fagbami suggested similar inferences regarding zoonotic DENV to those put forward by Rudnick and Smith in Malaysia (Fagbami, Monath, and Fabiyi, 1977). Moreover, recent phylogenetic evidence (Vasilakis, Tesh, and Weaver, 2008) confirmed that human contact with sylvatic DENV also took place in Ibadan, Nigeria during 4 years of DEN activity in the 1960's (Carey et al., 1971). Entomologic studies suggest that the linking vector between sylvatic DENV and human communities located within or near the forest may be *Ae. furcifer*, a gallery forest-dwelling mosquito that disperses into rural peridomestic habitats (Diallo et al., 2003; Diallo et al., 2005).

Our understanding of the DENV clinical presentation (DF or DHF/DSS) in humans has been greatly enhanced from the early 3rd century, when it was first associated with water-associated flying insects, to the 20th Century where their ecology and transmission cycle were elucidated. As described above, DENV have had a close relationship with humans for about 2,000 years, which only during the past few decades has intensified due to altered ecologic conditions, as well as human behavior (global commerce, urbanization, large population movements) and unstainable vector control programs. Currently, all DENV serotypes have reached nearly global hyperendemicity and will likely continue to cause epidemics of various intensities and pathogenic severity. Today, about a third of the global human population is at risk for DENV infection mostly in urban and periurban areas throughout the tropics. By current estimates, approximately 100 million DENV infections occur annually, leading to 500,000 cases of DHF and 20,000 deaths. Thus DENV have become the preeminent arboviral pathogens of humans.

4. The origins of dengue viruses

The origins of DENV have been the subject of speculation for decades. Phylogenetic relationships to other flaviviruses provide little insight because the closest relatives to DENV occur in several continents (Kuno et al., 1998). Gubler (Gubler, 1997a) hypothesized that endemic DENV evolved from sylvatic strains in Africa or Asia that utilize nonhuman primate hosts and gallery forest-dwelling *Aedes* vectors (not the endemic/epidemic vectors *Ae. aegypti* or *Ae. albopictus*). The sylvatic cycle is presumed to be ancestral because efficient interhuman transmission is thought to require a minimum human population size of 10,000-1 million, which did not exist until about 4,000 years ago when urban civilizations arose (Gubler, 1997a).

The first support for the hypothesis that DENV was originally zoonotic came from phylogenetic studies of DENV-2, which demonstrated that strains from sylvatic habitats in West Africa were distinct from all others (Rico-Hesse, 1990). Wang et al. (Wang et al.,

2000) provided more comprehensive phylogenetic support for the hypothesis of a sylvatic DENV origin, when they sequenced the complete E genes of DENV-1, -2 and -4 isolated in tropical forest habitats in Malaysia by Rudnick et al. during the 1960's and 1970's (Rudnick, 1965; Rudnick, Marchette, and Garcia, 1967), as well as DENV-2 from sylvatic cycles in West Africa (Robin et al., 1980). As in the previous studies (Rico-Hesse, 1990), the African sylvatic DENV-2 were distinct from all endemic/epidemic (henceforth referred to as endemic) isolates, differing by 19% in their nucleotide sequences (Fig. 2). The Malaysian sylvatic isolates of DENV-1, -2 and -4 were also distinct from both endemic isolates collected from humans and urban vectors, and from the African sylvatic strains; the Malaysian DENV-2 isolates differ from the epidemic strains by 17%, while the DENV-1 and -4 Malaysian sylvatic strains differ from their epidemic counterparts by about 7% and 14%, respectively. Although sylvatic DENV-3 was not isolated in Malaysia by Rudnick and colleagues, the presence of DENV-3 antibodies in non-human, canopy dwelling primates indicated that a sylvatic DENV-3 cycle probably exists there (Rudnick, 1978).

Using various methods and estimates of nucleotide substitution rates, various investigators have estimated that the endemic DENV-2 genotypes diverged from the sylvatic forms about 40-600 years ago (Twiddy, Holmes, and Rambaut, 2003; Vasilakis et al., 2007a; Wang et al., 2000). The African and Malaysian sylvatic lineages diverged slightly later, as did the sylvatic and epidemic DENV-1 and -4 lineages (Fig. 2). The greater diversity of sylvatic DENV in Malaysia compared to Africa suggests that an ancestor of all DENV arose in the former region (although the exact location cannot be determined due to limited sampling) and diverged in the relatively distant past into the 4 serotypes recognized today.

The 4 independent epidemic DENV emergence events, presumably involving host range changes from arboreal *Aedes* mosquitoes to *Ae. albopictus* and later to *Ae. aegypti (Gubler, 1997a)*, suggest that adaptation to new vectors and vertebrate hosts may have occurred repeatedly (Fig. 3). Because *Ae. aegypti* did not occur in Asia and Oceania at that time prior to the establishment of shipping trade, *Ae. albopictus* was probably the original human DENV vector. Acquisition of *Ae. aegypti* as a vector may have occurred only during the past few centuries as commerce via sailing ships distributed this mosquito throughout the tropics (Gubler, 1997a).

The emergence of distinct DENV serotypes, prior to emergence of the extant epidemic lineages, was most likely facilitated by the allopatric and perhaps ecological partitioning of ancestral sylvatic DENV strains in different non-human primate populations. This scenario is supported by the limited cross-protection against heterologous challenge exhibited by current strains of DENV. Maintenance of the current levels of antigenic and genetic diversity among the 4 DENV serotypes may be selected by immune enhancement, if each virus gains replication efficiency in the human and/or nonhuman primate host due to limited cross-reactive immunity (Ferguson, Anderson, and Gupta, 1999). Spatial-temporal travelling waves of DHF incidence, which have a three-year period in Thailand, appear to move radially at a speed of 148 km per month (Cummings et al., 2004). The resulting oscillations in DEN disease may be explained by immune enhancement without any external influences (Billings et al., 2007).

5. DENV transmission cycles and control of disease

Two distinct DENV transmission cycles occur: (1) Endemic DENV circulate among humans, which serve as both reservoir and amplification hosts, and peridomestic *Ae. Aegypti* and *Ae. albopictus*, with other *Aedes* spp. serving as secondary vectors. The efficiency of the endemic cycle, which is now completely independent both evolutionarily and ecologically from the ancestral, sylvatic cycles (see section on genetic relationships

below), is greatly enhanced by the ecology and behavior of *Ae. aegypti*. This species lays its eggs in water storage containers and refuse in close proximity to human dwellings, readily enters human habitations, and takes multiple bloodmeals during each gonotrophic cycle for both egg production and general nutrition. All of these behaviors increase vector competence and transmission to multiple human hosts (Harrington, Edman, and Scott, 2001; Weaver et al., 2004). Movement of people and their water storage containers during the 17^{th}-19^{th} centuries probably spread *Ae. aegypti* from its origin in Africa throughout the tropics. After World War II, this species increased in prevalence and distribution in Asia and the Pacific Islands. *Ae. aegypti* was partially eradicated from tropical America in the 1940s and 50s, but has now reinfested most of the neotropics (Gubler, 1997a). Although *Ae. aegypti* plays a greater role in urban transmission, *Ae. albopictus* and some other secondary *Aedes* spp. vectors are sometimes more susceptible to experimental infection (Gubler, 1988) and in some situations are more important vectors (Ali et al., 2003). *Ae. albopictus*, another treehole species of sylvatic origin albeit in Asia, has spread widely in the world since the 1970s including to the U.S., Latin America, tropical Africa, the Pacific Islands and Europe (Deubel and Murgue, 2001). Although less anthropophagic than *Ae. aegypti*, it is considered an important secondary vector of DENV and possibly of greater importance in the early historical stages of endemic dengue emergence. Considerable variation in susceptibility and transmission efficiency has also been demonstrated among geographic populations of both *Ae. aegypti* and *Ae. albopictus* (Gubler et al., 1979; Gubler and Rosen, 1976).

(2) Sylvatic DENV circulate among non-human primate reservoir hosts and several different *Aedes* mosquitoes in forested habitats of West Africa and Malaysia (Gubler, 1988; Gubler, 1994). While sylvatic DENV transmission cycles have received little study, some important features have been determined. In Malaysia, where all 4 serotypes of DENV are probably maintained in canopy-dwelling *Ae. niveus* mosquitoes and most likely non-human primates (Rudnick, 1965; Rudnick, 1978; Rudnick, 1984b; Rudnick, Marchette, and Garcia, 1967), DENV-1, -2 and -4 have been isolated from sentinel monkeys, and seroconversion has been demonstrated against DENV-1, -2 and -3. In West Africa, sylvatic DENV-2 has been isolated from the arboreal species Ae. africanus, Ae. leuteocephalus, Ae. opok, Ae. taylori and *Ae. furcifer* (Cordellier et al., 1983; Roche et al., 1983), as well as from 5 humans in eastern Senegal (Monlun et al., 1992; Robin et al., 1980; Saluzzo et al., 1986). All sylvatic isolates are genetically distinct from all endemic isolates, and are isolated evolutionarily (Gubler, 1997a; Rico-Hesse, 1990; Wang et al., 2000)(see discussion of genetics below). Intriguingly, although an African origin for *Ae. aegypti* is suggested by (i) the abundance in Africa of closely related *Aedes* (*Stegomyia*) spp., (ii) the lack of related *Stegomyia* subgenus *Aedes* mosquitoes in the Americas, and (iii) the existence only in Africa of sylvatic *Ae. aegypti* (*Ae. aegypti formosus*)(Carter, 1930; Christophers, 1960; Dyar, 1928; Powell, Tabachnick, and Arnold, 1980; Tabachnick and Powell, 1979), the sylvatic, African forms of DENV probably do not utilize *Ae. aegypti formosus* as an important vector. Indeed, this subspecies is relatively refractory to infection (Black et al., 2002; Diallo et al., 2008; Diallo et al., 2005).

Control of DENV spread relies primarily on reduction of vector populations by elimination of peridomestic water sources and insecticide applications. This strategy succeeded in most neotropical locations during the 1950s-1970s during campaigns to eliminate yellow fever, but was not sustained. *Ae. aegypti* has recently reinfested most neotropical and semi-neotropical locations, and DEN has returned with a vengeance (Gubler, 2002; Monath, 1994). Worldwide, changing lifestyles have enhanced *Ae. aegypti* densities by providing larval breeding sites for this anthropophilic and peridomestic species. Increases in air travel have resulted in the movement of virus strains among transmission foci, providing opportunities for sequential infection of populations by 2 or more serotypes, resulting in an increased incidence of DHF and DSS in many areas. No effective antiviral drugs are

available to treat dengue infections. Several promising DEN vaccine candidates have been developed, but are not yet available for general use. A particular challenge with DEN vaccination is avoiding immune enhancement, which could occur if a vaccine is not protective against all 4 serotypes (Guy and Almond, 2008).

6. Contributions of phylogenetics to the understanding of dengue epidemiology

Early genetic comparisons of DENV strains relied on T1-RNase-resistant oligonucleotide fingerprinting, also called RNA fingerprinting. Initial studies of the 4 DENV serotypes revealed few shared T1-resistent oligonucleotides (Vezza et al., 1980). Repik et al. (Repik et al., 1983) first delineated topotypes of DENV-1 by showing that isolates from the same geographic region were very similar, but differed from those from other areas. Trent et al. also identified topotypes of DENV-2, and showed that different topotypes are not selectively the cause of severe DEN disease in Thailand (Trent et al., 1989). Evolution of the DENV-2 virus genome from 1962-1986 was gradual, with a slow accumulation of changes in the pattern of oligonucleotides through time, suggesting a balance of genetic drift and natural selection. Similar results followed soon thereafter for DENV-4 (Henchal et al., 1986).

During the late 1980's, direct sequencing of viral RNA largely supplanted RNA fingerprinting for DENV molecular epidemiologic studies due to the greater efficiency and ability to characterize more precisely individual mutations. Rico-Hesse used RNA sequencing of DENV-1 and DENV-2 isolates to demonstrate additional evolutionary relationships between etiologic isolates of disease outbreaks, as well as the distinct nature of DENV-2 isolates from sylvatic cycles in West Africa (Rico-Hesse, 1990). Similar studies of NS1 gene sequences from DENV-2 strains revealed no correlation between disease severity, serological response, or year of isolation and NS1 nucleotide or amino acid sequences (Blok et al., 1991).

Phylogenetic studies of many mosquito-borne flaviviruses have revealed relatively long time periods between lineage divergence, suggesting a "boom and bust" pattern of intense diversification followed by a "pruning" of the tree via extinction of many lineages (Zanotto et al., 1996b). The best examples of this pattern are the four serotypes of DENV, which appear to be undergoing a period of rapid radiation, consistent with their rapid emergence since World War II (Holmes and Twiddy, 2003).

As the efficiency of viral RNA genome sequencing advanced with the advent of reverse transcription-polymerase chain reaction (RT-PCR) amplification and automated sequencing, more extensive analyses using longer sequences generated more robust phylogenies. Early studies of all 4 DENV serotypes demonstrated multiple lineages, often geographically-based, sometimes with implications for disease severity, as follows:

a. DENV-1

Early studies of DENV-1 evolution utilized direct RNA sequencing of 240 nucleotides across the E/NS1 gene junction. Five distinct genotypes were recognized, and these were later confirmed with complete E gene sequences showing a maximum nucleotide divergence of 6% among them (Goncalvez et al., 2002; Rico-Hesse, 1990).

Current phylogenies based on complete DENV-1 E gene sequences (Fig. 4) confirm the previously identified lineages as follows: (i) genotype I, representing strains from Southeast Asia, China and East Africa; (ii) genotype II, representing strains from Thailand collected in the 1950s and 1960s; (iii) genotype III, representing the sylvatic strain collected in Malaysia; (iv) genotype IV, representing strains from the West Pacific islands and Australia;

and (v) genotype V, representing all strains collected in the Americas, strains from West Africa, and a limited number of strains collected from Asia.

b. DENV-2

DENV-2 evolution using homologous 240 nt sequences. As with DENV-1, 5 distinct genotypes were recognized, including one comprising the West African sylvatic strains (Rico-Hesse, 1990). Similar analyses using complete E gene sequences produced more robust results with similar genotypic groupings (Lewis et al., 1993). Extensive analyses of DENV-2 strains from Thailand revealed the simultaneous circulation of many DENV-2 variants simultaneously, with both Thai genotypes isolated from both DF and DHF cases (Rico-Hesse et al., 1998). Although the 240 nt E/NS1 gene sequences produced trees with many low bootstrap values, probably reflecting few informative nucleotides in the relatively short sequences, the conclusion was drawn that only 4 robust DENV-2 genotypes could be identified. Later, an analysis of E gene sequences from 147 DENV-2 isolates extended the range of the newly defined “Cosmopolitan” genotype to nearly global distribution, and identified 2 new genotypes that appeared to be restricted to Asia (Twiddy et al., 2002).

Current phylogenies of DENV based on complete E gene sequences (Fig. 5) confirm 5 major genotypes: (i) the Asian genotype, or Asian genotype 1, representing strains from Malaysia and Thailand, and Asian genotype 2 representing strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines; (ii) the cosmopolitan genotype, representing strains of wide geographic distribution including Australia, East and West Africa, the Pacific and Indian ocean islands, the Indian subcontinent and the Middle East; (iii) the American genotype, representing strains from Latin America and older strains collected from the Caribbean, the Indian subcontinent and Pacific Islands in the 1950s and 1960s; (iv) the Southeast Asian/American genotype, representing strains from Thailand and Vietnam and strains collected in the Americas over the last 20 years; and (v) the sylvatic genotype, representing strains collected from humans, forest mosquitoes or sentinel monkeys in West Africa and Southeast Asia (Lewis et al., 1993; Rico-Hesse et al., 1997; Twiddy et al., 2002; Vasilakis, Tesh, and Weaver, 2008; Wang et al., 2000) (Fig. 5).

c. DENV-3

Focusing on the E gene, Lanciotti et al. (Lanciotti et al., 1994)identified geographically independent evolution of DENV-3 in 4 regions: subtype I was identified in Indonesia, Malaysia, the Philippines and the South Pacific islands; subtype II included strain from Thailand; subtype III consisted of viruses from Sri Lanka, India, Africa and Samoa; and subtype IV included of strains from Puerto Rico and Tahiti. Similar results were also obtained using NS3 gene sequences (Chow, Seah, and Chan, 1994).

Current DENV-3 phylogenies complete E gene sequences (Fig. 6) confirm the above described 4 genotypes plus the sylvatic genotype, as follows: (i) genotype I, representing strains from Indonesia, Malaysia, the Philippines and recent isolates from the South Pacific islands; (ii) genotype II, representing strains from Thailand, Vietnam and Bangladesh; (iii) genotype III, representing strains from Sri Lanka, India, Africa and Samoa. However, the complete genome phylogenetic analysis clearly includes the 1962 strain from Thailand within this genotype (Chao et al., 2005); and (iv) genotype IV, representing strains from Puerto Rico, Latin and central America and the 1965 Tahiti strain. Sylvatic strains of DENV-3 have not been isolated to date, but they are believed to exist in Malaysia, based on the seroconversion of sentinel monkeys (Rudnick, 1984a).

d. DENV-4

Initial analyses revealed that DENV-4 strains exhibited greater sequence conservation than the other DENV serotypes (92%) and 96-100% conservation in E protein amino acids. DEN-4 viruses were separated into two subtypes from: 1) the Philippines, Thailand and Sri Lanka, and; 2) Indonesia, Tahiti, the Caribbean, Central and South America (Lanciotti, Gubler, and Trent, 1997).

Current DENV-4 phylogenies complete E gene sequences (Fig. 7) delineate 4 major genotypes: (i) genotype I, representing strains from Thailand, the Philippines, Sri Lanka, and Japan (imported into Japan from Southeast Asia); (ii) genotype II, representing strains from Indonesia, Malaysia, Tahiti, the Caribbean and the Americas. Subsequent analysis with additional strains revealed putative evidence of intra-serotypic recombination amongst DENV-4 from independent ancestral lineages (most likely Indonesia 1976 and Malaysia 1969) may have contributed to the emergence of a distinct genotype, representing all Malaysian strains (AbuBakar, Wong, and Chan, 2002). Genotype II has become well established in the Caribbean since its introduction in the area in the early 1980s from Southeast Asia (Bennett et al., 2003; Foster et al., 2003); (iii) genotype III, representing recent Thai strains that are distinct from other Thai isolates (Klungthong et al., 2004); and (iv) genotype IV, representing the sylvatic strains from Malaysia.

e. Epidemic initiations and lineage replacements

The initiation of DEN epidemics has also been attributed to DENV strain introductions, as revealed by phylogenetics. Partial sequences of DENV-2 strains from the 1981 Cuban epidemic, the first in the Western hemisphere to include documented DHF cases, showed a close relationship to older DENV-2 strains from Asia, and more distant relationships to contemporary isolates from Jamaica and Vietnam. Partial genomic sequences derived from paraffin-embedded autopsy tissues of severe Cuban cases confirmed this DHF etiology (Sariol et al., 1999). DENV-4 was also first reported in the Americas in 1981. Bayesian coalescent studies revealed that, for both DENV-2 and DENV-4, the initial introductions were characterized by an exponential increase in the number of viral lineages, followed by constant genetic diversity (Carrington et al., 2005). DENV-4 apparently underwent a more rapid rate of population growth than DENV-2, allowing for a more efficient spread. Phylogenetic studies showed that DENV-2 outbreaks in Puerto Rico were associated with subtype IIIb, which was originally introduced from Asia. This lineage continued to circulate in Puerto Rico until 1994, when another was introduced from somewhere in the Western Hemisphere (Bennett et al., 2006). Positive selection has apparently acted on a several of amino acid sites in the E protein, some of which may have altered virulence based on their association with epidemics.

A similar history of DENV introduction and spread was reported from Brazil. Partial E/NS1 nucleotide sequences of DENV-1 and -2 isolates suggested that, since their initial introduction from 1981-1988, these lineages have continued to evolve without the introduction of new genotypes (Pires Neto et al., 2005).

A 1993-1994 Malaysian DEN epidemic was traced to the introduction of subtype II DENV-3 from Thailand, where it was isolated from 1962-1987. In contrast, earlier DENV-3 Malaysian isolates belonged to subtype I (Kobayashi et al., 1999). Similar studies in India suggest that genotype V of DENV-2 was replaced by genotype IV between 1967 and 1996 (Singh et al., 1999). DENV-3 strains isolated from Bangladesh in 2000 and 2001 were most closely related to recently emerged DENV-3 from neighboring Thailand and Myanmar, but distinct from those isolated in India and Sri Lanka (Podder et al., 2006). DENV-3, subtype III, which apparently originated in the Indian subcontinent, appears to have spread into

Africa during the 1980s and from Africa into Latin America during the 1990s. Furthermore, DENV-3, subtype III isolates from mild versus severe disease outbreaks are phylogenetically distinct, suggesting differences in virulence among subtype III clades (Messer et al., 2003). Recently, Indian DEN epidemics have been determined phylogenetically to be linked to DENV-3 subtype III, which apparently replaced the earlier circulating DENV-2 (subtype IV) (Dash et al., 2006).

A detailed investigation of a DEN epidemic that occurred in French Polynesia from August 1996 to April 1997 revealed the introduction of DENV-2 after 7 years of DENV-3 circulation. Index cases were detected close to the international airport of Tahiti, and accompanying seroepidemiologic studies suggested transmission primarily within houses (Deparis et al., 1998). A 1996-1998 Guatemalan DEN epidemic was caused by DENV-3 strains that were apparently imported from Asia, Africa, or from a Pacific island (Usuku et al., 2001). Several 2002 DENV-4 outbreaks in north Queensland, Australia, were traced phylogenetically to Thailand or Indonesia as their likely origins (Hanna et al., 2003). Two Australian outbreaks of DEN occurred in 2003-2004, one that spread from Cairns to Townsville, the other from the Torres Strait to Cairns. Phylogenetic analyses indicated that both epidemics were initiated by viremic travelers from Papua New Guinea (Hanna et al., 2006).

Studies of E gene sequences from 2000-2001 Venezuelan DENV-3 isolates revealed that they are closely related to genotype III isolates previously detected Panama and Nicaragua, which have since spread through Central America and Mexico. This genotype apparently replaced the genotype V strain that circulated in Venezuela in the late 1960s and 1970s (Uzcategui et al., 2003). Similar studies indicate that DENV-3 genotype III was introduced into Brazil from the Caribbean Islands at least twice, and then into Paraguay from Brazil on at least three occasions (Aquino et al., 2008; Aquino et al., 2006). Ecuadorian DENV strains have also been linked phylogenetically to isolates of Caribbean origin (Regato et al., 2008).

Nearly simultaneous 2001 outbreaks of DENV-1 in Myanmar and several distant sites in the Pacific were shown phylogenetically to be caused by 3 different genotypes (I, II, and III). These results suggested multiple introductions from a variety of locations in Asia (Nuegoonpipat et al., 2004). Phylogenetic studies of isolates from the Myanmar outbreak, involving over 15,000 cases of DHF/DSS and 192 deaths, incriminated 2 lineages of DENV-1 that had diverged from an earlier, now extinct, lineage sometime before 1998 (Thu et al., 2004). Further phylogenetic analyses indicated that the two new lineages did not arise from the previously circulating DENV-1 viruses nor did they imply any differences in the fitness of the pre- and post-2001 viral populations, suggesting that a stochastic interepidemic event led to the replacement (Myat Thu et al., 2005). More recently, DENV-1 outbreaks occurred in Hawaii and Tahiti in 2001-2002. Phylogenetic analyses indicated that most Hawaiian isolates were imported from Tahiti and belonged to subtype IV (Imrie et al., 2006). One Hawaiian isolate from a Hawaii resident with a travel history to Samoa was linked to a DENV-1 strain previously isolated from another visitor to Samoa, indicating that 2 distinct strains of DENV-1 were introduced into Hawaii in 2001. A 2004 Chinese DEN epidemic in was linked to DENV-1 importation via a traveler returning from Thailand (Xu et al., 2007).

Phylogenetic studies have also revealed the apparent extinction of DENV genotypes and replacements at several locations. Extensive phylogenetic studies of DENV from Puerto Rico revealed marked evolutionary shifts in DENV-4 populations. Although viral lineages tend to be temporally clustered there, the most common genotype at a given time and place apparently arises from a previously rare lineage (Bennett et al., 2003). Recent replacements may, in part, have resulted from positive selection of three amino acid replacements in the

NS2A gene. DENV-4 was introduced into the Caribbean on a single occasion in the early 1980s, and has readily dispersed in the region since, resulting in virus stains related more by time of isolation than by location (Foster et al., 2003).

The most extensive studies of DENV lineage replacements have been conducted in Thailand. Strains of DENV-2 circulating there prior to 1992 were apparently replaced by two new lineages that have not been identified elsewhere, suggesting their local evolution, and similar DENV-3 extinctions may have occurred there previously (Wittke et al., 2002). No evidence of positive selection in the DENV E gene was detected in either case. The authors speculated that, because these replacements occurred during interepidemic periods, genetic bottlenecks and genetic drift were responsible. DENV-1 also underwent a lineage turnover in Thailand, where a major clade replacement in genotype I occurred during the mid-1990s. The preceding increase in DENV-1 clade diversity was associated with a concomitant decrease in DENV-4 prevalence, while the subsequent clade replacement was associated with a decline in DENV-1 and an increase in DENV-4 (Zhang et al., 2005). The authors hypothesized that intraserotypic DENV diversification predominates at times of relative serotype abundance, and that clade replacements result from differential susceptibility to cross-reactive immunity from other serotypes. Modeling studies indicate that moderately cross-protective immunity gives rise to persistent out-of-phase oscillations similar to those observed between DENV-1 and -4, but that weak cross-protection (e.g. among DENV-1, -2, and -3) or cross-enhancement only produces in-phase patterns. Comprehensive analyses of DENV-2 revealed that 3 genotypes have circulated in Thailand, with Asian genotype predominating since 1991.

Recently, these comprehensive studies of DENV in Thailand have been extended to a smaller spatial scale by monitoring prospectively school children in Kamphaeng Phet during 2001 (Jarman et al., 2008). Phylogenetic studies of E gene sequences of DENV-1, -2, and -3 isolates revealed genetic variation in both time and space, with multiple DENV lineages circulating within individual schools, suggesting the frequent viral dispersal. Frequent introductions of DENV-2 sequences into the area occurred. The sequences tended to cluster within individual schools, but gene flow was also observed among nearby schools. Little sequence evolution was observed in DENV lineages within individual schools over this short timescale.

f. Genetic DENV correlates of DEN severity

Many investigators have compared the sequences of DENV isolates obtained from patients who experienced severe disease (DHF and/or DSS) with those isolated from cases of DEN fever. Most of these studies have identified genetic variation among strains, but not consistent sequences differences that correlated with disease severity (Blok et al., 1991; dos Santos et al., 2002; Mangada and Igarashi, 1998; Pandey et al., 2000; Raekiansyah et al., 2005; Sistayanarain et al., 1996; Uzcategui et al., 2001). One study of DENV-2 infections in northeastern Thailand reported that subtype III strains may result in milder disease irrespective of the immune status (primary or secondary DENV infection) compared with subtypes I or II, but this conclusion was based on a relatively small number of isolates (Pandey and Igarashi, 2000). Genetic studies of DENV-3 strains from a 2004 Indonesia epidemic indicated that they group with strains isolated in 1998 during a major epidemic in Sumatra, yet no newly-acquired amino acid changes were detected (Ong et al., 2008). These results suggested that a particular genotype with inherent epidemic potential reemerged in 2004.

The first strong evidence for genetic determinants of DEN severity came from studies of DENV-2, which indicated that the Asian genotype is associated with severe disease whereas sympatric American genotype isolates are not (Rico-Hesse et al., 1997). Detailed genomic

sequence analyses of 11 strains from these 2 genotypes revealed 6 deduced amino acid charge differences between them, in the prM, E, NS4B, and NS5 genes. In addition, consistent sequence differences observed within the 5′ and 3′ untranslated regions (UTRs) were predicted to change RNA secondary structures (Leitmeyer et al., 1999). The authors hypothesized that the primary determinants of severe DEN reside in (i) amino acid 390 of the E protein, which may alter interactions with cellular receptors; (ii) in nt 68-80 of the 5′ UTR, which may be involved in translation initiation; and (iii) in the upstream 300 nucleotides of the 3′ UTR, which may regulate viral replication via the formation of replicative intermediates.

The abovementioned phylogenetic evidence of selection in the Americas for the more virulent Southeast Asian DENV-2 lineage has been corroborated by experimental data. The southeast Asian DENV-2 lineage strains associated with DHF/SSS generally replicate more efficiently in human dendritic cells, which are probably important human targets for DENV replication, than the American lineage strains (Cologna, Armstrong, and Rico-Hesse, 2005). Furthermore, 15 DENV-2 strains of both lineages of were compared their ability to infect and disseminate in several populations of *Ae. aegypti (Armstrong and Rico-Hesse, 2003)*. Overall disseminated infection rates were higher for the SE Asian genotype strains, suggesting that both the human hosts and the endemic vector have selected for the more virulent SE Asian genotype strains.

5. Molecular evolution of dengue viruses: insights from phylogenetic and experimental studies

a. Selection pressures

In addition to elucidating patterns of DENV transmission and beginning to unravel determinants of severe disease, phylogenetic studies have also provided insights into their process of molecular evolution during natural transmission. As with nearly all arboviruses and other kinds of viruses as well, the identification of predominantly synonymous mutations during the natural evolution [see references above as well as (King et al., 2008)] of DENV implied that purifying selection (selection against most mutations, especially those that encode amino acid changes) dominated their evolution. Presumably, the DENV proteins have evolved to a high level of fitness for their alternate infection of mosquitoes and primates, which results in most amino acid changes being deleterious and purified quickly from the population by selection.

The first comprehensive attempt to identify selection pressures on DENV involved 147 DENV-2 strains (Twiddy et al., 2002). Comparisons of synonymous and nonsynonymous substitution rates in the E gene suggested that different DENV-2 genotypes have experienced different selection pressures. Maximum likelihood methods that evaluate differences in d_N/d_S ratios among codons suggested positive selection in the Cosmopolitan genotype and one of the two Asian genotypes. One mutation that appeared to be under positive selection, E-390, may affect transmissibility. However, no evidence of positive selection was detected during the emergence of the endemic DENV-2 lineage from the ancestral, sylvatic strains (Twiddy et al., 2002). Further studies with all 4 DENV serotypes identified several E gene codons in DENV-2, -3 and -4 but not -1 that apparently are subject to relatively weak positive selection (Twiddy, Woelk, and Holmes, 2002). Most of these sites were located in, or near to, potential T- or B-cell epitopes, suggesting positive immune selection. Although most other genes exhibited strong purifying selection, several positively selected amino acid substitutions were also identified in the NS2B and NS5 genes of DENV-2.

The effect of humoral immune selection on DENV evolution has received relatively little support. DENV-1 and -3 are reported to have lost cytotoxic lymphocyte epitopes during their evolution, based on computer epitope predictions and maximum parsimony analyses (Hughes, 2001).

b. Rates of DENV evolution

Early attempts to infer rates of DENV evolution employed simple genetic distance measurements and regression analyses (Lanciotti, Gubler, and Trent, 1997; Wang et al., 2000; Zanotto et al., 1996a). These methods generally yielded rate estimates of 6-8 × 10^{-4} subs/site/year. Newer, more sophisticated maximum likelihood methods applied to E gene sequences have produced similar estimates for DENV evolutionary rates and suggest that evolution generally approximates a molecular clock, although some minor rate differences occur (Twiddy, Holmes, and Rambaut, 2003). Rates estimated for the 4 DENV serotypes range from 4.55 × 10^{-4} for DENV-1 to 11.58 × 10^{-4} for genotype 3 of DENV-3. Statistical comparisons suggest that DENV-3 is evolving significantly faster than both DENV-1 and DENV-2. Comprehensive, genomic analyses of DENV-2 suggest that both the evolutionary rates and patterns of selection are similar for endemic versus sylvatic lineages, with the only exception being a uniquely high frequency of positive selection in the NS4B genes of sylvatic strains (Vasilakis et al., 2007a). These estimates are within a range determined for a variety of zoonotic arboviruses, and the alternating replication in arthropods and vertebrates, as well as the modulation of viral genome replication during persistent vector infections, may contribute to the slow rates of arboviruses (Hanley and Weaver, 2008).

c. DENV population sizes

The effects of population size on DENV has also been addressed by many phylogenetic studies. By comparing multiple DENV-2 isolates from humans infected both in 1980 and 1987, Sittisombut et al. (Sittisombut et al., 1997) first suggested that a genetic bottleneck and subsequent expansion of one or a limited number of subtype IIIa strains may have occurred during the intervening 6 years.

In addition to the epidemiological data indicating a dramatic increase in DENV circulation in many areas of the tropics, phylogenetic analyses have also documented increased genetic diversity consistent with viral population expansion. Combined with the growing evidence that DENV strains differ in virulence, this trend predicts the evolution of an expanded range of pathogenic properties (Holmes and Burch, 2000).

d. Recombination

The role of recombination in DENV evolution remains controversial. Phylogenetic methods such as bootscanning have been used to identify recombination in DENV-1 (Aaskov et al., 2007; AbuBakar, Wong, and Chan, 2002; Chen et al., 2008; Tolou et al., 2001; Uzcategui et al., 2001). The strongest evidence comes from a New Caledonian who was infected with both genotypes I and II, as well as a recombinant (Aaskov et al., 2007). Because recombination of flaviviruses has not been achieved experimentally despite concerted efforts (K.A. Hanley, personal communication), caution should be exercised when inferring conclusions about these putative recombination events. Several conditions should be met in order for natural recombination leading to the transmission of a recombinant strain to be conclusively confirmed. First, the recombinant crossover should be demonstrated in a single PCR amplicon following cloning to ensure that it occurs in a single cDNA molecule. Second, the recombination should be demonstrated repeatedly in clonal populations of viable virus (e.g. a plaque harvest or limited endpoint dilution isolate). And third, the recombinant should be maintained during post-recombination evolution (Tolou et al., 2001).

e. DENV quasispecies

Like other RNA viruses, DENV is believed to undergo low fidelity replication, resulting in virus populations described as quasispecies, or mutant swarms surrounding a master sequence (Domingo et al., 1985; Holland and Domingo, 1998). The presence of DENV quasispecies has been confirmed by sequencing multiple clones derived from RT-PCR products, including from unpassaged, natural isolates (Aviles et al., 2003; Wang et al., 2002). These quasispecies have been found to include significant proportions of genomes with stop codons in their E genes, as well as mixed genotypes and recombinants thereof (Craig et al., 2003). Using RT-PCR, concurrent mixed serotype (DENV-2 and DENV-3) human infections were detected in 2/21 patients studied in southern Taiwan in 2000 (Wang et al., 2003). A 2001 genetic DENV-1 variant containing a stop codon in the E gene, detected in both humans and mosquitoes from Myanmar, is reported to have spread to all individuals sampled (Aaskov et al., 2006). The maintenance of long-term transmission of these defective RNA genomes would require complementation via coinfection of host cells with DENV supplying a functional E gene. Therefore, high multiplicities of infection would need to be maintained at all stages of transmission and dissemination through both human hosts and mosquito vectors, which seems unlikely based on the bottlenecks at the stage of mosquito infection inferred from studies of other mosquito-borne flaviviruses (Scholle et al., 2004) or alphaviruses (Smith et al., 2008).

f. Constraints on DENV evolution imposed by mosquito-borne transmission

DENV are maintained by horizontal arthropod-borne (mosquito-borne or tick-borne) transmission among vertebrate hosts. As a consequence, DENV must replicate alternately in the very different environments created by invertebrate and vertebrate hosts. Flaviviruses, as well as other arthropod-borne viruses, exhibit a lower rate of sequence evolution than many other RNA viruses, such as poliovirus or HIV, that are maintained in single host transmission cycles (Jenkins et al., 2002; Parvin et al., 1986; Wolfs et al., 1990). The genetic stability of arboviruses presents a paradox, since it is known that the RNA-dependent RNA polymerase (RdRp) of RNA viruses does not possess proofreading activity (Holland et al., 1982; Steinhauer, Domingo, and Holland, 1992), and the absence of a repair mechanism leads to approximately one mutational event per genome replication. Thus, one could argue that the observed arbovirus stability could be attributed to their unique requirement for replication in divergent hosts, which could impose additional selective constraints on arboviruses compared to single-host viruses. Arboviruses may have adapted to replicate efficiently in either host through selection for a compromise genome, where optimal replication in one host involves a fitness tradeoff for the alternate host (Coffey et al., 2008; Cooper and Scott, 2001; Greene et al., 2005; Weaver et al., 1999; Weaver, Rico-Hesse, and Scott, 1992).

However, some experimental evidence does not support the notion that alternating host replication produces negative fitness trade-offs (Novella et al., 1999). Alternatively, the observed arbovirus stability could also be attributed to the establishment of persistent infections, a phenomenon most common in tick-borne arboviruses, where replication is probably modulated by RNA interference (Sanchez-Vargas et al., 2004). To date, many phylogenetic analyses suggest the presence of strong purifying selection on arboviruses in nature, supporting strong selective constraints on flavivirus evolution (Holmes, 2003; Jerzak et al., 2005; Klungthong et al., 2004; Woelk and Holmes, 2002). Consequently, arboviruses evolve at a slower rate than other RNA viruses transmitted by a single host (Jenkins et al., 2002).

As described above, DENV have been shown to circulate within hosts in nature as a population of closely related genomes around a dominant master genome sequence,

indicating that DENV are maintained as a quasispecies. Therefore the existence of extensive genetic diversity within DENV populations suggests that genome variants within these populations may play a role in viral fitness, replication and their ability to successfully adapt to new and dynamic environments.

A study by Chen (Chen, Wu, and Chiou, 2003), examined the genetic diversity of DENV-2 variants after they were subjected to serial passage in mammalian (Vero), mosquito (C6/36) or alternating passage between them. Unlike serial passage in Vero cells or alternating passage between Vero and C6/36 cells, serial passage (20 or 30 times) in C6/36 cells did not result in any nucleotide change, suggesting that mosquito cells have a minimal effect on DENV evolution. However, since the conclusions of this study were based upon consensus sequences of a limited fraction of the DENV genome (2,541 nucleotides of the E/NS1 region), rather that characterization of the mutant spectrum of examined region, no meaningful inferences could be derived about the role of mosquitoes in DENV evolution.

A more recent study (Vasilakis and Weaver, unpublished), examined whether the human-to-mosquito transmission cycle may constrain DENV evolution by imposing constraints on optimal replication in either host, if fitness tradeoffs occur. Passaged but uncloned, as well as clonal DENV were serially passaged either in one cell type to eliminate host alternation, or in an alternating passage series between vertebrate liver (Huh-7) and mosquito (C6/36 *Ae. albopictus*) cells. DENV that were allowed to specialize in single host cells (10 passages) exhibited fitness gains but fitness losses in the bypassed cells, and most viruses passaged in 10 alternating cycles exhibited detectable fitness gains in both cells. Detection of several amino acid changes that were common in both passage series suggested convergent evolution via positive selection. These data were consistent with the hypothesis that releasing arboviruses from the alternating host cell transmission and replication cycle will result in the acquisition of higher fitness for the retained host cell (vertebrate or invertebrate), than will occur in an alternating cell cycle. However, the data provided limited support to the hypothesis that DENV and other arboviruses must adopt a fitness compromise, where optimal replication in one host cell or involves a fitness tradeoff for the alternate host cell. Support for this hypothesis was mainly provided by the alternate passage of the uncloned viruses, whereas in the cloned viruses significant fitness increases were observed in both cell types. Two possible explanations could account for this observation: (i) acquisition of host cell-specific and/or amphi-cell-specific adaptations could account for the ability of the alternating host cell passaged cloned viruses to acquire fitness gains almost similar to those observed from exclusive passage in the single host cell line and (ii) fitness increases in the alternate passage of the cloned viruses may be attributed to a low starting fitness due to the genetic bottleneck effects of the biological cloning, a phenomenon that has been observed previously (Chao, 1990; Duarte et al., 1992; Duarte et al., 1993; Escarmis et al., 1996; Weaver et al., 1999).

Interestingly, exclusive DENV-2 passage in the vertebrate host cell or alternating host cells led to the emergence of a qualitatively and quantitatively different mutation spectrum than exclusive passage in the invertebrate host cell line, whereas in the former the mutant spectrum was distributed throughout the genome, in the latter mutations located exclusively within the non-structural genes and 3′ - NCR. These observations support the notion that selection occurs mainly within the vertebrate host (Ciota et al., 2008). However, an inherent limitation of utilizing consensus sequences to generate inferences with regard to the fitness of viral populations is that consensus sequences only reflect the majority nucleotide at any given position of the viral genome, and do not represent the true range of the quasispecies spectrum of a master fit sequence that dominates the ensemble. Consequently, it is impossible to account for mutations present at a low frequency in the quasispecies ensemble that remain undetected, since low frequency viral sequences within the mutant spectrum

may have dominant phenotypes. In fact, a recent West Nile virus (WNV) study demonstrated the importance of the mutant spectrum's size in the establishment of the adaptive phenotype (Ciota et al., 2007).

6. Research trends and needs

Genetic studies of DENV isolates from mosquitoes and patients have made strong progress toward understanding patterns of spread and disease. Considerable progress has also been made toward understanding the patterns of genotype abundance and replacements that sometimes lead to new epidemics and that may regulate transmission dynamics. The continued advances in the efficiency, speed and cost of DNA sequencing are now opening new opportunities to revisit many aspects of DENV epidemiology and evolution by examining DENV strains from natural hosts at the population level. For example, new "deep sequencing" technology can efficiently sequence a large number of RNA viral genomes from a single host to provide more complete information on the quasispecies rather than just determining consensus sequences from RT-PCR amplicons. This will enable a more thorough understanding of the population dynamics and sizes of DENV at various stages of infection and transmission, and could also lead to the identification of sequence variants, present as minority populations, that are associated with DHF/DSS.

Recent progress has also been made by retrospective genetic studies of DENV from the sylvatic cycles. This work, combined with experimental studies of replication in models for human infection and in mosquitoes, suggests that the sylvatic genotypes have the capability to cause human infection and diseases, and can readily reemerge in the future unless effective vaccination is developed and sustained (Vasilakis et al., 2008; Vasilakis et al., 2007a; Vasilakis et al., 2007b; Vasilakis, Tesh, and Weaver, 2008). However, understanding the ecology and epidemiology of sylvatic DENV is essential to understanding and predicting emergence, yet has been largely ignored. Except for the pioneering studies of Rudnick and colleagues in Malaysia during the 1970s (Rudnick, 1984b), nothing is known of the distribution and ecology of sylvatic DENV in Asia, and human infections have never been identified or characterized. Longitudinal mosquito surveillance in Senegal has provided information on the temporal patterns of sylvatic DENV-2 amplification there (Diallo et al., 2003), and human infections including small epidemics caused by sylvatic strains have been characterized in West Africa (Vasilakis, Tesh, and Weaver, 2008). However, the ecological contact between sylvatic cycles and human populations is poorly studied, and the range of human disease and, most importantly viremia, caused by sylvatic DENV strains is unknown. Therefore, advances in understanding DENV emergence and predicting locations and times with the greatest potential will require comprehensive, prospective epidemiological and ecological studies in enzootic locations of West Africa and Asia. Such studies might even lead to the identification of additional serotypes of DENV that have not yet made their way into the urban transmission cycle.

Finally, the prospect of effective tetravalent DENV vaccination (Guy and Almond, 2008), which could control or even eradicate the urban cycles that rely only on human reservoir hosts, heightens the need for understanding the ease with which the sylvatic strains can reemerge to reinitiate urban transmission cycles.

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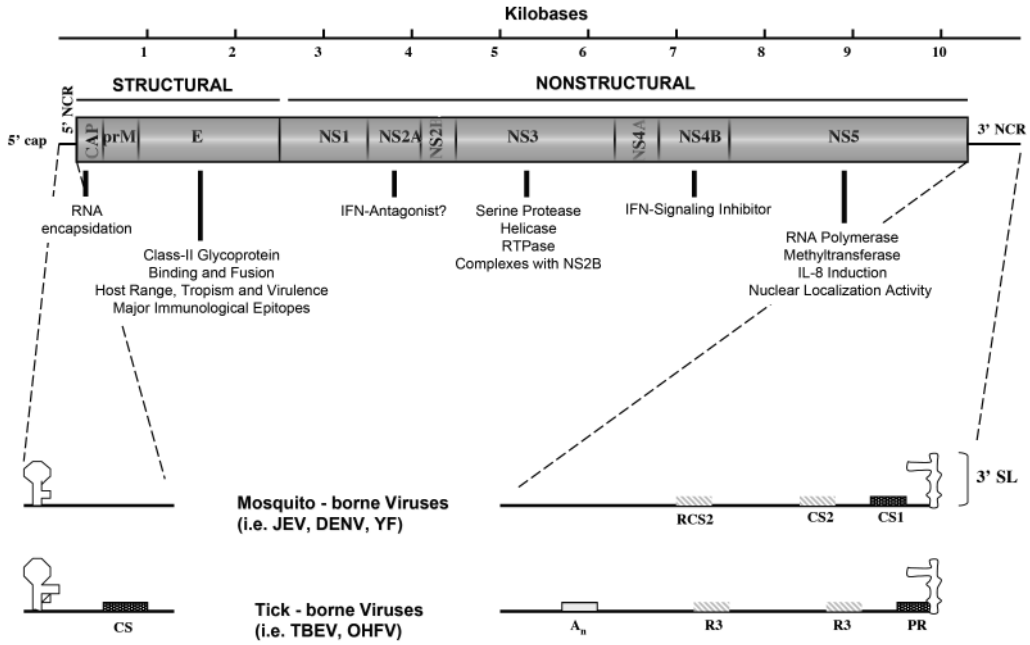

Fig. 1.
Cartoon depicting the DENV genome and the major functions of the gene products.

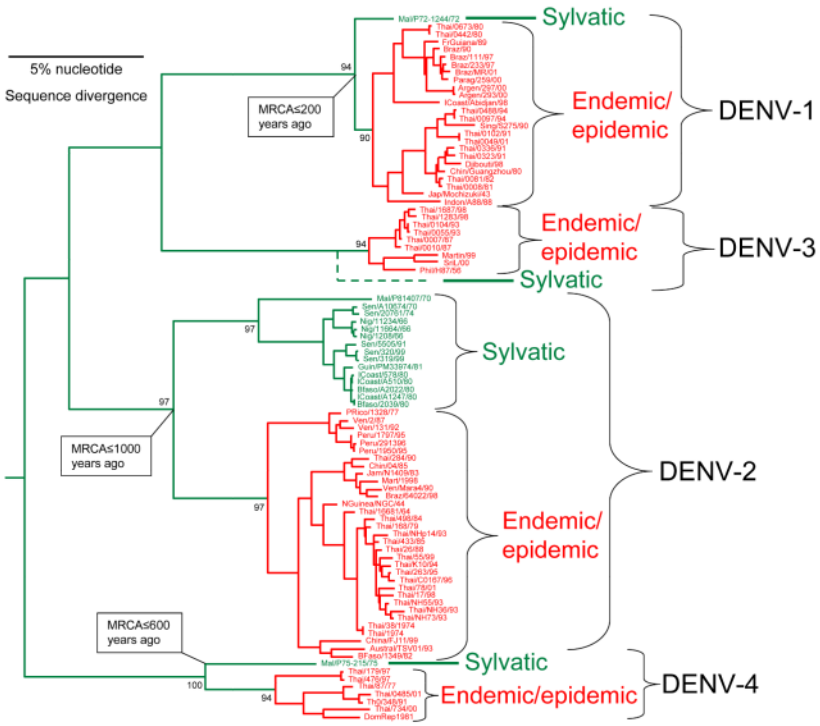

Fig. 2.

Phylogenetic tree of DENV strains from all 4 serotypes derived from complete open reading frames available in the GenBank library. The phylogeny was inferred using Bayesian analysis (one million reiterations) and all horizontal branches are scaled according to the number of substitutions per site. Bayesian probability values are shown for key nodes. Virus strains are coded by abbreviated country of collection/strain name/year of collection.

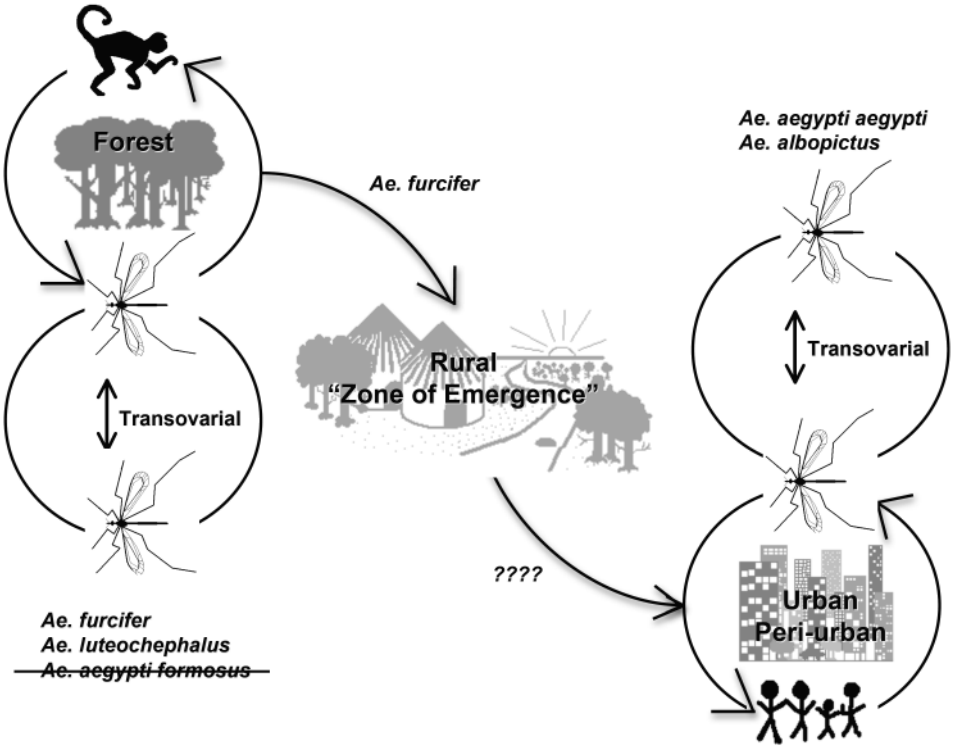

Fig. 3.
Cartoon depicting the hypothetical evolutionary history of endemic/epidemic DENV emergence from zoonotic transmission cycles.

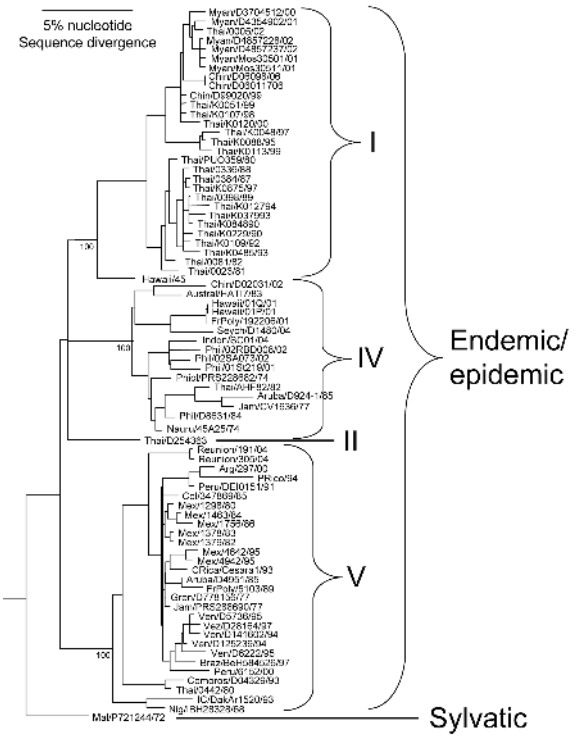

Fig. 4.

Phylogenetic relationships of DENV-1 strains from the GenBank library. The phylogeny was inferred based on complete E gene nucleotide sequences and Bayesian analysis (one million reiterations) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Bayesian probability values are shown for key nodes. Virus strains are coded by abbreviated country of collection/strain name/year of collection.

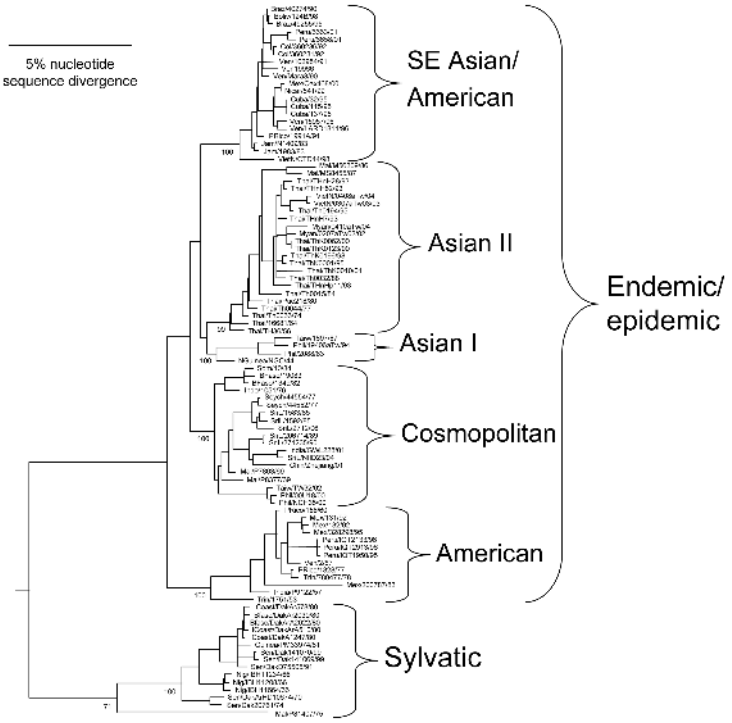

Fig. 5.

Phylogenetic relationships of DENV-2 strains from the GenBank library. The phylogeny was inferred based on complete E gene nucleotide sequences and Bayesian analysis (one million reiterations) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Bayesian probability values are shown for key nodes. Virus strains are coded by abbreviated country of collection/strain name/year of collection.

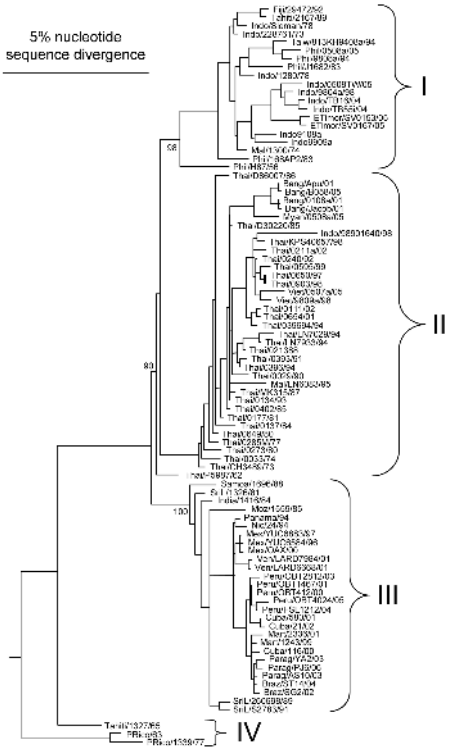

Fig. 6.

Phylogenetic relationships of DENV-3 strains from the GenBank library. The phylogeny was inferred based on complete E gene nucleotide sequences and Bayesian analysis (one million reiterations) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Bayesian probability values are shown for key nodes. Virus strains are coded by abbreviated country of collection/strain name/year of collection.

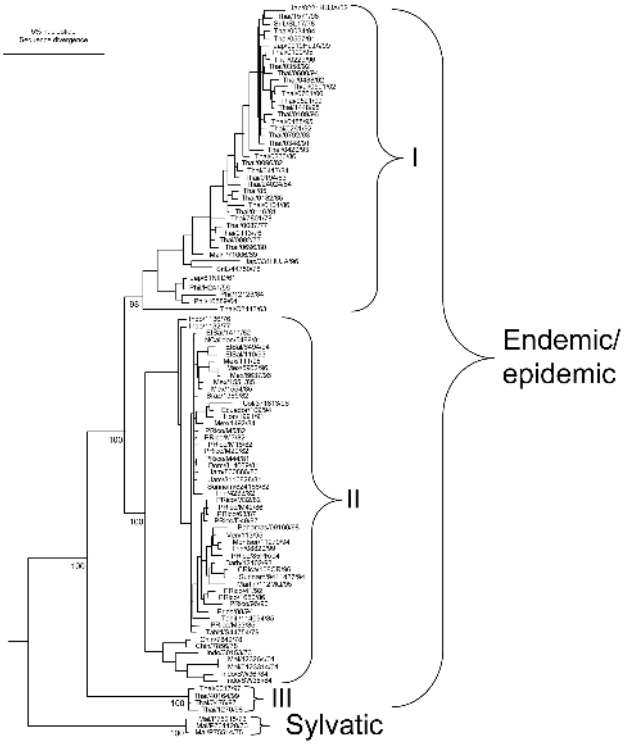

Fig. 7.

Phylogenetic relationships of DENV-4 strains from the GenBank library. The phylogeny was inferred based on complete E gene nucleotide sequences and Bayesian analysis (one million reiterations) using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Bayesian probability values are shown for key nodes. Virus strains are coded by abbreviated country of collection/strain name/year of collection.