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Heterogeneous Co-circulation of Multiple DENV-1 and DENV-2 Genotypes during the 2023 Vietnam Outbreak

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Abstract

Background

Dengue virus (DENV) remains a major public health burden in Vietnam, where repeated outbreaks occur against a background of complex serotype and genotype circulation. Previous outbreak investigations in Vietnam have often emphasized dominance of a single serotype, genotype, or lineage. We investigated the genomic diversity of DENV recovered from a hospital-based cohort during the 2023 outbreak.

Methods

Serum samples were collected from 148 patients admitted to hospitals in Vietnam during the 2023 dengue outbreak. DENV infection and serotype were determined by RT-PCR/qRT-PCR. A subset of DENV-positive samples was propagated at low passage using an FcγR-expressing BHK cell system to support viral recovery for whole-genome sequencing. Complete genome sequences were generated using next-generation sequencing and analyzed with reference sequences from GenBank. Phylogenetic analysis was performed using maximum likelihood methods, and non-synonymous substitutions in DENV-2 Cosmopolitan isolates were compared between historical and 2023 outbreak datasets.

Results

Among 148 samples, 90 were DENV-positive. Most infections were DENV-2 (80/90, 88.9%), followed by DENV-1 (9/90, 10.0%) and DENV-4 (1/90, 1.1%). Whole-genome sequencing was attempted for 53 isolates and yielded 47 complete DENV genomes. DENV-1 isolates belonged to genotypes I and IV, while DENV-2 isolates were dominated by the Cosmopolitan genotype with limited detection of Asian I. DENV-1 genotype I clustered with previously reported Vietnamese strains, whereas genotype IV grouped more closely with non-local reference sequences. DENV-2 Cosmopolitan isolates clustered with Vietnamese and regional Southeast Asian sequences. Comparative analysis identified shared and 2023-associated non-synonymous substitutions distributed across E, NS1, NS3 and NS5, with the largest number of substitutions observed in NS5.

Conclusions

These findings indicate heterogeneous co-circulation of DENV-1 and DENV-2 genotypes during the 2023 outbreak and support the use of whole-genome surveillance to monitor dengue genotype diversity beyond serotype-level reporting.

Background

Dengue virus (DENV) has remained a persistent public health challenge in Vietnam since its first recorded outbreak in 1963 [1]. The 2023 epidemic season occurred during a year of exceptional dengue activity globally and was associated with a marked increase in reported cases in Vietnam [2]. Although DENV-2 was reported as the dominant serotype during this period, serotype-level surveillance alone may obscure the underlying genotype and lineage structure of circulating viruses.

Historically, major dengue outbreaks in Vietnam and neighboring countries have often been described through the emergence, re-emergence, or dominance of a specific serotype, genotype, or lineage. For example, the 2022 outbreak in southern Vietnam was attributed mainly to a newly introduced lineage within the DENV-2 Cosmopolitan genotype [3,6]. In northern Vietnam, molecular studies from recent outbreaks have identified DENV-1 genotype I, DENV-4 genotype I, and co-circulation of DENV-2 Asian I and Cosmopolitan genotypes [4,5]. Similar DENV-2 genotype dynamics have been documented in Thailand and other parts of mainland Southeast Asia [7,8]. Together, these observations suggest repeated regional movement and turnover of DENV lineages.

Genotype diversity within a serotype may have epidemiological and immunological relevance. The DENV envelope (E) protein is a major target of neutralizing antibodies, and within-serotype genetic divergence can contribute to antigenic variation [10–15]. However, the interpretation of genotype diversity requires caution when functional neutralization, clinical severity, and vaccine breakthrough data are not directly measured. Genomic surveillance nevertheless provides an essential framework for detecting genotype co-circulation, lineage persistence, and potential regional exchange.

This study used whole-genome sequencing and phylogenetic analysis to characterize DENV recovered during the 2023 outbreak from a hospital-based cohort in Vietnam. We focused on the distribution of DENV-1 and DENV-2 genotypes and on non-synonymous substitutions in the predominant DENV-2 Cosmopolitan genotype. The objective was to determine whether the 2023 outbreak was consistent with a single dominant lineage or with more heterogeneous co-circulation of multiple DENV genotypes.

Methods

Ethics approval and clinical samples

This study was approved by the Research Ethics Committee of the Faculty of Medicine of the University of Tokyo (2023313NI-(2)), and by the National Institute of Hygiene and Epidemiology Institutional Review Board in Bio Medical Research (IRB-VN01057/IORG0008555, No. NIHE IRB-28/2024). Written informed consent was obtained from participants or their legal guardians according to the approved protocol.

A total of 148 serum samples were collected from patients admitted to hospitals in Vietnam during the 2023 dengue outbreak, predominantly from Vung Tau General Hospital. Samples were screened for DENV RNA by reverse transcription polymerase chain reaction (RT-PCR), and infecting serotypes were identified by real-time quantitative reverse transcription PCR (qRT-PCR) [30]. Samples were transported

to Japan for further molecular analysis under the applicable institutional and material-transfer procedures.

Virus titration and low-passage virus recovery

To evaluate viral infectivity and determine suitability for downstream isolation, serum samples were serially diluted in minimum essential medium supplemented with 2% fetal bovine serum and inoculated onto FcγR-negative and FcγR-expressing baby hamster kidney (BHK) cell lines. After adsorption for 1 h at 37°C with 5% CO2, maintenance medium was added and cells were incubated for 5–7 days. Plates were fixed with formaldehyde and stained with methylene blue to visualize plaque formation [31].

Because viral titers can decrease during storage and international transport, low-passage antibody-dependent enhancement (ADE)-based propagation was used to recover sufficient infectious virus for whole-genome sequencing [19]. Each serum sample was diluted in medium containing a dengue cross-reactive mouse monoclonal antibody to facilitate immune-complex formation, incubated at 4°C, and inoculated onto FcγR-expressing BHK cells. Supernatants were collected after 5–7 days for downstream RNA extraction [29,31,32]. In this manuscript, this method is described only as a virus-recovery approach; viral-load and ADE-related analyses are not included here.

RNA extraction and qRT-PCR

Viral RNA was extracted from culture supernatants using Zymo Research kits according to the manufacturer's protocol and eluted in RNase-free water. qRT-PCR was performed to quantify viral genome copies and to ensure sufficient input for library preparation. Viral titers were estimated using standard curves generated from serial dilutions of quantified dengue virus RNA standards [30].

Whole-genome sequencing

Among 90 DENV-positive samples, 53 were selected for whole-genome sequencing based on viral genome copy number, patient age-group representation, and serotype diversity. Extracted RNA was reverse-transcribed using random hexamers, and double-stranded cDNA was prepared for library construction [33]. cDNA products were purified with magnetic beads, and libraries were prepared using the Illumina Nextera XT DNA Library Preparation Kit according to the manufacturer's instructions. Library quality and fragment-size distribution were assessed before sequencing. Normalized pooled libraries were sequenced using the Illumina MiSeq platform to generate paired-end reads [34,35].

Phylogenetic analysis

Forty-seven of 53 sequenced isolates yielded complete genomes suitable for analysis. Sequencing reads were mapped to DENV-1 (GenBank accession MW792695) or DENV-2 (GenBank accession MW788986)

reference genomes using Geneious Prime [36]. Approximately 100–120 complete genome reference sequences per serotype were downloaded from GenBank to represent global and regional diversity. Multiple sequence alignments were generated using MAFFT. Maximum-likelihood phylogenetic trees were constructed in MEGA with 500 bootstrap replicates and visualized using iTOL [37,38].

Mutational analysis of DENV-2 Cosmopolitan isolates

Only genomes with sufficient coverage and minimal ambiguous nucleotides were retained for mutational analysis. Analyses were restricted to isolates classified as DENV-2 Cosmopolitan genotype. Consensus sequences were grouped into historical and 2023 outbreak datasets according to phylogenetic characterization, and non-synonymous substitutions were compared between these datasets.

Consensus sequences were aligned using MAFFT, and the annotated DENV-2 reference genome NC_001474.2 was used for coding-sequence annotation and amino-acid substitution analysis [39]. Genetic variations were identified using the Find Variations/SNPs function in Geneious Prime. Variants were translated according to annotated coding regions, and synonymous and non-synonymous substitutions were determined by comparing codon and amino-acid changes [25]. Non-synonymous substitutions observed in both historical and 2023 isolates were classified as shared substitutions, whereas substitutions detected only in 2023 isolates were classified as 2023-specific substitutions. Frequencies were calculated as the proportion of genomes in each dataset carrying the substitution. No formal statistical hypothesis testing was conducted.

Use of AI-assisted technologies

AI-assisted tools, including ChatGPT and Grammarly, were used to assist with language editing during manuscript preparation. The authors reviewed and revised the output to ensure accuracy and clarity. No AI-assisted tools were used for data generation, data analysis, data interpretation, or figure preparation.

Results

Study population and serotype distribution

Of 148 serum samples collected during the 2023 outbreak, 90 (60.8%) were DENV-positive by RT-PCR/qRT-PCR. Most samples were collected in Vung Tau (146/148, 98.7%), with two samples from Da Nang. The mean patient age was 29.6 years (SD 16.5; range 1–88), and most patients were adults (> 12 years old; 122/148, 82.4%). The mean duration of illness before sampling was 2.1 days (SD 1.1). Among DENV-positive samples, DENV-2 was the dominant serotype (80/90, 88.9%), followed by DENV-1 (9/90, 10.0%) and DENV-4 (1/90, 1.1%); no DENV-3 infections were detected (Table 1).

Table 1
Distribution of demographic characteristics and infecting serotypes in the study population.

Variable	Frequency	Percent
No. of patients (n = 148)		
DENV-positive	90	60.81
DENV-negative	58	39.19
Province		
Vung Tau	146	98.65
Da Nang	2	1.35
Age, years, mean ± SD (minimum-maximum)	29.6 ± 16.5 (1–88)	
Children (< 12 years old)	26	17.57
Adults (> 12 years old)	122	82.43
Days ill, mean ± SD	2.1 ± 1.1	
Infecting serotype (qRT-PCR, n = 90)		
DENV-1	9	10.00
DENV-2	80	88.89
DENV-3	0	0.00
DENV-4	1	1.11

Whole-genome sequencing and phylogenetic characterization

Whole-genome sequencing was performed on 53 selected DENV-1, DENV-2, and DENV-4 isolates. Forty-seven isolates yielded high-quality complete genomes suitable for phylogenetic analysis. Complete genomes generated in this study were analyzed together with representative DENV-1 and DENV-2 reference genomes from GenBank.

DENV-1 isolates from the 2023 outbreak clustered within genotypes I and IV (Fig. 1). Most DENV-1 isolates (approximately 86%) belonged to genotype I and grouped with previously reported Vietnamese strains, consistent with sustained local circulation. A smaller subset (approximately 14%) belonged to genotype IV and clustered more closely with non-local reference sequences, consistent with sporadic introduction or broader regional circulation.

DENV-2 isolates were dominated by the Cosmopolitan genotype, with a smaller proportion belonging to Asian I genotype (Fig. 2). DENV-2 Cosmopolitan isolates clustered with previously reported Vietnamese

and regional Southeast Asian strains, supporting continued local circulation together with regional viral exchange. The concurrent detection of DENV-1 genotype I/IV and DENV-2 Cosmopolitan/Asian I indicates a heterogeneous genomic landscape during the 2023 outbreak.

Shared non-synonymous substitutions in DENV-2 Cosmopolitan isolates

Because DENV-2 Cosmopolitan was the predominant genotype among sequenced DENV-2 isolates, subsequent mutational analyses focused on historical and 2023 DENV-2 Cosmopolitan genomes. Fourteen shared non-synonymous substitutions were detected across the E, NS1, NS3, and NS5 proteins (Table 2). Several substitutions, including NS1-Q2735R, NS1-R2936K, NS3-K5081R, NS3-K6317R, NS5-L8230V, and NS5-K10241R, were detected at high frequency in both historical and 2023 isolates, suggesting persistence within the circulating Cosmopolitan lineage. Other substitutions, including E-Q1092H, E-H1381N, and NS3-S5555N, were more frequently detected among 2023 isolates, suggesting ongoing diversification within the lineage.

Table 2
Shared non-synonymous substitutions identified in historical and 2023 DENV-2 Cosmopolitan isolates. Positions are shown as genome nucleotide positions according to the annotated DENV-2 reference genome used for variant calling. Frequencies represent the proportion of genomes in each dataset carrying the substitution.

Protein	Genome nucleotide position	Amino acid change	Historical (%)	2023 (%)
E	1092	Q→H	50.00%	100.00%
E	1381	H→N	69.20%	100.00%
E	1426	I→V	100.00%	100.00%
E	2320	I→V	54.50%	100.00%
NS1	2659	S→T	33.30%	100.00%
NS1	2735	Q→R	100.00%	100.00%
NS1	2936	R→K	100.00%	100.00%
NS3	5081	K→R	100.00%	100.00%
NS3	5555	S→N	33.30%	100.00%
NS3	6317	K→R	100.00%	100.00%
NS5	8230	L→V	100.00%	100.00%
NS5	9451	V→I	65.00%	83.30%
NS5	9596	S→N	85.00%	83.30%
NS5	10241	K→R	100.00%	100.00%

2023-specific substitutions in DENV-2 Cosmopolitan isolates

Twenty-four non-synonymous substitutions were detected exclusively among 2023 DENV-2 Cosmopolitan isolates (Table 3). These substitutions were distributed across E (n = 5), NS1 (n = 5), NS3 (n = 4), and NS5 (n = 10). Most were detected in all analyzed 2023 isolates, suggesting that they represent characteristic signatures of the sampled 2023 Cosmopolitan viruses. Notable substitutions included E-E1075K, E-K1312E, NS1-T2770A, NS3-A4999T, NS5-R8857G, and NS5-K9968T. Several NS5 substitutions were detected at lower frequencies, indicating additional intra-lineage variation among the 2023 isolates.

Table 3

Non-synonymous substitutions unique to 2023 DENV-2 Cosmopolitan isolates. Positions are shown as genome nucleotide positions according to the annotated DENV-2 reference genome used for variant calling. Frequencies represent the proportion of 2023 genomes carrying the substitution.

Protein	Genome nucleotide position	Amino acid change	2023 (%)
E	1075	E→K	100.00%
E	1312	K→E	100.00%
E	1321	V→I	100.00%
E	2140	I→F	100.00%
E	2297	I→T	100.00%
NS1	2770	T→A	100.00%
NS1	2803	S→L	100.00%
NS1	2814	Q→H	100.00%
NS1	2942	K→R	100.00%
NS1	2954	F→S	100.00%
NS3	4999	A→T	100.00%
NS3	5078	R→K	100.00%
NS3	5338	V→I	100.00%
NS3	5902	I→V	100.00%
NS5	7583	I→T	91.70%
NS5	7613	S→N	100.00%
NS5	7862	R→K	100.00%
NS5	8857	R→G	100.00%
NS5	9244	G→R	83.30%
NS5	9278	K→R	100.00%
NS5	9383	G→V	100.00%
NS5	9392	G→A	83.30%
NS5	9968	K→T	100.00%
NS5	10047	D→E	83.30%

Protein-wise summary showed that NS5 contained the largest number of non-synonymous substitutions (n = 14), followed by E (n = 9), NS1 (n = 8), and NS3 (n = 7) (Table 4). NS5 also contained the highest number of 2023-specific substitutions (n = 10), suggesting that this genomic region accumulated the greatest number of changes within the comparison performed here.

Table 4
Protein-wise distribution of shared and 2023-specific non-synonymous substitutions in DENV-2 Cosmopolitan isolates.

Protein	Shared substitutions (n)	2023-specific substitutions (n)	Total (n)
E	4	5	9
NS1	3	5	8
NS3	3	4	7
NS5	4	10	14

Discussion

This study provides whole-genome evidence for heterogeneous co-circulation of multiple DENV-1 and DENV-2 genotypes during the 2023 dengue outbreak in Vietnam. Although DENV-2 was the dominant serotype in the sampled cohort, genomic analysis revealed concurrent circulation of DENV-1 genotypes I and IV and DENV-2 Cosmopolitan and Asian I genotypes. This pattern contrasts with several prior outbreak descriptions that emphasized dominance of a single genotype or lineage, including the 2022 southern Vietnam DENV-2 outbreak driven mainly by a newly introduced Cosmopolitan lineage [3,6].

DENV-1 genotype I accounted for most DENV-1 genomes in this study and clustered with previously reported Vietnamese strains, supporting sustained local circulation. The detection of DENV-1 genotype IV, although limited, is notable because genotype IV has been associated with broader regional and inter-regional circulation in Asia and island settings [23]. Because the number of DENV-1 genotype IV sequences in this cohort was small, source attribution should not be inferred without broader phylogeographic analysis. Nevertheless, its detection indicates that DENV-1 diversity during the 2023 outbreak was not limited to a single genotype.

Among DENV-2 genomes, the predominance of the Cosmopolitan genotype is consistent with recent reports from Vietnam and neighboring countries [3–8]. Co-circulation of DENV-2 Cosmopolitan and Asian I genotypes has previously been reported in Hanoi and nearby provinces during the 2019–2020 epidemic [4], and related genotype dynamics have been observed in Thailand and southern China [7–9]. The findings from the present cohort therefore fit a broader mainland Southeast Asian pattern of recurrent DENV movement, genotype turnover, and regional exchange.

Comparative mutational analysis identified shared and 2023-associated non-synonymous substitutions in DENV-2 Cosmopolitan isolates. These substitutions were concentrated in E, NS1, NS3, and NS5, which

encode proteins involved in viral entry, immune interaction, and replication [10,26,27]. The largest number of substitutions was observed in NS5, the viral polymerase and methyltransferase. These observations suggest ongoing diversification within the sampled DENV-2 Cosmopolitan lineage. However, this study was not designed to determine the functional effects of individual substitutions, and the observed mutations should not be interpreted as evidence of altered fitness, virulence, immune escape, or vaccine breakthrough without experimental or epidemiological validation.

The public-health implication of these findings is that serotype-level surveillance may be insufficient in settings where multiple genotypes or lineages co-circulate. Whole-genome surveillance provides higher resolution for distinguishing local persistence from potential introductions, tracking genotype turnover, and identifying genetic changes that may warrant further phenotypic investigation. Such information is particularly relevant for dengue-endemic countries with frequent human mobility and intense regional connectivity.

This study has several limitations. First, the number of complete genomes was modest (n = 47), limiting the resolution of genotype-frequency estimates and mutational comparisons. Second, the samples were hospital-based and predominantly collected in Vung Tau (98.7%) and therefore should not be interpreted as representing nationwide DENV diversity in Vietnam. Third, detailed clinical severity, vaccination, vector, and environmental metadata were not available for integrated analysis. Fourth, no time-scaled phylogeographic analysis was performed, so regional source attribution cannot be concluded. Finally, the biological significance of the identified substitutions remains to be determined through structural, antigenic, replication, and neutralization studies.

Conclusions

The 2023 dengue outbreak cohort analyzed in this study showed heterogeneous co-circulation of DENV-1 and DENV-2 genotypes, including DENV-1 genotypes I and IV and DENV-2 Cosmopolitan and Asian I. The predominance of DENV-2 Cosmopolitan together with additional genotype diversity and 2023-associated substitutions highlights the need for whole-genome surveillance to complement routine serotype-level monitoring. Expanded sampling across Vietnam and neighboring countries, together with functional and clinical analyses, will be important for clarifying the epidemiological and biological significance of the genomic diversity observed here.

Abbreviations

ADE
antibody-dependent enhancement
BHK
baby hamster kidney
DENV
dengue virus

E
envelope protein
FBS
fetal bovine serum
MAFFT
multiple alignment using fast Fourier transform
MEGA
Molecular Evolutionary Genetics Analysis
MTA
material transfer agreement
NCBI
National Center for Biotechnology Information
NGS
next-generation sequencing
NS
non-structural protein
qRT-PCR
quantitative reverse transcription polymerase chain reaction
RT-PCR
reverse transcription polymerase chain reaction
WGS
whole-genome sequencing.

Declarations

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of the Faculty of Medicine of the University of Tokyo (2023313NI-(2)), and by the National Institute of Hygiene and Epidemiology Institutional Review Board in Bio Medical Research (IRB-VN01057/IORG0008555, No. NIHE IRB-28/2024). Written informed consent was obtained from participants or their legal guardians according to the approved protocol.

Consent for publication

Not applicable.

Competing interests

M.L.M. and L.M.Y are members of the Editorial Board of Tropical Medicine and Health. The remaining authors declare that they have no competing interests.

Authors' information

Not applicable.

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Author Contribution

M.L.M. conceptualized and designed the study. D.C.M.A., V.B.T.H., Y.S.L.A., K.H., T.T.T.N., L.K.H.N., and M.L.M. developed and performed the methodology. D.C.M.A., V.B.T.H., M.J., A.B., H.H., T.T.T.N., L.K.H.N., N.L.N., T.T.H.P., and M.L.M. conducted the investigation. V.B.T.H., T.T.T.N., L.K.H.N., N.L.N., and T.T.H.P. collected samples and performed initial characterization. D.C.M.A., M.J., A.B., and H.H. performed virus titration in BHK cells. D.C.M.A., V.B.T.H., M.J., A.B., and H.H. performed virus isolation. V.B.T.H., D.C.M.A., M.J., A.B., and H.H. performed RNA extraction and qRT-PCR. D.C.M.A., M.J., and M.L.M. performed whole-genome sequencing and data curation. D.C.M.A. performed formal analysis and drafted the manuscript. K.I.B. and M.L.M. reviewed and edited the manuscript. M.L.M. and M.T.Q.L. acquired funding. M.L.M., L.M.Y., and M.T.Q.L. provided resources. M.L.M. supervised the study. All authors read and approved the final manuscript.

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Data Availability

The DENV genome sequences generated in this study will be deposited in a public nucleotide sequence repository before publication. Additional de-identified data supporting the findings of this study are available from the corresponding author upon reasonable request and subject to applicable institutional and material-transfer requirements.

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Figures

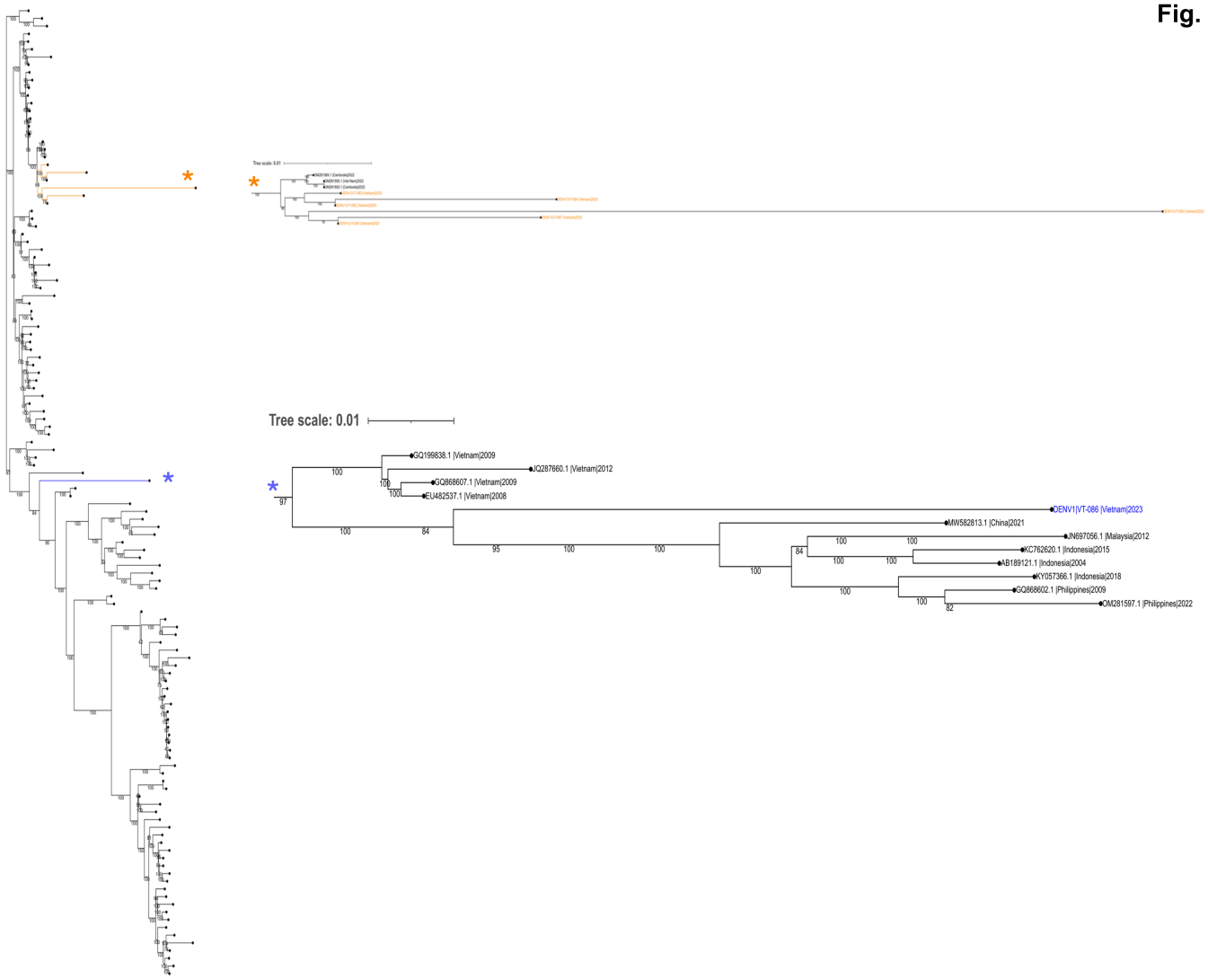


Figure 1

Maximum-likelihood phylogenetic tree of DENV-1 complete genomes from the 2023 Vietnam outbreak. Phylogenetic relationships were inferred using complete genome sequences generated in this study (n = 7) together with reference sequences retrieved from GenBank. Genotype classification is indicated by colored clades. Most 2023 isolates cluster within genotype I and group closely with previously reported Vietnamese strains, while a smaller subset clusters within genotype IV and aligns more closely with non-local reference sequences. Bootstrap support values (≥50%) are shown at key nodes. DENV-1 genotype I and genotype IV clades are indicated by colored branches, and enlarged clades are marked with corresponding asterisks. The scale bar indicates nucleotide substitutions per site.



Figure 2

Maximum-likelihood phylogenetic tree of DENV-2 complete genomes from the 2023 Vietnam outbreak. Phylogenetic analysis was performed using complete genome sequences from this study (n = 40) together with representative global reference sequences from GenBank. Most Vietnamese DENV-2 isolates clustered within the Cosmopolitan genotype, whereas the remaining isolates belonged to Asian I genotype. Clustering with previously reported Vietnamese and regional Southeast Asian strains supports continued local circulation and regional viral exchange. Bootstrap values (≥50%) are shown at key nodes. DENV-2 Asian I and Cosmopolitan clades are indicated by colored branches, and enlarged clades are marked with corresponding asterisks. The scale bar represents nucleotide substitutions per site.