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Molecular genotyping of dengue viruses by phylogenetic analysis of the sequences of individual genes

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The prevalence of four serotypes of dengue virus (DENV) has risen dramatically in recent years accompanied by an increase in viral genetic diversity. The evolution of DENV has had a major impact on their virulence for humans and on the epidemiology of dengue disease around the world. In order to perform disease surveillance and understand DENV evolution and its effects on virus transmission and disease, an efficient and accurate method for genotype identification is required. Phylogenetic analysis of viral gene sequences is the method used most commonly, with envelope (E) gene the most frequently selected target. To determine which gene might be suitable targets for genotyping DENV, phylogenetic analysis was performed on 10 individual coding genes plus the 3′-non-translated region (3′NTR) for 56 geographically divergent DENV strains representing all identified genotypes. These were reflected in eleven maximum likelihood phylogenetic trees. Based on the bootstrap values (over 90%) supporting the major nodes, the best target genes were identified for each serotype: for DENV-1, the sequences of all coding genes except non-structural gene 4A (NS4A), for DENV-2, PrM/M, E, NS1, NS3, NS4A and NS5, for DENV-3, all coding genes and the 3′NTR, and for DENV-4, C, PrM/M, E, NS1, NS2A, NS2B, NS4A and NS5.

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1. Introduction

Dengue (DEN) is one of the most globally important emerging and re-emerging infectious diseases. It is caused by a single-stranded, positive-sense RNA virus, named DEN virus (DENV) in the genus *Flavivirus* and the family *Flaviviridae*. The DEN viruses consist of four antigenically related but genetically distinct serotypes, DENV-1, DENV-2, DENV-3 and DENV-4. The viral genome is approximately 11 kb in length containing a single open reading frame that encodes three structural proteins, capsid (C), premembrane/membrane (PrM/M), and envelope (E) and seven non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, flanked by 5′- and 3′-non-translated regions (5′NTR/3′NTR).

DENV infection of humans can be asymptomatic or cause undifferentiated fever, dengue fever (DF), or life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). By the end of the 20th century, the four DENV serotypes and their primary mosquito vector, *Aedes aegypti*, had spread to nearly all countries of the tropics and sub-tropics. It is estimated that each year more than 2.5 billion people are at risk for DENV infection and more than 100 countries experience endemic DENV transmission (Gubler and Meltzer, 1999; World Health Organization, 1999, 2002). Co-circulation of multiple DENV serotypes, genotypes and clades in the same community has become common (Klungthong et al., 2004; Loroño-Pino et al., 1999; Rico-Hesse, 2003; Zhang et al., 2005, 2006). This increasing global spread and co-circulation of multiple DENV serotypes together with the error-prone nature of the viral RNA-dependent RNA polymerase have led to increasing genetic diversity of these viruses and the evolution of new genotypes within each serotype (AbuBakar et al., 2002; Bennett et al., 2006; Foster et al., 2003; Holmes and Burch, 2000; Klungthong et al., 2004; Salda et al., 2005; Uzcategui et al., 2001, 2003). The evolution of DENV genotypes may lead to phenotypic changes in the viruses that alter their potential to cause outbreaks of severe or attenuated disease (Grenfell et al., 2004). Understanding DENV variation is especially important as we continue to understand better the mechanisms behind its pathogenesis.

DENV surveillance and disease diagnosis typically rely only on serologic assays such as ELISA, which are often unable to determine the infecting serotype, especially after secondary infections. Recently, reverse transcription-polymerase chain reaction

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Table 1
DENV sequences used in this analysis

Serotypes	Country of origin	Isolation (years)	Genotypes	Genebank accession #
DENV-1	ThD1-0442/80[a]	1980	III	AY732477
	ThD1-0673/80[a]	1980	III	AY732474
	ThD1-0008/81[a]	1981	I	AY732483
	ThD1-0081/82[a]	1982	I	AY732481
	ThD1-0323/91[a]	1991	I	AY732478
	ThD1-0336/91[a]	1991	I	AY732477
	ThD1-0097/94[a]	1994	I	AY732480
	ThD1-0488/94[a]	1994	I	AY732475
	ThD1-0049/01[a]	2001	I	AY732482
	ThD1-0102/01[a]	2001	I	AY732479
	Philippines/74	1974	II	AF425627
	Indonesia A88	1988	II	AB074761
	Tahiti/01	2001	II	AB111070
	Indonesia 02	2002	II	AB111075
DENV-2	ThD2-0038/74[a]	1974	Asian I	DQ181806
	ThD2-0168/79[a]	1979	Asian I	DQ181805
	ThD2-0498/84[a]	1984	Asian I	DQ181804
	ThD2-0263/95[a]	1995	Asian I	DQ181800
	ThD2-0017/98[a]	1998	Asian I	DQ181799
	ThD2-0055/99[a]	1999	Asian I	DQ181798
	ThD2-0078/01[a]	2001	Asian I	DQ181797
	ThD2-0433/85[a]	1985	Asian I	DQ181803
	ThD2-0026/88[a]	1988	Asian I	DQ181802
	ThD2-0284/90[a]	1990	Asian/American	DQ181801
	Jamaica N1409/83	1983	Asian/American	M20558
	Martinique/98	1998	Asian/American	AF208496
	DominicanDR59/01	2001	Asian/American	AB122022
	AustraliaTSV01/93	1993	Cosmopolitan	AY037116
	Indonesia98900663/98	1998	Cosmopolitan	AB189122
	ChinaFJ11/99	1999	Cosmopolitan	AF359579
	IndonesiaBA05i/04	2004	Cosmopolitan	AY858035
	Tonga/74	1974	American	AY744147
	Columbia/86	1986	American	AY702040
	Venezuela2/87	1987	American	AF100465
	Venezuela131/92	1992	American	AF100469
	NewGuineaC	1944	Asian II	AF038403
DENV-3	ThD3-0055/93[a]	1993	II	AY676351
	ThD3-0104/93[a]	1993	II	AY676350
	ThD3-1283/98[a]	1998	II	AY676349
	ThD3-1687/98[a]	1998	II	AY676348
	ThD3-0007/87[a]	1987	II	AY676353
	ThD3-0010/87[a]	1987	II	AY676352
	Indonesia/78	1978	I	AY648961
	Tahiti/89	1989	I	AY744677
	Martinique/99	1999	III	AY099337
	Sri Lanka1266/00	2000	III	AY099336
	Puerto Rico/63	1963	IV	AY146762
	Puerto Rico/77	1977	IV	AY146761
DENV-4	ThD4-0087/77[a]	1977	I	AY618991
	ThD4-0348/91[a]	1991	I	AY618990
	ThD4-0017/97[a]	1997	III	AY618989
	ThD4-0476/97[a]	1997	III	AY618988
	ThD4-0734/00[a]	2000	II	AY618993
	ThD4-0485/01[a]	2001	I	AY618992
	China Guangzhou B5	NA[b]	I	AF289029
	Dominica/81	1981	II	AF326573

[a] "Th" represents Thailand.
[b] Year of isolation not available.

(RT-PCR) and real-time quantitative RT-PCR have been used successfully to identify DENV serotypes. However, increasing viral intra-genetic diversity requires an effective method for genotype identification. Recent studies have shown that DEN viruses can be classified as being of epidemiologically low, medium, or high impact (Rico-Hesse, 1990). For example, some DENV genotypes tend to be associated only with sylvatic cycles and with little or low transmission to humans, whereas, other genotypes tend to be associated with DF outbreaks only, and yet other genotypes have been associated with DHF and DSS. The evolution of viral genotypes is based on the accumulation of genetic mutations in the viruses over time and epidemiologically related strains show higher similarity than unrelated strains within the same species. Thus, genotyping allows the discrimination of micro-organisms below the species level. The most widely accepted method for accurate DENV genotype determination involves the phylogenetic analysis of gene sequences (mainly E gene) by the maximum likelihood (ML) method incorporating transition/transversion rate calculations and bootstrap (statistical) support for the branching patterns (Hillis, 1998; Lemmon and

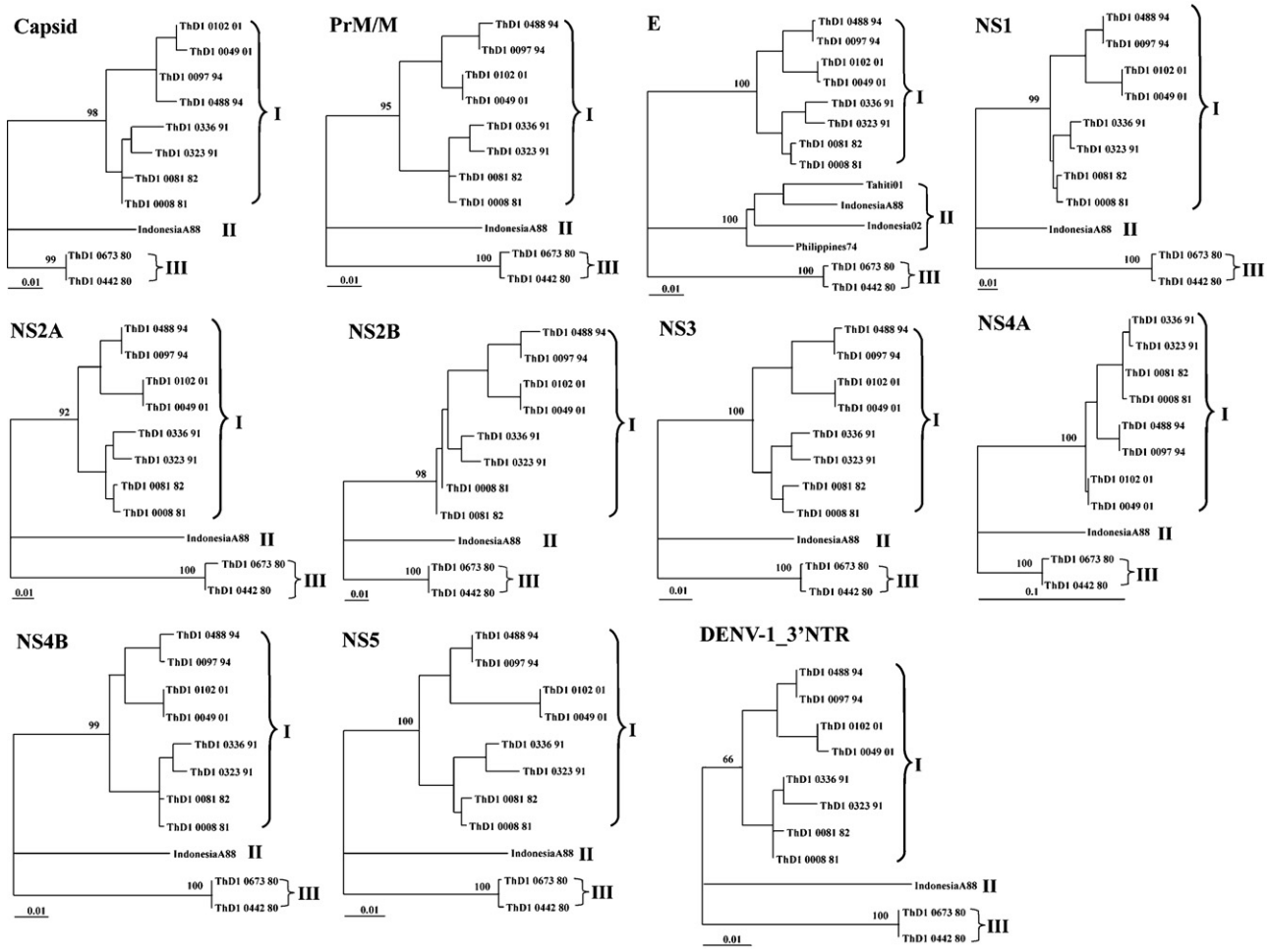

Fig. 1. Maximum likelihood (ML) phylogenetic trees derived from 10 coding genes and 3′NTR of DENV-1. Three human genotypes of DENV-1 were reflected in all 11 ML trees shown as I (Asia and Oceania), II (South Asia and Oceania), and III (Latin America, Caribbean, Asia and Africa). The bootstrap supporting values were shown at major nodes on the tree. The trees were rooted by the two strains of genotype III and all horizontal branch lengths were drawn to scale.

Milinkovitch, 2002). However, the rationale for this approach is not well documented.

Recent comparative analysis of 32 complete genomic sequences of DENV strains sampled in Thailand over the past three decades revealed nucleotide substitutions in most regions of the viral genome except for the 5′NTR; especially in the NS genes (Zhang et al., unpublished data). This observation raises the question: which gene is the most suitable target for genotyping DENV? To answer this question, phylogenetic analysis was performed on individual gene sequences of 56 DENV isolates to determine whether targeting genes other than E, including NS genes, might increase the efficiency and accuracy of DENV genotype determination and identify newly evolved or previously unidentified genotypes.

2. Materials and methods

2.1. Viruses

Nucleotide sequence data sets used in this study were composed of 56 complete genomic sequences representing all known genotypes of the four DENV serotypes including 32 Thai DENV isolates sequenced (details described in Klungthong et al., 2004; Zhang et al., 2005, 2006), and 24 additional complete DENV sequences obtained from GenBank (since not many complete genomic sequences are available in the GenBank, those available for representative genotypes of the four serotypes were used). The GenBank accession numbers for all 56 DENV sequences are shown in Table 1.

2.2. Genotyping method

Phylogenetic analysis was performed gene-by-gene on the sequences of the coding region and the 3′NTR of 32 Thai DENV strains and 24 DENV strains representing the global genetic variability (all known genotypes) of each serotype. Because the 5′NTR is highly conserved among the four Thai DENV serotypes it was not included in the analysis. Sequence alignments were performed using the ClustalX program (Thompson et al., 1994) which resulted in alignments of each gene sequence for each of the four DENV serotypes. Maximum likelihood phylogenetic trees were then estimated for all four data sets using PAUP* software (Swofford, 2003). To determine the support for particular groupings on the phylogenetic trees, bootstrap re-sampling analysis was performed using 1000 replicate neighbor-joining trees estimated by the ML substitution model.

3. Results

To determine which genes are suitable for intra-serotype identification, phylogenetic analysis was performed separately on the

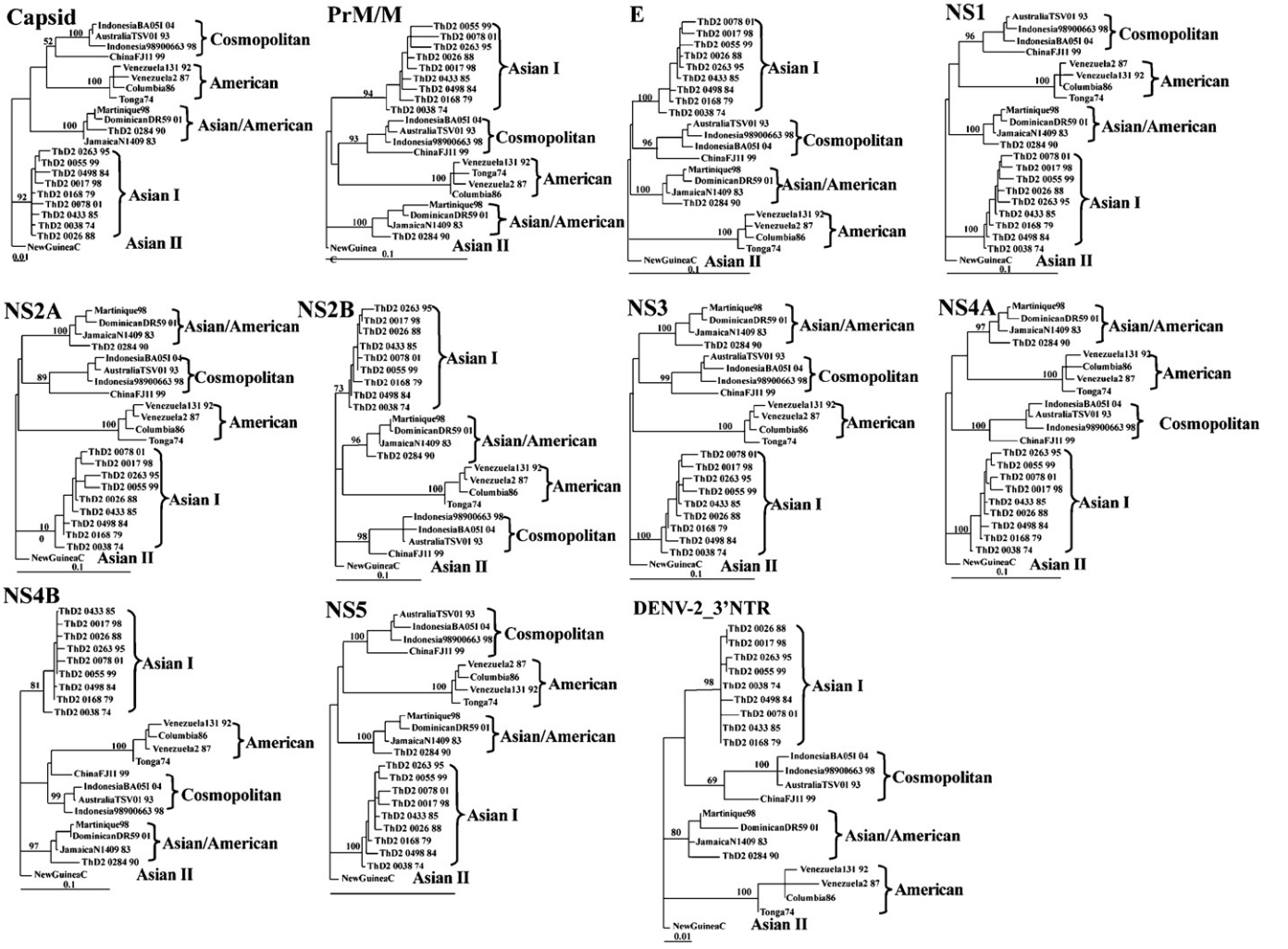

Fig. 2. Maximum likelihood (ML) phylogenetic trees derived from 10 coding genes and 3′NTR of DENV-2. The five genotypes of DENV-2 were reflected in all 11 ML trees shown as I (Asian I), II (Asian II), III (Asian/American), IV (Cosmopolitan), and V (American). The bootstrap supporting values were shown at the major nodes on the tree. The trees were rooted by one strain of genotype II and all horizontal branch lengths were drawn to scale.

sequences of the three structural and seven non-structural genes as well as the 3′NTR. Eleven phylogenetic trees were generated by the ML method for each DENV serotype (1 tree/1 gene). The bootstrap value was added to each major node. The closer the bootstrap value is to 100% at the nodes, the more accurate the genotype identification. A clade supported by a bootstrap value of at least 90% is considered to signify a high degree of confidence.

3.1. Genotyping DENV-1

In the phylogenetic analysis of DENV-1 all three established genotypes were reflected in eleven ML trees derived from each gene (Fig. 1). The ML trees generated from the E (1485 bp), NS3 (1857 bp) and NS5 (2697 bp) genes were each supported by a bootstrap value of 100% at all major nodes, while the ML trees derived from the C (342 bp), PrM (498 bp), NS1 (1056 bp), NS2A (654 bp), NS2B (390 bp) and NS4B (747 bp) genes were each supported by bootstrap values of greater than 92% at the major nodes (Fig. 1). Although the bootstrap value at the major nodes on the ML tree of the NS4A gene was 100%, the genotype I strains ThD1-0488-94 and ThD1-0097-94 fell into a different clade from the one obtained for the other 10 ML trees (Fig. 1). Additionally, although all genotypes were identified on the ML tree of the 3′NTR, the bootstrap value supporting one major node was only 66% rather than 90% or greater. Therefore, all ML trees of the DENV-1 showed similar topology except the tree for the NS4A gene, and bootstrap values greater than 92% at all major nodes except for the 3′NTR, which suggests that all genes, except the NS4A gene and 3′NTR, can be used for the genotyping of DENV-1.

3.2. Genotyping DENV-2

In the analysis of DENV-2 all five established genotypes were reflected in eleven ML trees with similar topology, but only the ML tree derived from the NS5 gene was supported by a bootstrap value of 100% at all four major nodes (Fig. 2). The ML trees derived from the PrM/M, E, NS1, NS3 and NS4A genes had three of four major nodes supported by a bootstrap value of 100% and the fourth node supported by bootstrap values ranging from 92 to 99% (Fig. 2). However, the ML trees generated from the C, NS2A, NS2B, and NS4B genes and the 3′NTR had one or more major nodes supported by a bootstrap value of less than 89% (Fig. 2). The phylogenetic analysis of each individual gene with over 90% bootstrap value supporting the major nodes suggests that the sequences of the PrM/M, E, NS1, NS3, NS4A and NS5 genes may be the most suitable for the genotyping of DENV-2.

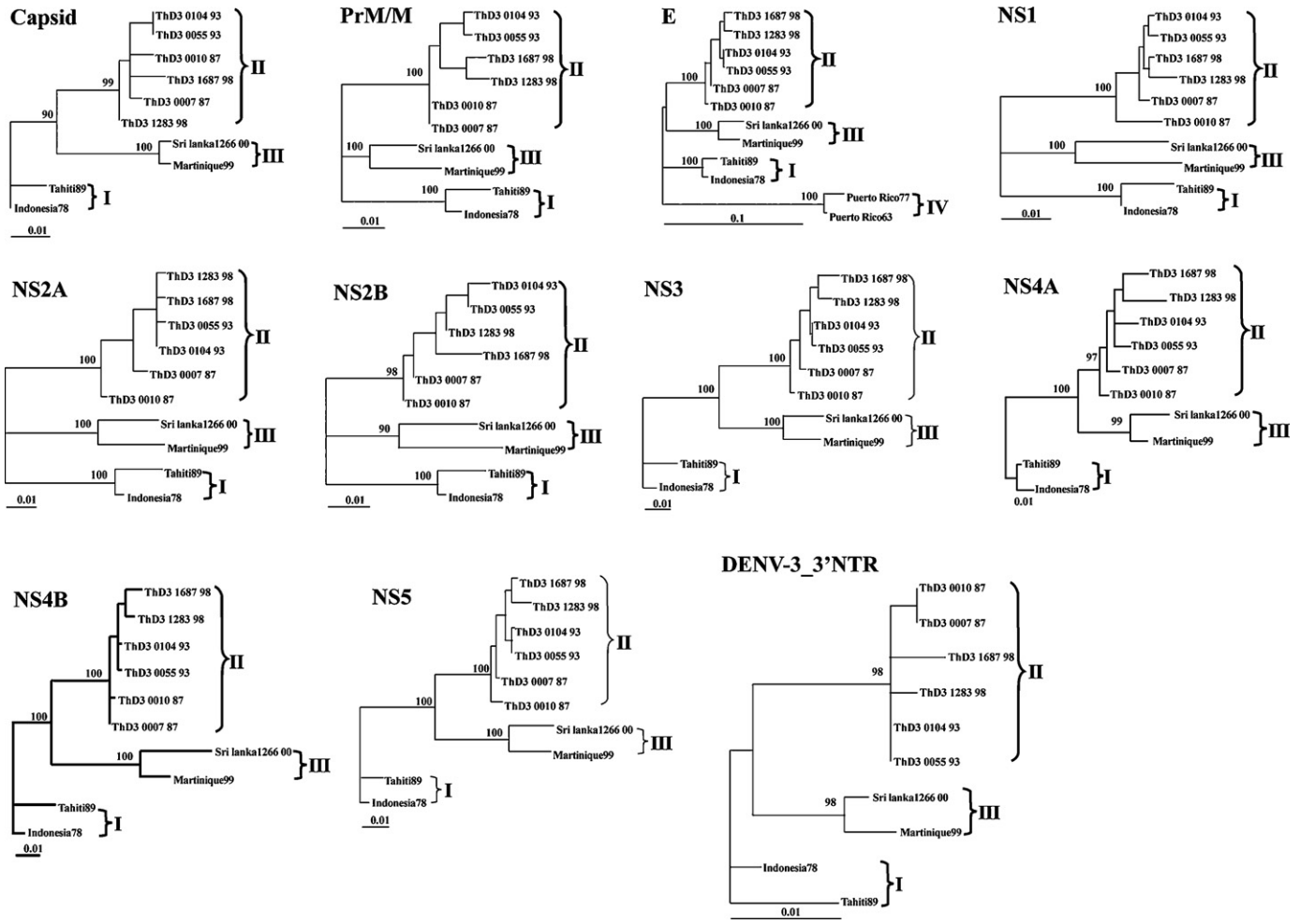

Fig. 3. Maximum likelihood (ML) phylogenetic trees derived from 10 coding genes and the 3′NTR of DENV-3. The three human genotypes of DENV-3 were reflected in all 11 ML trees shown as I (South-East Asia and Oceania), II (South-East Asia) and III (South Asia and Latin America) except the tree of the E gene in which four genotypes (IV, Latin America and Caribbean) were reflected. The bootstrap supporting values were shown at the major nodes on the tree. The trees were rooted by the two strains of genotype I and all horizontal branch lengths were drawn to scale.

3.3. Genotyping DENV-3

In the analysis of DENV-3 three (genotype I, II, and III) of four established genotypes were reflected in eleven ML trees with similar topology and over 90% bootstrap values supporting all major nodes. For genotype IV strains (isolated in Puerto Rico) only the *E* gene sequence was available from GenBank. Therefore, the genotype IV strains were reflected only in a single ML tree for the E gene, but not the ten other ML trees for the other genes. The trees derived from the sequences of the PrM, E, NS1, NS2A, NS3, NS4B and NS5 genes were all supported by a bootstrap value of 100% at the major nodes, while the ML trees derived from the C, NS2B and NS4A genes and the 3′NTR all had three major nodes supported by bootstrap values ranging from 90 to 100% (Fig. 3), which indicated that all coding genes and 3′NTR sequences were good targets for the genotyping of DENV-3.

3.4. Genotyping DENV-4

For the analysis of DENV4, complete genomic sequences were unavailable from GenBank for sylvatic strains used as an out-group. Therefore, only three of the four established DENV-4 genotypes were reflected in the ML trees. The ML trees derived from the C, PrM/M, E, NS1, NS2A, NS2B, NS4A and NS5 genes showed similar topology and were supported by bootstrap values of 90% to 100% at each major node (Fig. 4). However, one strain (ThD4-0734-00) fell into genotype I in the ML trees derived from the NS3 and the NS4B genes as well as the 3′NTR, whereas it fell into genotype II in the ML trees derived from the other eight genes. Phylogenetic analysis the individual genes of DENV-4 indicated that the C, PrM/M, E, NS1, NS2A, NS2B, NS4A, and NS5 genes may all be good target sequences for genotyping.

4. Discussion

Phylogenetic analysis performed on gene sequences obtained directly from patients' sera provides a rapid approach for discriminating among DEN viruses according to serotype, genotype and clade. This molecular epidemiological typing technique is used widely and is accepted for the genotyping of DEN viruses. Thus far, most studies performing phylogenetic analysis of DENV, using mainly nucleotide sequences of the E gene, have identified three genotypes for DENV-1, five for DENV-2, and four for both DENV-3 and DENV-4 (Klungthong et al., 2004; Lanciotti et al., 1994, 1997; Lewis et al., 1993; Rico-Hesse, 1990, 2003; Zhang et al., 2005, 2006).

In the present study, phylogenetic analyses were performed on the nucleotide sequences of individual coding and non-coding genes from 56 geographically divergent DENV isolates represent-

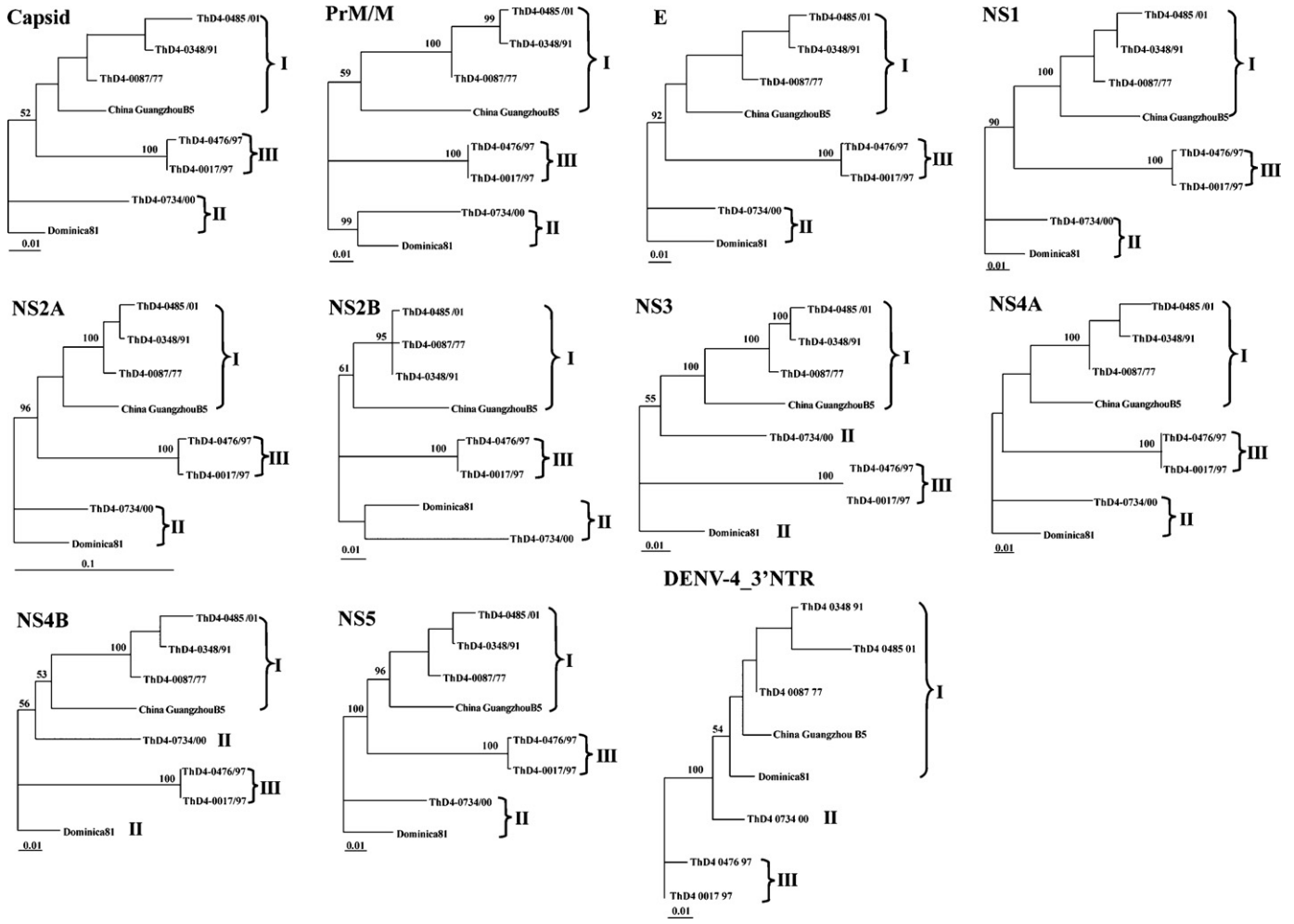

Fig. 4. Maximum likelihood (ML) phylogenetic trees derived from 10 coding genes and 3′NTR of DENV-4. The three human genotypes of DENV-4 were reflected in all 11 ML trees shown as I (Asia), II (South-East Asia, Latin America and Caribbean) and III (Thailand). The bootstrap supporting values were shown at the major nodes on the tree. The trees were rooted by the two strains of genotype II and all horizontal branch lengths were drawn to scale.

ing all known genotypes of the four DENV serotypes. The results support genotypes identified previously and provide additional information on the suitability and usefulness of each individual gene region for the molecular genotyping of DENV. They suggest that viral evolution into differing genetic groups reflects differences in both structural and NS genes, with the result that most structural and NS gene sequences in addition to the E gene can be used for genotyping and clade identification of the majority of the DENV isolates analyzed. There were, however, some exceptions. For example, one Thai DENV-4 strain (ThD4-0734-00) gave a contradictory result, since it fell into genotype I in the ML trees constructed for NS3, NS4B, and the 3′-NTR, but into genotype II for the other 8 ML trees. This finding could be explained if strain ThD4-0734-00 is an inter-genotypic recombinant because multiple co-circulating DENV genotypes of the same serotype in one community increases the possibility of concurrent infection with multiple genotypes in one individual. A recombinant genotype has been reported in the case of a DENV isolate (Craig et al., 2003; Holmes et al., 1999; Tolou et al., 2001; Uzcategui et al., 2001; Worobey et al., 1999), in which case it would not negate the utility of these genes for genotyping DENV-4 strains. Of course, the nature of this unusual virus needs to be investigated further. Additionally, two DENV-1 strains (ThD1-0488-94 and ThD4-0097-94) fell into one clade from the ML tree derived from the NS4A gene but a different clade for the other 10 ML trees (Fig. 1). This might be due to the accumulation of sufficient number of amino acid mutations in the NS4A gene leading to difficulty in identifying accurately the clade using this gene. The sequences of the NS4A gene for these two strains showed that amino acid residue 93 of NS4A has mutated from Valine to Alanine, which resulted in a different topology for the NS4A ML tree. The 3′NTR could be used only for genotyping DENV-3 but not for genotyping accurately DENV-1, DENV-2 and DENV-4 due to one or two low bootstrap values supporting major nodes, leading to a different topology for the ML tree for DENV-4.

Since several genes can be used to genotype DEN viruses by phylogenetic analysis, the target sequence(s) can be selected based on the study objectives. For example, if the aim of the study is only virus classification, then selection of a short gene sequence with high bootstrap values supporting the major nodes is probably the most efficient choice. However, if in addition to genotype identification the target gene will also be used for the study of viral evolution and selection pressure analysis, then those genes in which mutations have the most direct effects on biologically important functions, such as virus binding, membrane fusion, replication, transcription, and translation, may be more appropriate targets. For example, the E protein, as the major surface antigen of DENV is subject to constant immunological selective pressure from antibodies and T-cells in the human host. It could also be a target for non-immunological selective pressures as the virus moves between the human host and the mosquito vector. Sequential sequencing of

the E gene from viral isolates collected during high and low epidemic periods could be useful for addressing the question of how mutations in the E gene correlate with virus transmission rates and population immunity. The NS3 protein is a multifunctional enzyme with separate active sites involved in viral RNA replication and capping: helicase, nucleoside 5′-triphosphatase (NTPase) and RNA 5′-triphosphatase (RTPase) (Benarroch et al., 2004). The NS5 protein has N-terminal methyltransferase activity spanning amino acid residues 1–296, which has been expressed as a soluble active enzyme, and C-terminal RNA-dependent RNA-polymerase activity residing in residues 273–900 (Egloff et al., 2002; Pryor et al., 2007; Yap et al., 2007). Therefore, mutations occurring in these genes could have significant effects on virus replication, translation, maturation, and infectivity.

In conclusion, based on the bootstrap values supporting the major nodes of 44 ML trees derived from three structural and seven NS genes as well as the 3′NTR, the most suitable targets for genotyping DENV were identified. For genotyping DENV-1 any of nine coding genes (excepting NS4A) could be used. For DENV-2 PrM/M, E, NS1, NS3, NS4A and NS5 were useful targets. For DENV-3 all 10 coding genes and the 3′NTR could be used. Finally, for DENV-4, C, PrM/M, E, NS1, NS2A, NS2B, NS4A and NS5 were all suitable targets for genotyping.

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References

AbuBakar, S., Wong, P.F., Chan, Y.F., 2002. Emergence of dengue virus type 4 genotype IIA in Malaysia. J. Gen. Virol. 83, 2437–2442.

Benarroch, D., Selisko, B., Locatelli, G.A., Maga, G., Romette, J.L., Canard, B., 2004. The RNA helicase, nucleotide 5′-triphosphatase, and RNA 5′-triphosphatase activities of dengue virus protein NS3 are Mg^{2+}-dependent and require a functional Walker B motif in the helicase catalytic core. Virology 328, 208–218.

Bennett, S.N., Holmes, E.C., Chirivella, M., Rodriguez, D.M., Beltran, M., Vorndam, V., Gubler, D.J., McMillan, W.O., 2006. Molecular evolution of dengue 2 virus in Puerto Rico: positive selection in the viral envelope accompanies clade reintroduction. J. Gen. Virol. 87, 885–893.

Craig, S., Thu, H.M., Lowry, K., Wang, X.F., Holmes, E.C., Aaskov, J., 2003. Diverse dengue type 2 virus populations contain recombinant and both parental viruses in a single mosquito host. J. Virol. 77, 4463–4467.

Egloff, M.P., Benarroch, D., Selisko, B., Romette, J.L., Canard, B., 2002. An RNA cap (nucleoside-2′-*O*-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J. 21, 2757–2768.

Foster, J.E., Bennett, S.N., Vaughan, H., Vorndam, V., McMillan, W.O., Carrington, C.V., 2003. Molecular evolution and phylogeny of dengue type 4 virus in the Caribbean. Virology 306, 126–134.

Grenfell, B.T., Pybus, O.G., Gog, J.R., Wood, J.L., Daly, J.M., Mumford, J.A., Holmes, E.C., 2004. Unifying the epidemiological and evolutionary dynamics of pathogens. Science 303, 327–332.

Gubler, D.J., Meltzer, M., 1999. Impact of dengue/dengue hemorrhagic fever on the developing world. Adv. Virus Res. 53, 35–70.

Hillis, D.M., 1998. Taxonomic sampling, phylogenetic accuracy, and investigator bias. Syst. Biol. 47, 3–8.

Holmes, E.C., Burch, S.S., 2000. The causes and consequences of genetic variation in dengue virus. Trends Microbiol. 8, 74–77.

Holmes, E.C., Worobey, M., Rambaut, A., 1999. Phylogenetic evidence for recombination in dengue virus. Mol. Biol. Evol. 16, 405–409.

Klungthong, C., Zhang, C., Mammen Jr., M.P., Ubol, S., Holmes, E.C., 2004. The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Virology 329, 168–179.

Lanciotti, R.S., Gubler, D.J., Trent, D.W., 1997. Molecular evolution and phylogeny of dengue-4 viruses. J. Gen. Virol. 78 (Pt 9), 2279–2284.

Lanciotti, R.S., Lewis, J.G., Gubler, D.J., Trent, D.W., 1994. Molecular evolution and epidemiology of dengue-3 viruses. J. Gen. Virol. 75 (Pt 1), 65–75.

Lemmon, A.R., Milinkovitch, M.C., 2002. The metapopulation genetic algorithm: an efficient solution for the problem of large phylogeny estimation. Proc. Natl. Acad. Sci. U.S.A. 99, 10516–10521.

Lewis, J.A., Chang, G.J., Lanciotti, R.S., Kinney, R.M., Mayer, L.W., Trent, D.W., 1993. Phylogenetic relationships of dengue-2 viruses. Virology 197, 216–224.

Loroño-Pino, M.A., Cropp, C.B., Farfán, J.A., Vorndam, A.V., RodrÍguez-Angulo, E.M., Rosado-Paredes, E.P., Flores-Flores, L.F., Beaty, B.J., Gubler, D.J., 1999. Common occurrence of concurrent infections by multiple dengue virus serotypes. Am. J. Trop. Med. Hyg. 61, 725–730.

Pryor, M.J., Rawlinson, S.M., Butcher, R.E., Barton, C.L., Waterhouse, T.A., Vasudevan, S.G., Bardin, P.G., Wright, P.J., Jans, D.A., Davidson, A.D., 2007. Nuclear localization of dengue virus nonstructural protein 5 through its importing α/β-recognized nuclear localization sequences is integral to viral infection. Traffic 8, 795–807.

Rico-Hesse, R., 1990. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology 174, 479–493.

Rico-Hesse, R., 2003. Microevolution and virulence of dengue viruses. Adv. Virus Res. 59, 315–341.

Salda, L.T., Parquet, M.D., Matias, R.R., Natividad, F.F., Kobayashi, N., Morita, K., 2005. Molecular epidemiology of dengue 2 viruses in the Philippines: genotype shift and local evolution. Am. J. Trop. Med. Hyg. 73, 796–802.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), Version 4. Sinauer Associates, Sunderland, MA.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tolou, H.J., Couissinier-Paris, P., Durand, J.P., Mercier, V., de Pina, J.J., de Micco, P., Billoir, F., Charrel, R.N., de Lamballerie, X., 2001. Evidence for recombination in natural populations of dengue virus type 1 based on the analysis of complete genome sequences. J. Gen. Virol. 82, 1283–1290.

Uzcategui, N.Y., Camacho, D., Comach, G., Cuello de Uzcategui, R., Holmes, E.C., Gould, E.A., 2001. Molecular epidemiology of dengue type 2 virus in Venezuela: evidence for in situ virus evolution and recombination. J. Gen. Virol. 82, 2945–2953.

Uzcategui, N.Y., Comach, G., Camacho, D., Salcedo, M., Cabello de Quintana, M., Jimenez, M., Sierra, G., Cuello de Uzcategui, R., James, W.S., Turner, S., Holmes, E.C., Gould, E.A., 2003. Molecular epidemiology of dengue virus type 3 in Venezuela. J. Gen. Virol. 84, 1569–1575.

World Health Organization, 1999. Strengthening implementation of the global strategy for dengue fever and dengue hemorrhagic fever, prevention and control. Report of the Informal Consultation, WHO HQ, Geneva, pp. 18–20.

World Health Organization, 2002. Dengue and dengue hemorrhagic fever. Geneva, Switzerland, Fact sheet no. 117.

Worobey, M., Rambaut, A., Holmes, E.C., 1999. Widespread intra-serotype recombination in natural populations of dengue virus. Proc. Natl. Acad. Sci. U.S.A. 96, 7352–7357.

Yap, T.L., Xu, T., Chen, Y.L., Malet, H., Egloff, M.P., Canard, B., Vasudevan, S.G., Lescar, J., 2007. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1 85-angstrom resolution. J. Virol. 81, 4753–4765.

Zhang, C., Mammen Jr., M.P., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Monkongdee, P., Nimmannitya, S., Kalayanarooj, S., Holmes, E.C., 2005. Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. J. Virol. 79, 15123–15130.

Zhang, C., Mammen Jr., M.P., Chinnawirotpisan, P., Klungthong, C., Rodpradit, P., Nisalak, A., Vaughn, D.W., Nimmannitya, S., Kalayanarooj, S., Holmes, E.C., 2006. Structure and age of genetic diversity of dengue virus type 2 in Thailand. J. Gen. Virol. 87, 873–883.